NANOPARTICLE MANUFACTURING

BACKGROUND

[0001] Polymer nanoparticle systems have become an important drug delivery modality. In nanoparticle formulations of drugs, biodegradable polymers are commonly used as a matrix to carry a payload (*e.g.*, a therapeutic or diagnostic agent). Diverse approaches have been applied in order to produce polymer nanoparticles containing a payload. However, improved approaches are both desirable and needed.

SUMMARY

[0002] The present disclosure provides nanoparticle compositions in which individual nanoparticles comprise polymers and (one or more) payloads, and optionally may comprise a coating, *etc.*; the present disclosure also provides various technologies for making, using, and/or characterizing such nanoparticle compositions (and/or components and/or intermediates thereof).

[0003] Among other things, the present disclosure identifies the source of at least one problem in certain polymer nanoparticle technologies, particularly when utilized to prepare compositions for delivering and/or otherwise including one or more complex payloads (*e.g.*, protein, carbohydrate, lipid and/or nucleic acid mixtures, crude samples, cellular extracts, *etc.*) and/or one or more fragile payloads (*e.g.,* that maybe particularly susceptible to degradation and/or inactivation, *etc.* when exposed to particular conditions; in some embodiments, nucleic acids, particularly RNA and most particularly longer RNAs such as mRNAs, polypeptides, and/or saccharides may be considered to be "fragile" payloads).

[0004] Alternatively or additionally, according to various embodiments, provided technologies display certain advantages and/or solve one or more problems associated with conventional nanoparticle technologies, specifically including certain nanoparticle manufacturing technologies.

[0005] For example, in some embodiments, the present disclosure provides technologies for manufacturing nanoparticles (*e.g.*, comprised of polymers and including payloads and/or coating agents as described herein) that involve few steps and/or particularly gentle reagents and

conditions. In some embodiments, provided manufacturing technologies involve simple procedures requiring fewer steps than conventional nanoparticle production methods. In some embodiments, provided manufacturing technologies do not involve harsh manufacturing conditions (*e.g.*, high temperature, pressure and/or shear force). Among other things, therefore, the present disclosure provides technologies that may be particularly suitable and/or may offer particular advantages, for use with fragile (*e.g.*, susceptible to damage from energy input and/or exposure to during manufacturing) and/or complex payloads (*e.g.,* wherein individual nanoparticles may contain one or more payloads and/or may be coated with one or more coating agents, and further wherein one or more such payloads and/or coating agents may itself be or comprise a complex [*e.g.,* multi-component, and/or crude] composition). In some embodiments, technologies described herein may be particularly useful for the manufacturing of nanoparticles (*e.g.*, comprised of polymers and including payloads and/or coating agents as described herein) at a greater scale than conventional methodologies.

[0006] In some embodiments, provided manufacturing technologies utilize and/or benefit from attributes of non-solvent systems (*e.g.*, non-solvent systems of polymers, payloads, and/or coating agents).

[0007] In some embodiments, the present disclosure provides insights regarding material behaviors at fluid interfaces and provide strategies for harnessing such insights in production technologies. For example, in certain embodiments, the present disclosure provides and/or utilizes mild mixing at a fluid interface of a heterogeneous, layered two-fluid system. The present disclosure teaches that mixing which is (a) mild, as described herein; and/or (b) occurs at the interface can be particularly useful and effective and can overcome various challenges otherwise encountered in nanoparticle production. Moreover, the present disclosure demonstrates that such processes can achieve surprising results, including for example, remarkable uniformity in nanoparticle production (e.g., in average size and/or in polydispersity (see, for example, Example 10, which exemplifies a heterogeneous, layered two-fluid process referred to herein as a "Tequila Sunrise" process).

[0008] In some embodiments, provided technologies achieve nanoparticle compositions in which payloads are not significantly surface exposed (*e.g.*, not exposed to the environment surrounding the nanoparticle). In some embodiments, provided technologies for manufacturing nanoparticles allow for production of nanoparticles encapsulating one or more payloads, such that

encapsulated payloads are substantially wholly encapsulated. In some embodiments, payloads are considered to be "encapsulated" when they are not detectable as "free" (e.g., when nanoparticles have not been disrupted); in some such embodiments, at least 80% or at least 85% or at least 90% of payload is encapsulated. For example, in some embodiments, assessment of total payload utilized in nanoparticle manufacturing, and of "free" payload detectable when nanoparticles have not been disrupted reveal that no more than about 10%, 15%, or 20% of the total payload is detected as "free" payload.

[0009] In various embodiments, provided technologies embody, permit, and/or achieve one or more surprising features such as, for example, efficiency and/or simplicity of nanoparticle production, conditions amenable to fragile payloads, production of complex nanoparticle compositions (*e.g.,* wherein individual nanoparticles may contain one or more payloads and/or may be coated with one or more coating agents, and further wherein one or more such payloads and/or coating agents may itself be or comprise a complex [*e.g.,* multi-component, and/or crude] composition), production of nanoparticle compositions characterized by desirable attributes such as, for example, one or more of desirable particle average size and/or size distribution, particular zeta potential, particular immunomodulatory effects, particular release characteristics, *etc.*

[0010] In some embodiments, provided nanoparticle compositions can achieve immune modulation. For example, among other things, the present disclosure documents stimulation of Th1-type immune reactions with nanoparticle compositions containing lipids (*e.g.,* an *E. coli* lipid extract and/or lipopolysaccharide) on their surfaces. Without wishing to be bound by any particular theory, we propose that such nanoparticle compositions may be viewed by a recipient's immune system as analogous to bacterial agents (*e.g.*, to bacterial cells). The present disclosure proposes and demonstrates that this ability to direct a Th1-type immune response to an administered nanoparticle composition presents an opportunity to shift or otherwise bias a recipient's immune response to one or more antigens included in the nanoparticle composition toward such a Th1-type response; such an effect is particularly useful in the treatment of allergy to an encapsulated allergen. The present disclosure documents effectiveness of this approach with encapsulated peanut allergens, which are well known to trigger particularly potent (*e.g.*, anaphylactic) immune responses in certain allergic individuals. Those skilled in the art, reading the present disclosure, therefore, will appreciate that success encapsulating peanut allergens (including in the form of a crude peanut extract), and furthermore modulating a subject's

immune response to them, provides significant evidence that comparable results can be achieved with other allergens (including specifically other food allergens, but also allergens generally.

[0011] In some embodiments, provided nanoparticle compositions may be used as and/or incorporated into pharmaceutical composition(s) (*e.g.,* into dosage forms); in some embodiments, provided nanoparticle compositions are amenable to formulation for delivery via any of a variety of routes such as, for example, mucosal, oral, parenteral, topical and/or transdermal *etc.*

[0012] In particular embodiments, provided nanoparticles are amenable to oral administration and/or are administered orally *e.g.,* into the mouth. In some embodiments, provided nanoparticles are amenable to mucosal delivery and/or are administered buccally (*e.g.,* via the oral mucosa). In some embodiments, provided nanoparticles are administered sublingually.

[0013] In some embodiments, provided nanoparticle compositions are suitable for formulation into any of a variety of liquid, solid, or gel compositions including, for example, dispersions, emulsions, solutions, *etc.*, tablets, capsules, gums, lozenges, suppositories, *etc.*; and/or incorporation into any of a variety of devices such as, for example, patches, syringes, *etc.*

[0014] In some embodiments a device (*e.g.,* a patch, roller, *etc.*) may be or comprise a plurality of needles (*e.g.,* microneedles).

[0015] In some embodiments a formulation comprising nanoparticles as described herein may be stable to storage. In some embodiments, stability to storage refers to stability of the nanoparticle structure of the formulation – *e.g.,* as may be reflected, for example, in one or more characteristics such as average particle size, polydispersity etc. Alternatively or additionally, in some embodiments, stability to storage refers to stability of individual nanoparticles, *e.g.,* their ability to encapsulate payload, such as an allergen.

[0016] In some embodiments, a formulation comprising nanoparticles may be stored at room temperature. In some embodiments, such a formulation may be stored under cooling, *e.g.,* at a temperature below about 4°C. In some embodiments, such a formulation may be stored at a temperature at or below about 0°C, -20°C, -80°C, -196°C, *etc.* (*i.e.*, under freezing conditions). In some embodiments, a formulation comprising nanoparticles may be stored in a conventional freezer; in some embodiments, such freezer may undergo periodic defrost cycles.

[0017] In some embodiments, the present disclosure provides nanoparticle compositions including at least one antigen substantially co-localized with at least one adjuvant agent; in some

embodiments, provided nanoparticle compositions are characterized in that administration to a subject in need thereof achieves a desired immunological effect in the subject with respect to the antigen. In some embodiments, provided nanoparticle compositions may be designed and constructed to deliver both an antigen and an adjuvant to a population of a subject's cells (*e.g.*, immune cells, [*e.g.*, antigen presenting cells ("APCs")]).

[0018] In various embodiments, the present disclosure provides technologies for manufacturing a population of nanoparticles.

[0019] In some embodiments, provided manufacturing technologies comprise steps of (i) providing a first preparation, which comprises a payload (*e.g.*,. a hydrophilic payload) in a first aqueous solvent system and a second preparation, which comprises a polymer (*e.g.*, a hydrophobic polymer) in a second solvent system, wherein the second solvent system is non-aqueous, and the polymer is not fully (and in many embodiments is not significantly) soluble in the first aqueous solvent system; (ii) combining the first and second preparations to form a mixture that comprises the payload and the polymer in a combined solvent system; and (iii) adding a non-solvent system to the mixture, so that a population of nanoparticles comprising the payload and the polymer is formed in a nanoparticle suspension (*e.g.*, wherein the non-solvent system is a non-solvent of the polymer and the payload) (*e.g.*, wherein the non-solvent system precipitates the payload and the polymer, so that each of the nanoparticles comprises the payload and the polymer).

[0020] In some embodiments, the present disclosure provides technologies in which a nanoparticle preparation is manufactured by (i) providing a first liquid preparation, which comprises a payload (e.g., a fragile payload) in a first aqueous solvent system and a second liquid preparation, which comprises a polymer (e.g., a hydrophobic polymer) in a second solvent system; (ii) combining the first and second preparations to form a mixture that comprises the payload and the polymer in a combined solvent system, and (iii) adding a liquid non-solvent system to the mixture, so that a population of nanoparticles comprising the payload and the polymer is formed (*e.g.*, wherein the method does not involve energy input) (*e.g.*, wherein the non-solvent system does not significantly degrade the payload, or decrease one or more biological or pharmaceutical activities of the payload) (*e.g.*, wherein one or more biological or pharmaceutical activities of fragile payload are substantially same before and after the step of adding).

[0021] Alternatively or additionally, in some embodiments, the present disclosure provides manufacturing technologies that include mild mixing at a fluid interface (*e.g.,* in a heterogeneous, layered two-fluid system).

[0022] In some embodiments, the present disclosure provides nanoparticle manufacturing technologies in which (i) payload materials and polymer materials are combined in the presence of a solvent/antisolvent system; typically at least the payload material(s) are sufficiently hydrophilic to be provided in water or other aqueous system (the present disclosure provides an insight that use of an organic antisolvent can reduce payload loss during the encapsulation process); and (ii) combined materials are mixed in an intentionally heterogeneous, layered two-fluid process, that typically involves mild mixing (quite different from conventional teachings of desirability or even necessity of intense mixing to homogenize a solvent/antisolvent mixture) at the fluid interface. The present disclosure demonstrates that this approach achieves surprising and remarkable consistency in nanoparticle size (e.g., average size) and/or polydispersity. Furthermore, the approach is scalable and, thanks to its gentle conditions, is particularly useful for the incorporation of fragile payloads (e.g., nucleic acid, polypeptide and/or carbohydrate (*e.g.*, polysaccharide) payloads).

[0023] In certain embodiments, provided nanoparticle manufacturing technologies may include one or more homogenization steps.

[0024] In certain embodiments, provided nanoparticle manufacturing technologies may utilize one or more stabilizers. For example, in some embodiments, deoxycholate may be utilized (e.g., being included at least in a homogenization step).

[0025] In some embodiments, provided nanoparticle manufacturing technologies may include one or more concentration and/or purification steps. In some embodiments, provided technologies utilize one or more tangential flow filtration (TFF) steps. Among other things, the present disclosure provides an insight that, particularly when TFF is utilized, if a stabilizing agent is desired, deoxycholate is a particularly useful stabilizing agent (and/or that other standard stabilizing agents, such as polyvinyl alcohol, PVA, may be less useful or not useful and, in fact, may damage a TFF membrane.

[0026] In some embodiments, provided nanoparticle manufacturing technologies achieve a ratio of payload to polymer in the nanoparticles that is between about 0.1 to about 0.9 of the ratio of payload to polymer in the original mixture from which nanoparticles are precipitated.

[0027] As noted, in some embodiments, provided technologies include one or more steps that remove solvent (e.g., the combined solvent/antisolvent system).

[0028] In some embodiments, provided manufacturing technologies utilize a stabilizing agent. In some embodiments, a stabilizing may be or comprise PVA. In some embodiments, however, particularly when one or more TFF steps is utilized, PVA is not used. In some embodiments, particularly in embodiments that utilize one or more TFF steps, deoxycholate is utilized as a stabilizing agent.

[0029] In some embodiments, provided manufacturing technologies include one or more steps of purifying nanoparticles (*e.g.*, by one or more of filtration, (*e.g.*, tangential flow filtration), sonication, dilution).

[0030] In some embodiments, provided technologies include one or more steps of drying nanoparticles.

[0031] In some embodiments, a nanoparticle preparation in accordance with the present disclosure (e.g., manufactured as described herein) has a mean size within a range of approximately 100-500 nm. In some embodiments, mean size is within a range of about 225 nm to about 450 nm. In many embodiments, mean size is determined by dynamic light scattering.

[0032] In some embodiments, a provided nanoparticle preparation has a mean diameter within a range of about 50 nm to about 150 nm.

[0033] In some embodiments, provided nanoparticle compositions include a payload selected from the group consisting of polypeptides, nucleic acids, carbohydrates (*e.g.*, polysaccharides), and combinations thereof. In many embodiments, a payload is or comprises a polypeptide. In many embodiments, a payload is or comprises a nucleic acid; in some such embodiments, a payload is or comprises a long nucleic acid (e.g., a gene therapy vector, an mRNA, etc); in some embodiments, a payload is or comprises a partly or wholly single stranded nucleic acid).

[0034] In some embodiments, a payload is or comprises a RNA. In some embodiments, and RNA payload is an mRNA. In some embodiments, an RNA payload has a length within a range of about 15 to about 3,000,000 residues. In some embodiments, an RNA payload has a length within a range of about 500 to 50000 residues. In some embodiments, an RNA payload has a length within a range of about 1000 to about 10000 residues. In some embodiments, an RNA payload is an mRNA encoding a polypeptide having a length within a range of about 50 to about 5000 amino acids; in some embodiments, such encoded polypeptide has a length within a range

of about 100 to about 3000 amino acids; in some embodiments, such encoded polypeptide polypeptide has a length within a range of about 200 to about 1500 amino acids.

[0035] In some embodiments, a provided nanoparticle preparation is manufactured using one or more relatively complex components (e.g., a payload that is a relatively crude extract or combination of components).

[0036] In some embodiments, a payload is or comprises one or more antigens. In some embodiments, an antigen is an allergic antigen, an infectious antigen, and/or a disease-associated (e.g., a cancer-associated) antigen.

[0037] In some embodiments, provided manufacturing technologies utilize a solvent system that comprises water and DMSO. In some such embodiments, a volume ratio of water and DMSO is within a range of 1:99 to 20:80, or 1:99 to 10:90.

[0038] In some embodiments, provided technologies utilize an anti-solvent system (which may in some embodiments be referred to as a non-solvent system). In some embodiments, an anti-solvent system is or comprises an alcohol. In some embodiments, an anti-solvent system is or comprises propanol, ethanol, methanol, or combination thereof. In some embodiments, an anti-solvent is or comprises IPA.

[0039] In some embodiments, provided nanoparticles include (e.g., are manufactured) from a polymer that is or comprises Poly (lactic-co-glycolic acid) (PLGA or PLG) .

[0040] In some embodiments, provided nanoparticles utilize (e.g., are manufactured from) a polymer preparation (e.g., a PLG preparation) where the polymer has a molecular weight within a range of 5,000 – 5,000,000 Daltons.

[0041] In some embodiments, a provided nanoparticle composition includes one or more payloads on (*e.g.,* in some embodiments attached to; in some embodiments otherwise associated) nanoparticle surface(s).

[0042] In some embodiments, the present disclosure provides vaccine compositions comprising a nanoparticle population comprising one or more payloads, or precursor(s) thereof, that activate antigen-specific T cells, for example wherein such one or more payloads is/are displayed (or, if a nucleic acid, may encode an agent that is displayed) by an MHC class I complex or an MHC class II complex.

[0043] In some embodiments, a provided vaccine composition comprises an immune adjuvant. In some embodiments, an immune adjuvant is provided from one or more bacterial sources. In

some embodiments, an immune adjuvant comprises a cellular lysate (e.g., microbial lysate) or a cellular lysate fraction. In some embodiments, an immune adjuvant is a mucosal immune adjuvant.

[0044] In some embodiments, a provided vaccine comprises a pore forming toxin.

[0045] In some embodiments, the present disclosure provides a vaccine composition comprising first and/or second nanoparticle populations, wherein the first nanoparticle population comprises a first payload, or precursor(s) thereof, that activates first antigen-specific T cells; and the second nanoparticle population comprises a second payload, or precursor(s) thereof, that activates second antigen-specific T cells. In some embodiments, the first payload is displayed by (or encodes an agent that is displayed by) an MHC class I complex. Alternatively or additionally, in some embodiments, the second payload is displayed by (or encodes an agent that is displayed by) an MHC class II complex. In some such embodiments, the first and second nanoparticle populations are included in a same composition.

[0046] In some embodiments, the present disclosure provides a method comprising steps of administering to a subject in need thereof a nanoparticle composition comprising a nanoparticle population having one or more payloads, or precursor(s) thereof, that activate antigen-specific T cells, wherein the nanoparticle composition is administered orally, sublingually, or buccally.

[0047] In some embodiments, the present disclosure provides a method comprising steps of administering to a subject in need thereof a nanoparticle composition comprising a first nanoparticle population having a first payload, or precursor(s) thereof, that activate a first antigen-specific T cell, and a second nanoparticle population having a second payload, or precursor(s) thereof, that activate a second antigen-specific T cell, wherein the nanoparticle composition is administered orally, sublingually or buccally.

[0048] In another aspect, the present disclosure provides a method comprising steps of (i) administering to a subject in need thereof a first nanoparticle composition comprising a first nanoparticle population having a first payload, or precursor(s) thereof, that activate a first antigen-specific T cell; and administering to the subject a second nanoparticle composition comprising a second nanoparticle population having a second payload, or precursor(s) thereof, that activate a second antigen-specific T cell, wherein the first and/or second nanoparticle compositions are administered orally, sublingually or buccally.

[0049] In some embodiments, the present disclosure provides a nanoparticle preparation prepared by the methods provided herein, and the nanoparticle preparation comprises a plurality of nanoparticles, each of which comprises a payload *(e.g.*, a hydrophilic payload) in a polymer.

DEFINITIONS

[0050] In order for the present disclosure to be more readily understood, certain terms are first defined below. Additional definitions for the following terms and other terms are set forth throughout the specification.

[0051] *Administration:* As used herein, the term "administration" refers to administration of a composition to a subject. Administration may be by any appropriate route. For example, in some embodiments, administration may be bronchial (including by bronchial instillation), buccal (*e.g.*, between teeth and cheek, includes lower and upper teeth), enteral, interdermal, intra-arterial, intradermal, intragastric, intramedullary, intramuscular, intranasal, intraperitoneal, intrathecal, intravenous, intraventricular, mucosal, nasal, oral, rectal, subcutaneous, sublingual, topical, tracheal (including by intratracheal instillation), transdermal, vaginal and vitreal.

[0052] *Aggregation:* The term "aggregation", as used herein, refers to the formation of higher molecular weight entities, rather than the desired, defined species of nanoparticles. Aggregation may pose a problem during nanoparticle formation and/or manufacturing, e.g., as described herein. Aggregation may involve entities "sticking" or "clumping" together. In some embodiments, aggregation is prevented and/or reduced during the nanoparticle formation and/or manufacturing, e.g., as described herein. In some embodiments, aggregates (e.g., entities formed by the process of aggregation) are excluded from nanoparticle compositions comprising desired, defined species of nanoparticles. Aggregates may comprise nanoparticles and/or components of nanoparticles as described herein. Aggregates may comprise biological material such as, for example, protein, DNA, and/or RNA. Aggregates may be defined by size (e.g., as characterized by molecular weight and/or diameter). In some embodiments, aggregates are greater than or equal to 1000, 1250, 1500, 1750, 2000, 2500, or 3000 nm in diameter. In some embodiments, aggregates are excluded via methods such as size exclusion chromatography.

[0053] *Allergen:* The term "allergen", as used herein, refers to those antigens that induce an allergic reaction. In some embodiments, an allergen is or comprises a polypeptide. In some embodiments, an allergen is or comprises a carbohydrate (*e.g.*, polysaccharide). In some

embodiments, an allergen is or comprises a small molecule. In some embodiments, an allergen is selected from the group consisting of food allergens, drug allergens, environmental allergens, insect venoms, animal allergens, and latex.

[0054] *Allergic reaction*: The phrase "allergic reaction," as used herein, has its art-understood meaning and refers to an IgE-mediated immune response to an antigen. When an antigen induces IgE antibodies, they will bind to IgE receptors on surfaces of basophils and mast cells. Subsequent exposures to the antigen trigger cross-linking of such surface-bound anti-allergen IgEs, which trigger release of histamine from stores within the cells. This histamine release triggers the allergic reaction. Typically, an allergic reaction involves one or more of the cutaneous (*e.g.*, urticaria, angioedema, pruritus), respiratory (*e.g.*, wheezing, coughing, laryngeal edema, rhinorrhea, watery/itching eyes), gastrointestinal (*e.g.*, vomiting, abdominal pain, diarrhea), and/or cardiovascular (*e.g.*, if a systemic reaction occurs) systems. For the purposes of the present disclosure, an asthmatic reaction is considered to be a form of allergic reaction. In some embodiments, allergic reactions are mild; typical symptoms of a mild reaction include, for example, hives (especially over the neck and face) itching, nasal congestion, rashes, watery eyes, red eyes, and combinations thereof. In some embodiments, allergic reactions are severe and/or life threatening; in some embodiments, symptoms of severe allergic reactions (*e.g.*, anaphylactic reactions) are selected from the group consisting of abdominal pain, abdominal breathing sounds (typically high-pitched), anxiety, chest discomfort or tightness, cough, diarrhea, difficulty breathing, difficulty swallowing, dizziness or light-headedness, flushing or redness of the face, nausea or vomiting, palpitations, swelling of the face, eyes or tongue, unconsciousness, wheezing, and combinations thereof. In some embodiments, allergic reactions are anaphylactic reactions. In some embodiments, allergic reactions are defined as a disorder characterized by an adverse local or general response from exposure to one or more allergens. In some embodiments, allergic reactions may be graded by a "toxicity grading" system, that will be known to those of skill in the art. For example, in some embodiments, a grading system (such as NCI-CTCAD v 4.03), will be used to grade allergic reactions, such as a system described in **Table 1** and/or **Table 2.**

Table 1. Allergic Reaction Grading System.

Grade 1 - Mild	**Grade 2 - Moderate**	**Grade 3 – Severe**	**Grade 4 – Life-threatening**	**Grade 5 - Death**
Transient flushing or rash,	Intervention or infusion interruption indicated;	Prolonged (*e.g.*, not rapidly responsive to symptomatic	Life-threatening	Death

drug fever <38 degrees C (<100.4 degrees F); intervention not indicated	responds promptly to symptomatic treatment (*e.g.*, antihistamines, NSAIDS, narcotics); prophylactic medications indicated for <=24 hrs	medication and/or brief interruption of infusion); recurrence of symptoms following initial improvement; hospitalization indicated for clinical sequelae (*e.g.*, renal impairment, pulmonary infiltrates)	consequences; urgent intervention indicated	

Table 2. Allergic Reaction Grading System.

Grade 1 - Mild	**Grade 2 - Moderate**	**Grade 3 – Severe**	**Grade 4 - Life threatening**	**Grade 5 - Death**
Transient or mild discomforts (<48 hours), no or minimal medical intervention/therapy required. These symptoms may include pruritus, swelling or rash, abdominal discomfort or other transient symptoms.	Symptoms that produce mild to moderate limitation in activity some assistance may be needed; no or minimal intervention/therapy is required. Hospitalization is possible. These symptoms may include persistent hives, wheezing without dyspnea, abdominal discomfort/ increased vomiting or other symptoms	Marked limitation in activity, some assistance usually required; medical intervention/therapy required, hospitalization is possible. Symptoms may include Bronchospasm with dyspnea, severe abdominal pain, throat tightness with hoarseness, transient hypotension among others. parenteral medication(s) are usually indicated.	Extreme limitation in activity, significant assistance required; significant medical/therapy. Intervention is required; hospitalization is probable. Symptoms may include persistent hypotension and/or hypoxia with resultant decreased level of consciousness associated with collapse and/or incontinence or other life threatening symptoms.	Death

[0055] *Allergy*: The term "allergy", as used herein, refers to a condition characterized by an IgE-mediated immune response to particular antigens. In some embodiments, the antigens are ones that do not elicit an IgE-mediated immune response in many or most individuals. In some embodiments, the term "allergy" is used to refer to those situations where an individual has a more dramatic IgE-mediated immune response when exposed to a particular antigen than is typically observed by members of the individual's species when comparably exposed to the same antigen. Thus, an individual who is suffering from or susceptible to "allergy" is one who experiences or is at risk of experiencing an allergic reaction when exposed to one or more allergens. In some embodiments, symptoms of allergy include, for example, presence of IgE antibodies, reactive with the allergen(s) to which the individual is allergic, optionally above a particular threshold, in blood or serum of the individual. In some embodiments, symptoms of allergy include development of a wheal/flare larger than a control wheal/flare when a preparation of the antigen is injected subcutaneously under the individual's skin. In some embodiments, an

individual can be considered susceptible to allergy without having suffered an allergic reaction to the particular allergen in question. For example, if the individual has suffered an allergic reaction, and particularly if the individual has suffered an anaphylactic reaction, to a related allergen (*e.g.*, one from the same source or one for which shared allergies are common), that individual may be considered susceptible to allergy to (and/or to an allergic or anaphylactic reaction to) the relevant allergen. Similarly, if members of an individual's family react to a particular allergen, the individual may be considered to be susceptible to allergy to (and/or to an allergic and/or anaphylactic reaction to) that allergen.

[0056] *Amino acid:* As used herein, the term "amino acid," in its broadest sense, refers to any compound and/or substance that can be incorporated into a polypeptide chain, *e.g.*, through formation of one or more peptide bonds. In some embodiments, an amino acid has the general structure H_2N–C(H)(R)–COOH. In some embodiments, an amino acid is a naturally-occurring amino acid. In some embodiments, an amino acid is a synthetic amino acid; in some embodiments, an amino acid is a D-amino acid; in some embodiments, an amino acid is an L-amino acid. "Standard amino acid" refers to any of the twenty standard L-amino acids commonly found in naturally occurring peptides. "Nonstandard amino acid" refers to any amino acid, other than the standard amino acids, regardless of whether it is prepared synthetically or obtained from a natural source. In some embodiments, an amino acid, including a carboxy- and/or amino-terminal amino acid in a polypeptide, can contain a structural modification as compared with the general structure above. For example, in some embodiments, an amino acid may be modified by methylation, amidation, acetylation, and/or substitution as compared with the general structure. In some embodiments, such modification may, for example, alter the circulating half-life of a polypeptide containing the modified amino acid as compared with one containing an otherwise identical unmodified amino acid. In some embodiments, such modification does not significantly alter a relevant activity of a polypeptide containing the modified amino acid, as compared with one containing an otherwise identical unmodified amino acid. As will be clear from context, in some embodiments, the term "amino acid" is used to refer to a free amino acid; in some embodiments it is used to refer to an amino acid residue of a polypeptide.

[0057] *Alloantigen*: The term "alloantigen", as used herein, refers to an antigen associated with allorecognition and/or graft rejection (*e.g.*, an antigen against which a rejection immune response

is directed). In general, alloantigens are agents that are present in or on tissue from one individual (*e.g.*, a donor individual) of a particular species, but not in or on tissue from another individual (*e.g.*, a recipient individual, for example who is genetically different from the donor individual) of the species, so that transfer of tissue from the donor individual to the recipient individual risks and/or results in a rejection immune response. In general, an antigen may be or include any chemical entity such as, for example, a small molecule, a nucleic acid, a polypeptide, a carbohydrate, a lipid, *etc*. In some embodiments, an alloantigen is or comprises a polypeptide. A variety of polypeptides are known in the art whose amino acid sequences can vary between and among individuals of the same species such that they might act as alloantigens.

[0058] *Allorecognition*: The term "allorecognition", as used herein, typically refers to an immune response mounted by the immune system of an individual (*i.e.*, a recipient) who receives a tissue graft from another individual (*i.e.*, a donor, who for example is genetically distinct from the recipient individual) of the same species, which immune response involves recognition of an alloantigen on the grafted tissue. Typically, allorecognition involves T cell recognition of the alloantigen. In many embodiments, T cells recognize an alloantigen peptide, for example, encoded by a polymorphic gene whose sequence differs between the donor and recipient individuals.

[0059] *Anaphylactic antigen:* The phrase "anaphylactic antigen", as used herein, refers to an antigen (*e.g.*, an allergen) that is recognized to present a risk of anaphylactic reaction in allergic individuals when encountered in its natural state, under normal conditions. For example, for the purposes of the present disclosure, pollens and animal danders or excretions (*e.g.*, saliva, urine) are not considered to be anaphylactic antigens. On the other hand, certain food antigens, insect antigens, drugs, and rubber (*e.g.*, latex) antigens latex are generally considered to be anaphylactic antigens. Exemplary anaphylactic antigens include those to which reactions are so severe as to create a risk of death (*e.g.*, nuts, seeds, and fish).

[0060] *Anaphylactic reaction:* The phrase "anaphylactic reaction," (*e.g.*, "anaphylaxis") as used herein, refers to a severe, whole body allergic reaction to an allergen, characterized by pathological responses in multiple target organs, *e.g.*, airway, skin digestive tract, and cardiovascular system. As noted above, symptoms of severe allergic reactions such as anaphylactic reactions typically develop quickly, often within minutes of exposure to the allergen, and can include, for example, abdominal pain, abdominal breathing sounds (typically

high-pitched), anxiety, chest discomfort or tightness, cough, diarrhea, difficulty breathing, difficulty swallowing, dizziness or light-headedness, flushing or redness of the face, nausea or vomiting, palpitations, swelling of the face, eyes or tongue, unconsciousness, wheezing, and combinations thereof. Particular signs of anaphylaxis may include, for example, abnormal heart rhythm (arrhythmia), fluid in the lungs (pulmonary edema), hives, low blood pressure, mental confusion, rapid pulse, skin that is blue from lack of oxygen or pale (*e.g.*, from shock), swelling (angioedema) in the throat that may be severe enough to block the airway, swelling of the eyes and/or face, weakness, wheezing. The most severe anaphylactic reactions can result in loss of consciousness and/or death. In some embodiments, anaphylactic reactions may be defined as a disorder characterized by an acute inflammatory reaction resulting from the release of histamine and histamine-like substances from mast cells, causing a hypersensitivity immune response. Clinically, anaphylaxis may present with breathing difficulty, dizziness, hypotension, cyanosis and/or loss of consciousness and may lead to death. In some embodiments, a grading system (such as NCI-CTCAD v 4.03), will be used to grade anaphylactic reactions, such as a system described in **Table 3:**

Table 3. Staging System of Severity of Anaphylaxis

Stage	Characterized By
1. *Mild (skin & subcutaneous tissues, GI, &/or mild respiratory)*	Flushing, urticaria, periorbital or facial angioedema; mild dyspnea, wheeze or upper respiratory symptoms; mild abdominal pain and/or emesis
2. *Moderate* (mild symptoms + features suggesting moderate respiratory, cardiovascular or GI symptoms)	Marked dysphagia, hoarseness and/or stridor; shortness of breath, wheezing & retractions; crampy abdominal pain, recurrent vomiting and/or diarrhea; and/or mild dizziness
3. *Severe* (hypoxia, hypotension, or neurological compromise)	Cyanosis or $SpO_2 \leq 90\%$ at any stage, hypotension, confusion, collapse, loss of consciousness; or incontinence

[0061] In some embodiments, anaphylactic reactions may be diagnosed according to the following criteria, wherein an anaphylactic reaction is likely to have occurred or be occurring when any one of the three following sets of criteria are fulfilled:

1. Acute onset of an illness (minutes to hours) with involvement of:
    - Skin/mucosal tissue (*e.g.*, generalized hives, itch or flush, swollen lips/tongue/uvula) AND

- Airway compromise (*e.g.*, dyspnea, stridor, wheeze/ bronchospasm, hypoxia, reduced PEF) AND/OR
- Reduced BP or associated symptoms (*e.g.*, hypotonia, syncope, incontinence)

2. Two or more of the following that occur rapidly after exposure to the allergen (minutes to hours):

- Skin/mucosal tissue (*e.g.*, generalized hives, itch/flush, swollen lips/tongue/uvula)
- Airway compromise (*e.g.*, dyspnea, stridor wheeze/bronchospasm, hypoxia, reduced PEF)
- Reduced BP or associated symptoms (*e.g.*, hypotonia, syncope, incontinence)
- Persistent GI symptoms (*e.g.*, nausea, vomiting, crampy abdominal pain)

3. Reduced BP after exposure to the allergen (minutes to hours):

- Infants and Children: low systolic BP (age-specific) or > 30% drop in systolic BP*
- Adults: systolic BP < 90 mm Hg or > 30% drop from their baseline

[0062] In some embodiments, low systolic BP for children is defined as < 70 mmHg from 1 month to 1 year; less than (70 mmHg + [2 x age]) from 1-10 years; and < 90 mmHg from age 11-17 years. In some embodiments, isolated skin or mucosal lesions following the ingestion of a food constitute a "food-induced allergic reaction".

[0063] *Animal:* As used herein, the term "animal" refers to any member of the animal kingdom. In some embodiments, "animal" refers to humans, at any stage of development. In some embodiments, "animal" refers to non-human animals, at any stage of development. In some embodiments, the non-human animal is a mammal (*e.g.*, a rodent, a mouse, a rat, a rabbit, a monkey, a dog, a cat, a sheep, cattle, a primate, and/or a pig). In some embodiments, animals include, but are not limited to, mammals, birds, reptiles, amphibians, fish, and/or worms. In some embodiments, an animal may be a transgenic animal, genetically-engineered animal, and/or a clone.

[0064] *Antigen*: The term "antigen", as used herein, refers to an agent that elicits an immune response; and/or (ii) an agent that binds to a T cell receptor (*e.g.*, when presented by an MHC molecule) or to an antibody (*e.g.*, produced by a B cell). In some embodiments, an antigen elicits a humoral response (*e.g.*, including production of antigen-specific antibodies); in some embodiments, an antigen elicits cellular response (*e.g.*, involving T-cells whose receptors

specifically interact with the antigen). In general, an antigen may be or include any chemical entity such as, for example, a small molecule, a nucleic acid, a polypeptide, a carbohydrate, a lipid, *etc*. In some embodiments, an antigen is or comprises a polypeptide. Those of ordinary skill in the art will appreciate that, in general, an antigen may be provided in isolated or pure form, or alternatively may be provided in crude form (*e.g.*, together with other materials, for example in an extract such as a cellular extract or other relatively crude preparation of an antigen-containing source). In some embodiments, antigens utilized in accordance with the present disclosure are provided in a crude form. In some embodiments, an antigen is a recombinant antigen.

[0065] *Antigen presenting cell:* The phrase "antigen presenting cell" or "APC," as used herein, has its art understood meaning referring to cells which process and/or present antigen(s) to T-cells. Exemplary antigen presenting cells include dendritic cells, macrophages and certain activated epithelial cells. In some embodiments, an antigen presenting cell is a cell that processes and/or presents antigen(s) to a particular T-cell population (*e.g.*, to T-cells of a particular type and/or T-cells that may be present in and/or localized to a particular site). Alternatively or additionally, in some embodiments, an antigen presenting cell may be a member of a particular cell population (*e.g.*, a particular type of cell and/or a member of a cell population that is present in and/or localized to a particular site). To give but one example, in some embodiments, an antigen presenting cell may present antigen(s) to a T-cell population that is present in and/or localized to a particular site and/or may itself be present in and/or localized to a particular site. Those of ordinary skill will appreciate, for example, that TLR2/TLR4-expressing dendritic cells have been described as particularly prevalent in the microenvironment within certain oral mucosal sites (*see*, for example Allam, *et al.,* Tolerogenic T cells, Th1/Th17 cytokines and TLR2/TLR4 expressing dendritic cells predominate the microenvironment within distinct oral mucosal sites. *Allergy* 66: 532, 2011).

[0066] *Approximately:* As used herein, the term "approximately" and "about" is intended to encompass normal statistical variation as would be understood by those of ordinary skill in the art. In certain embodiments, the term "approximately" or "about" refers to a range of values that fall within 25%, 20%, 19%, 18%, 17%, 16%, 15%, 14%, 13%, 12%, 11%, 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, 1%, or less in either direction (greater than or less than) of the stated

reference value unless otherwise stated or otherwise evident from the context (except where such number would exceed 100% of a possible value).

[0067] *Associated with:* Two events or entities are "associated" with one another, as that term is used herein, if the presence, level and/or form of one are correlated with that of the other. For example, a particular entity (*e.g.*, polypeptide) is considered to be associated with a particular disease, disorder, or condition, if its presence, level and/or form correlates with incidence of and/or susceptibility of the disease, disorder, or condition (*e.g.*, across a relevant population). In some embodiments, two or more entities are "associated" with one another if they interact, directly or indirectly, so that they are and remain in physical proximity with one another.

[0068] *Autoantigen*: As used herein, the term "autoantigen" is used to refer to antigens produced by an individual that are recognized by the immune system of that individual. In some embodiments, an autoantigen is one whose recognition by the individual's immune system is associated with an autoimmune disease, disorder or condition. In general, an autoantigen may be or include any chemical entity such as, for example, a small molecule, a nucleic acid, a polypeptide, a carbohydrate, a lipid, *etc*. In some embodiments, an autoantigen is or comprises a polypeptide. Those of skill in the art are familiar with a variety of agents, including polypeptides, that can act as autoantigens, and particular that are recognized in immune reactions associated with autoimmunity diseases, disorders and/or conditions.

[0069] *Biocompatible:* The term "biocompatible", as used herein, refers to materials that do not cause significant harm to living tissue when placed in contact with such tissue, *e.g.*, *in vivo*. In certain embodiments, materials are "biocompatible" if they are not toxic to cells. In certain embodiments, materials are "biocompatible" if their addition to cells *in vitro* results in less than or equal to 20% cell death, and/or their administration *in vivo* does not induce significant inflammation or other such adverse effects.

[0070] *Biodegradable*: As used herein, the term "biodegradable" refers to materials that, are broken down in biological systems. The degradation may occur inside cells, *e.g.*, where cellular machinery, such as by enzymatic degradation, by hydrolysis, and/or by combinations thereof is active, or it may occur elsewhere in vivo by means of, *e.g.*, hydrolysis or enzymatic action. In either case, the resultant degradation components do not cause significant toxic effects on the cells. In certain embodiments, components generated by breakdown of a biodegradable material are biocompatible and therefore do not induce significant inflammation and/or other adverse

effects *in vivo*. In some embodiments, biodegradable polymer materials break down into their component monomers. In some embodiments, breakdown of biodegradable materials (including, for example, biodegradable polymer materials) involves hydrolysis of ester bonds. Alternatively or additionally, in some embodiments, breakdown of biodegradable materials (including, for example, biodegradable polymer materials) involves cleavage of urethane linkages. Exemplary biodegradable polymers include, for example, polymers of hydroxy acids such as lactic acid and glycolic acid, including but not limited to poly(hydroxyl acids), poly(lactic acid)(PLA), poly(glycolic acid)(PGA), poly(lactic-co-glycolic acid)(PLG or PLGA), and copolymers with PEG, polyanhydrides, poly(ortho)esters, polyesters, polyurethanes, poly(butyric acid), poly(valeric acid), poly(caprolactone), poly(hydroxyalkanoates, poly(lactide-co-caprolactone), blends and copolymers thereof. Many naturally occurring polymers are also biodegradable, including, for example, proteins such as albumin, collagen, gelatin and prolamines, for example, zein, and carbohydrates (*e.g.*, polysaccharides) such as alginate, cellulose derivatives and polyhydroxyalkanoates, for example, polyhydroxybutyrate blends and copolymers thereof. Those of ordinary skill in the art will appreciate or be able to determine when such polymers are biocompatible and/or biodegradable derivatives thereof (*e.g.*, related to a parent polymer by substantially identical structure that differs only in substitution or addition of particular chemical groups as is known in the art).

[0071] *Biologically active:* As used herein, the phrase "biologically active" refers to a substance that has activity in a biological system (*e.g.*, in a cell (*e.g.*, isolated, in culture, in a tissue, in an organism), in a cell culture, in a tissue, in an organism, *etc.*). For instance, a substance that, when administered to an organism, has a biological effect on that organism, is considered to be biologically active. It will be appreciated by those skilled in the art that often only a portion or fragment of a biologically active substance is required (*e.g.*, is necessary and sufficient) for the activity to be present; in such circumstances, that portion or fragment is considered to be a "biologically active" portion or fragment.

[0072] *Carrier:* As used herein, refers to a diluent, adjuvant, excipient, or vehicle with which a composition is administered. In some exemplary embodiments, carriers can include sterile liquids, such as, for example, water and oils, including oils of petroleum, animal, vegetable or synthetic origin, such as, for example, peanut oil, soybean oil, mineral oil, sesame oil and the like. In some embodiments, carriers are or include one or more solid components. For example,

in some embodiments, a carrier may be or comprise a bead, film, rod, or similarly structured component.

[0073] *Cellular lysate:* As used herein, the term "cellular lysate" or "cell lysate" refers to a fluid containing contents of one or more disrupted cells (*i.e.*, cells whose membrane has been disrupted). In some embodiments, a cellular lysate includes both hydrophilic and hydrophobic cellular components. In some embodiments, a cellular lysate is a lysate of one or more cells selected from the group consisting of plant cells, microbial (*e.g.*, bacterial or fungal) cells, animal cells (*e.g.*, mammalian cells), human cells, and combinations thereof. In some embodiments, a cellular lysate is a lysate of one or more abnormal cells, such as cancer cells. In some embodiments, a cellular lysate is a crude lysate in that little or no purification is performed after disruption of the cells, which generates a "primary" lysate. In some embodiments, one or more isolation or purification steps are performed on the primary lysate. However, the term "lysate" refers to a preparation that includes multiple cellular components and not to pure preparations of any individual component.

[0074] *Combination therapy:* As used herein, the term "combination therapy" refers to those situations in which a subject is simultaneously exposed to two or more therapeutic agents. In some embodiments, such agents are administered simultaneously; in some embodiments, such agents are administered sequentially; in some embodiments, such agents are administered in overlapping regimens.

[0075] *Corresponding to*: As used herein, the term "corresponding to" is often used to designate the position/identity of a residue in a polymer, such as an amino acid residue in a polypeptide or a nucleotide residue in a nucleic acid. Those of ordinary skill will appreciate that, for purposes of simplicity, residues in such a polymer are often designated using a canonical numbering system based on a reference related polymer, so that a residue in a first polymer "corresponding to" a residue at position 190 in the reference polymer, for example, need not actually be the 190^{th} residue in the first polymer but rather corresponds to the residue found at the 190^{th} position in the reference polymer; those of ordinary skill in the art readily appreciate how to identify "corresponding" amino acids, including through use of one or more commercially-available algorithms specifically designed for polymer sequence comparisons.

[0076] *Derivative:* As used herein, the term "derivative" refers to a structural analogue substance that is produced or formed from another substance of similar structure in one or more steps. In

some embodiments, a derivative refers to a second chemical substance related structurally to a first chemical substance and theoretically derivable from the first chemical substance. Examples of cellulose derivatives include, but are not limited to, cellulose esters (such as organic and inorganic esters), cellulose ethers (such as alkyl, hydroxyalkyl and carboxyalkyl ethers), sodium carboxymethyl cellulose and cellulose acetate. Examples of cellulose organic esters include, but are not limited to cellulose acetate, cellulose triacetate, cellulose propionate, cellulose acetate propionate and cellulose acetate butyrate. Examples of cellulose inorganic esters include, but are not limited to, cellulose nitrate and cellulose sulfate. Examples of cellulose alkyl ethers include, but are not limited to, methylcellulose, ethylcellulose and ethyl methyl cellulose. Examples of cellulose hydroxyalkyl ethers include, but are not limited to, hydroxyethyl cellulose, hydroxypropyl cellulose, hydroxyethyl methyl cellulose, hydroxypropyl methyl cellulose and ethyl hydroxyethyl cellulose. Examples of cellulose carboxyalkyl ethers include, but are not limited to, carboxymethyl cellulose.

[0077] *Dosage form:* As used herein, the term "dosage form" refers to a physically discrete unit of a therapeutic agent for administration to a subject. Each unit contains a predetermined quantity of active agent. In some embodiments, such quantity is a unit dosage amount (or a whole fraction thereof) appropriate for administration in accordance with a dosing regimen that has been determined to correlate with a desired or beneficial outcome when administered to a relevant population (*i.e.*, with a therapeutic dosing regimen).

[0078] *Dosing regimen:* As used herein, the term "dosing regimen" refers to a set of unit doses (typically more than one) that are administered individually to a subject, typically separated by periods of time. In some embodiments, a given therapeutic agent has a recommended dosing regimen, which may involve one or more doses. In some embodiments, a dosing regimen comprises a plurality of doses each of which are separated from one another by a time period of the same length; in some embodiments, a dosing regimen comprises a plurality of doses and at least two different time periods separating individual doses. In some embodiments, a dosing regimen is correlated with a desired or beneficial outcome when administered across a relevant population (*i.e.*, is a therapeutic dosing regimen).

[0079] *Encapsulated:* The term "encapsulated" is used herein to refer to substances that are completely surrounded by another material.

[0080] *Expression*: As used herein, "expression" of a nucleic acid sequence refers to one or more of the following events: (1) production of an RNA template from a DNA sequence (*e.g.*, by transcription); (2) processing of an RNA transcript (*e.g.*, by splicing, editing, 5' cap formation, and/or 3' end formation); (3) translation of an RNA into a polypeptide or protein; and/or (4) post-translational modification of a polypeptide or protein.

[0081] *Functional:* As used herein, the term "functional" is used to refer to a form or fragment of an entity that exhibits a particular property and/or activity.

[0082] *Graft rejection:* The term "graft rejection" as used herein, refers to rejection of tissue transplanted from a donor individual to a recipient individual. In some embodiments, graft rejection refers to an allograft rejection, wherein the donor individual and recipient individual are of the same species. Typically, allograft rejection occurs when the donor tissue carries an alloantigen against which the recipient immune system mounts a rejection response. In some embodiments, graft rejection refers to a xenograft rejection, wherein the donor and recipient are of different species. Typically, xenograft rejection occurs when the donor species tissue carries a xenoantigen against which the recipient species immune system mounts a rejection response.

[0083] *Homology:* As used herein, the term "homology" refers to the overall relatedness between polymeric molecules, *e.g.*, between nucleic acid molecules (*e.g.*, DNA molecules and/or RNA molecules) and/or between polypeptide molecules. In some embodiments, polymeric molecules are considered to be "homologous" to one another if their sequences are at least 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 99% identical. In some embodiments, polymeric molecules are considered to be "homologous" to one another if their sequences are at least 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 99% similar (*e.g.*, containing residues with related chemical properties at corresponding positions). For example, as is well known by those of ordinary skill in the art, certain amino acids are typically classified as similar to one another as "hydrophobic" or "hydrophilic" amino acids, and/or as having "polar" or "non-polar" side chains. Substitution of one amino acid for another of the same type may often be considered a "homologous" substitution. Typical amino acid categorizations are summarized below:

Alanine	Ala	A	nonpolar	neutral	1.8
Arginine	Arg	R	polar	positive	-4.5
Asparagine	Asn	N	polar	neutral	-3.5
Aspartic acid	Asp	D	polar	negative	-3.5
Cysteine	Cys	C	nonpolar	neutral	2.5

Glutamic acid	Glu	E	polar	negative	-3.5
Glutamine	Gln	Q	polar	neutral	-3.5
Glycine	Gly	G	nonpolar	neutral	-0.4
Histidine	His	H	polar	positive	-3.2
Isoleucine	Ile	I	nonpolar	neutral	4.5
Leucine	Leu	L	nonpolar	neutral	3.8
Lysine	Lys	K	polar	positive	-3.9
Methionine	Met	M	nonpolar	neutral	1.9
Phenylalanine	Phe	F	nonpolar	neutral	2.8
Proline	Pro	P	nonpolar	neutral	-1.6
Serine	Ser	S	polar	neutral	-0.8
Threonine	Thr	T	polar	neutral	-0.7
Tryptophan	Trp	W	nonpolar	neutral	-0.9
Tyrosine	Tyr	Y	polar	neutral	-1.3
Valine	Val	V	nonpolar	neutral	4.2

Ambiguous Amino Acids	**3-Letter**	**1-Letter**
Asparagine or aspartic acid	Asx	B
Glutamine or glutamic acid	Glx	Z
Leucine or Isoleucine	Xle	J
Unspecified or unknown amino acid	Xaa	X

As will be understood by those skilled in the art, a variety of algorithms are available that permit comparison of sequences in order to determine their degree of homology, including by permitting gaps of designated length in one sequence relative to another when considering which residues "correspond" to one another in different sequences. Calculation of the percent homology between two nucleic acid sequences, for example, can be performed by aligning the two sequences for optimal comparison purposes (*e.g.*, gaps can be introduced in one or both of a first and a second nucleic acid sequences for optimal alignment and non-corresponding sequences can be disregarded for comparison purposes). In certain embodiments, the length of a sequence aligned for comparison purposes is at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, or substantially 100% of the length of the reference sequence. The nucleotides at corresponding nucleotide positions are then compared. When a position in the first sequence is occupied by the same nucleotide as the corresponding position in the second sequence, then the molecules are identical at that position; when a position in the first sequence is occupied by a similar nucleotide as the corresponding position in the second sequence, then the molecules are similar at that position. The percent

homology between the two sequences is a function of the number of identical and similar positions shared by the sequences, taking into account the number of gaps, and the length of each gap, which needs to be introduced for optimal alignment of the two sequences. Representative algorithms and computer programs useful in determining the percent homology between two nucleotide sequences include, for example, the algorithm of Meyers and Miller (CABIOS, 1989, 4: 11-17), which has been incorporated into the ALIGN program (version 2.0) using a PAM120 weight residue table, a gap length penalty of 12 and a gap penalty of 4. The percent homology between two nucleotide sequences can, alternatively, be determined for example using the GAP program in the GCG software package using an NWSgapdna.CMP matrix.

[0084] *Human*: In some embodiments, a human is an embryo, a fetus, an infant, a child, a teenager, an adult, or a senior citizen.

[0085] *Hydrophilic:* As used herein, the term "hydrophilic" and/or "polar" refers to a tendency to mix with, or dissolve easily in, water.

[0086] *Hydrophobic:* As used herein, the term "hydrophobic" and/or "non-polar", refers to a tendency to repel, *not* combine with, or an inability to dissolve easily in, water.

[0087] *Identity:* As used herein, the term "identity" refers to the overall relatedness between polymeric molecules, *e.g.*, between nucleic acid molecules (*e.g.*, DNA molecules and/or RNA molecules) and/or between polypeptide molecules. In some embodiments, polymeric molecules are considered to be "substantially identical" to one another if their sequences are at least 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 99% identical. As will be understood by those skilled in the art, a variety of algorithms are available that permit comparison of sequences in order to determine their degree of homology, including by permitting gaps of designated length in one sequence relative to another when considering which residues "correspond" to one another in different sequences. Calculation of the percent identity between two nucleic acid sequences, for example, can be performed by aligning the two sequences for optimal comparison purposes (*e.g.*, gaps can be introduced in one or both of a first and a second nucleic acid sequences for optimal alignment and non-corresponding sequences can be disregarded for comparison purposes). In certain embodiments, the length of a sequence aligned for comparison purposes is at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, or substantially 100% of the length of the reference sequence. The nucleotides at corresponding nucleotide positions are then compared.

When a position in the first sequence is occupied by the same nucleotide as the corresponding position in the second sequence, then the molecules are identical at that position. The percent identity between the two sequences is a function of the number of identical positions shared by the sequences, taking into account the number of gaps, and the length of each gap, which needs to be introduced for optimal alignment of the two sequences. Representative algorithms and computer programs useful in determining the percent identity between two nucleotide sequences include, for example, the algorithm of Meyers and Miller (CABIOS, 1989, 4: 11-17), which has been incorporated into the ALIGN program (version 2.0) using a PAM120 weight residue table, a gap length penalty of 12 and a gap penalty of 4. The percent identity between two nucleotide sequences can, alternatively, be determined for example using the GAP program in the GCG software package using an NWSgapdna.CMP matrix.

[0088] *Infection:* As used herein, the term "infection" refers to the invasion of a host organism's body by a disease-causing organism that multiplies in the host. Symptoms of an infection may result from action of toxins produced by the disease-causing organism and/or be reaction of host tissues to the organisms and/or to toxins they produce.

[0089] *Isolated:* As used herein, the term "isolated" refers to a substance and/or entity that has been (1) separated from at least some of the components with which it was associated when initially produced (whether in nature and/or in an experimental setting), and/or (2) produced, prepared, and/or manufactured by the hand of man. Isolated substances and/or entities may be separated from about 10%, about 20%, about 30%, about 40%, about 50%, about 60%, about 70%, about 80%, about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, about 99%, or more than about 99% of the other components with which they were initially associated. In some embodiments, isolated agents are about 80%, about 85%, about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, about 99%, or more than about 99% pure. As used herein, a substance is "pure" if it is substantially free of other components. In some embodiments, as will be understood by those skilled in the art, a substance may still be considered "isolated" or even "pure", after having been combined with certain other components such as, for example, one or more carriers or excipients (*e.g.*, buffer, solvent, water, *etc.*); in such embodiments, percent isolation or purity of the substance is calculated without including such carriers or excipients.

[0090] *Nanoparticle:* As used herein, the term "nanoparticle" refers to a particle having at least one dimension (*e.g.*, diameter) of less than 1000 nanometers. In some embodiments, a nanoparticle may have at least two dimensions of less than 1000 nanometers (nm). In some embodiments, a nanoparticle has at least two dimensions of less than 300 nm. In some embodiments, a nanoparticle has at least two dimensions of less than 100 nm. In some embodiments, one or more measuring techniques may be used to calculate mean size (*e.g.*, hydrodynamic diameter) of a nanoparticle or population of nanoparticles. For example, in some embodiments, for nanoparticles with sizes less than 600 nm size may be determined by dynamic light scattering with size being reported as z-average diameter calculated by a deconvolution program. In some embodiments, for particles with average sizes greater than 600 nm the average size may be determined from electron microscopy measurements of the particles where more than 200 particles are counted and the z-average diameter is reported. In some embodiments, a nanoparticle will have no dimension of more than 1000 nanometers.

[0091] *Nanoparticle composition:* As used herein, the term "nanoparticle composition" refers to a composition that contains at least one nanoparticle and at least one additional agent or ingredient. In some embodiments, a nanoparticle composition may be characterized by a particular distribution of particle sizes.

[0092] *Nucleic acid:* As used herein, the term "nucleic acid," in its broadest sense, refers to any compound and/or substance that is or can be incorporated into an oligonucleotide chain. In some embodiments, a nucleic acid is a compound and/or substance that is or can be incorporated into an oligonucleotide chain via a phosphodiester linkage. As will be clear from context, in some embodiments, "nucleic acid" refers to individual nucleic acid residues (*e.g.*, nucleotides and/or nucleosides); in some embodiments, "nucleic acid" refers to an oligonucleotide chain comprising individual nucleic acid residues. In some embodiments, a "nucleic acid" is or comprises RNA; in some embodiments, a "nucleic acid" is or comprises DNA. In some embodiments, a nucleic acid is, comprises, or consists of one or more natural nucleic acid residues. In some embodiments, a nucleic acid is, comprises, or consists of one or more nucleic acid analogs. In some embodiments, a nucleic acid analog differs from a nucleic acid in that it does not utilize a phosphodiester backbone. For example, in some embodiments, a nucleic acid is, comprises, or consists of one or more "peptide nucleic acids", which are known in the art and have peptide bonds instead of phosphodiester bonds in the backbone, are considered within the scope of the

present disclosure. Alternatively or additionally, in some embodiments, a nucleic acid has one or more phosphorothioate and/or 5'-N-phosphoramidite linkages rather than phosphodiester bonds. In some embodiments, a nucleic acid is, comprises, or consists of one or more natural nucleosides (*e.g.*, adenosine, thymidine, guanosine, cytidine, uridine, deoxyadenosine, deoxythymidine, deoxyguanosine, and deoxycytidine). In some embodiments, a nucleic acid is, comprises, or consists of one or more nucleoside analogs (*e.g.*, 2-aminoadenosine, 2-thiothymidine, inosine, pyrrolo-pyrimidine, 3-methyl adenosine, 5-methylcytidine, C-5 propynyl-cytidine, C-5 propynyl-uridine, 2-aminoadenosine, C5-bromouridine, C5-fluorouridine, C5-iodouridine, C5-propynyl-uridine, C5-propynyl-cytidine, C5-methylcytidine, 2-aminoadenosine, 7-deazaadenosine, 7-deazaguanosine, 8-oxoadenosine, 8-oxoguanosine, O(6)-methylguanine, 2-thiocytidine, methylated bases, intercalated bases, and combinations thereof). In some embodiments, a nucleic acid comprises one or more modified sugars (*e.g.*, 2'-fluororibose, ribose, 2'-deoxyribose, arabinose, and hexose) as compared with those in natural nucleic acids. In some embodiments, a nucleic acid has a nucleotide sequence that encodes a functional gene product such as an RNA or protein. In some embodiments, a nucleic acid includes one or more introns. In some embodiments, nucleic acids are prepared by one or more of isolation from a natural source, enzymatic synthesis by polymerization based on a complementary template (*in vivo* or *in vitro*), reproduction in a recombinant cell or system, and chemical synthesis. In some embodiments, a nucleic acid is at least 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, 500, 600, 700, 800, 900, 1000, 1500, 2000, 2500, 3000, 3500, 4000, 4500, 5000 or more residues long.

[0093] *Non-solvent:* As used herein the term "non-solvent" is used in reference to a particular substance and refers to a liquid system (which may be a single liquid or mixture of liquids) in which the substance is relatively insoluble. In some embodiments, a liquid system is considered to be a "non-solvent" with respect to a particular substance if the substance does not dissolve in the liquid at room temperature and under atmospheric conditions and/or without investment of mechanical, electrical, or other energy, for example, to a weight/volume percent above about 1, 0.5, or 0.1. In some embodiments, a liquid system is considered to be a "non-solvent" with respect to a particular substance if the substance aggregates in, coagulates in, or precipitates from the liquid, and/or cannot readily be maintained in solution in the liquid.

[0094] *Patient:* As used herein, the term "patient" or "subject" refers to a human or any non-human animal (*e.g.*, mouse, rat, rabbit, dog, cat, cattle, swine, sheep, horse or primate) to whom therapy is administered. In many embodiments, a patient is a human being. In some embodiments, a patient is a human presenting to a medical provider for diagnosis or treatment of a disease, disorder or condition. In some embodiments, a patient displays one or more symptoms or characteristics of a disease, disorder or condition. In some embodiments, a patient does not display any symptom or characteristic of a disease, disorder, or condition. In some embodiments, a patient is someone with one or more features characteristic of susceptibility to or risk of a disease, disorder, or condition.

[0095] *Payload:* As used herein, the term "payload" refers to an entity for delivery as described herein. In some embodiments, a payload may be or comprise a biologically active agent (*e.g.*, a therapeutically active agent). In some embodiments, a payload may be or comprise one or more carbohydrates, lipids, metals, nucleic acids, polypeptides, small molecules and/or combinations thereof. In some embodiments, a payload may be or comprise a complex agent (*e.g.*, may comprise a plurality of, and/or combination(s) of one or more different materials – *e.g.,* carbohydrates, lipids, nucleic acids, proteins, small molecules, *etc.*; *e.g.,* may be or comprise a mixture, a crude sample, a cellular extract, *etc*., and/or a combination or mixture of any with one or more other agents or substances).

[0096] *Pharmaceutically acceptable:* The term "pharmaceutically acceptable" as used herein, refers to agents that, within the scope of sound medical judgment, are suitable for use in contact with tissues of human beings and/or animals without excessive toxicity, irritation, allergic response, or other problem or complication, commensurate with a reasonable benefit/risk ratio.

[0097] *Polypeptide:* The term "polypeptide", as used herein, generally has its art-recognized meaning of a polymer of at least three amino acids. In some embodiments, the term is used to refer to specific functional classes of polypeptides, such as, for example, autoantigen polypeptides, nicotinic acetylcholine receptor polypeptides, alloantigen polypeptides, *etc.* For each such class, the present specification provides several examples of amino acid sequences of known exemplary polypeptides within the class; in some embodiments, such known polypeptides are reference polypeptides for the class. In such embodiments, the term "polypeptide" refers to any member of the class that shows significant sequence homology or identity with a relevant reference polypeptide. In many embodiments, such member also shares significant activity with

the reference polypeptide. For example, in some embodiments, a member polypeptide shows an overall degree of sequence homology or identity with a reference polypeptide that is at least about 30-40%, and is often greater than about 50%, 60%, 70%, 80%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more and/or includes at least one region (*i.e.*, a conserved region, often including a characteristic sequence element) that shows very high sequence identity, often greater than 90% or even 95%, 96%, 97%, 98%, or 99%. Such a conserved region usually encompasses at least 3-4 and often up to 20 or more amino acids; in some embodiments, a conserved region encompasses at least one stretch of at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15 or more contiguous amino acids.

[0098] *Protein:* As used herein, the term "protein" refers to a polypeptide (*i.e.*, a string of at least three amino acids linked to one another by peptide bonds). Proteins may include moieties other than amino acids (*e.g.*, may be glycoproteins, proteoglycans, *etc.*) and/or may be otherwise processed or modified. Those of ordinary skill in the art will appreciate that a "protein" can be a complete polypeptide chain as produced by a cell (with or without a signal sequence) or can be a characteristic portion thereof. Those of ordinary skill will appreciate that a protein can sometimes include more than one polypeptide chain, for example linked by one or more disulfide bonds or associated by other means. Polypeptides may contain L-amino acids, D-amino acids, or both and may contain any of a variety of amino acid modifications or analogs known in the art. Useful modifications include, *e.g.*, terminal acetylation, amidation, methylation, *etc.* In some embodiments, proteins may comprise natural amino acids, non-natural amino acids, synthetic amino acids, and combinations thereof. The term "peptide" is generally used to refer to a polypeptide having a length of less than about 100 amino acids, less than about 50 amino acids, less than 20 amino acids, or less than 10 amino acids. In some embodiments, proteins are antibodies, antibody fragments, biologically active portions thereof, and/or characteristic portions thereof.

[0099] *Precipitation*: As used herein, the term "precipitation" refers to the formation of a solid in a solution.

[0100] *Refractory:* As used herein, the term "refractory" refers to any subject that does not respond with an expected clinical efficacy following the administration of provided compositions as normally observed by practicing medical personnel.

[0101] *Sample:* As used herein, the term "sample" typically refers to an aliquot of material obtained or derived from a source of interest, as described herein. In some embodiments, a source of interest is a biological or environmental source. In some embodiments, a source of interest may be or comprise a cell or an organism, such as a microbe, a plant, or an animal (e.g., a human). In some embodiments, a source of interest is or comprises biological tissue or fluid. In some embodiments, a biological tissue or fluid may be or comprise amniotic fluid, aqueous humor, ascites, bile, bone marrow, blood, breast milk, cerebrospinal fluid, cerumen, chyle, chime, ejaculate, endolymph, exudate, feces, gastric acid, gastric juice, lymph, mucus, pericardial fluid, perilymph, peritoneal fluid, pleural fluid, pus, rheum, saliva, sebum, semen, serum, smegma, sputum, synovial fluid, sweat, tears, urine, vaginal secretions, vitreous humor, vomit, and/or combinations or component(s) thereof. In some embodiments, a biological fluid may be or comprise an intracellular fluid, an extracellular fluid, an intravascular fluid (blood plasma), an interstitial fluid, a lymphatic fluid, and/or a transcellular fluid. In some embodiments, a biological fluid may be or comprise a plant exudate. In some embodiments, a biological tissue or sample may be obtained, for example, by aspirate, biopsy (e.g., fine needle or tissue biopsy), swab (e.g., oral, nasal, skin, or vaginal swab), scraping, surgery, washing or lavage (e.g., brocheoalvealar, ductal, nasal, ocular, oral, uterine, vaginal, or other washing or lavage). In some embodiments, a biological sample is or comprises cells obtained from an individual. In some embodiments, a sample is a "primary sample" obtained directly from a source of interest by any appropriate means. In some embodiments, as will be clear from context, the term "sample" refers to a preparation that is obtained by processing (e.g., by removing one or more components of and/or by adding one or more agents to) a primary sample. For example, filtering using a semi-permeable membrane. Such a "processed sample" may comprise, for example nucleic acids or proteins extracted from a sample or obtained by subjecting a primary sample to one or more techniques such as amplification or reverse transcription of nucleic acid, isolation and/or purification of certain components, *etc.* In some embodiments, a sample may be a "crude" sample in that it has been subjected to relatively little processing and/or is complex in that it includes components of relatively varied chemical classes.

[0102] *Small molecule:* As used herein, the term "small molecule" has its art-understood meaning of being an organic compound that is typically is less than about 5 kilodaltons (kD) in size. In some embodiments, a small molecule is less than about 4 kD, 3 kD, about 2 kD, or about

1 kD. In some embodiments, a small molecule is less than about 800 daltons (D), about 600 D, about 500 D, about 400 D, about 300 D, about 200 D, or about 100 D. In some embodiments, a small molecule has a molecular weight that is less than about 2000 g/mol, less than about 1500 g/mol, less than about 1000 g/mol, less than about 800 g/mol, or less than about 500 g/mol. In some embodiments, small molecules are non-polymeric (e.g., are not polymers such as, for example, not nucleic acids, polypeptides, polysaccharides, etc).

[0103] *Stable:* The term "stable," when applied to compositions herein, means that the compositions maintain one or more aspects of their physical structure (*e.g.*, size range and/or distribution of particles) over a period of time. In some embodiments, a stable nanoparticle composition is one for which the average particle size, the maximum particle size, the range of particle sizes, and/or the distribution of particle sizes (*i.e.*, the percentage of particles above a designated size and/or outside a designated range of sizes) is maintained for a period of time under specified conditions. In some embodiments, a stable provided composition is one for which a biologically relevant activity is maintained for a period of time. In some embodiments, the period of time is at least about one hour; in some embodiments the period of time is about 5 hours, about 10 hours, about one (1) day, about one (1) week, about two (2) weeks, about one (1) month, about two (2) months, about three (3) months, about four (4) months, about five (5) months, about six (6) months, about eight (8) months, about ten (10) months, about twelve (12) months, about twenty-four (24) months, about thirty-six (36) months, or longer. In some embodiments, the period of time is within the range of about one (1) day to about twenty-four (24) months, about two (2) weeks to about twelve (12) months, about two (2) months to about five (5) months, *etc.* For example, if a population of nanoparticles is subjected to prolonged storage, temperature changes, and/or pH changes, and a majority of the nanoparticles in the composition maintain a diameter within a stated range, the nanoparticle composition is stable. In some embodiments, a stable composition is stable at ambient conditions. In some embodiments, a stable composition is stable under biologic conditions (*i.e.*, 37° C in phosphate buffered saline).

[0104] *Subject:* As used herein, the term "subject" refers to a human or any non-human animal (*e.g.*, mouse, rat, rabbit, dog, cat, cattle, swine, sheep, horse or primate). A human includes pre and post-natal forms. In many embodiments, a subject is a human being. A subject can be a patient, which refers to a human presenting to a medical provider for diagnosis or treatment of a

disease. A subject can be afflicted with or is susceptible to a disease or disorder but may or may not display symptoms of the disease or disorder.

[0105] *Sublingual:* As used herein, the term "sublingual" refers to the route of administration where a substance is placed in the oral cavity (*e.g.*, sublingual (*e.g.*, buccal mucosal space)) to be absorbed through the oral mucosa. In some embodiments, sublingual administration may be or comprise buccal mucosal administration.

[0106] *Substantially:* As used herein, the term "substantially" refers to the qualitative condition of exhibiting total or near-total extent or degree of a characteristic or property of interest. One of ordinary skill in the biological arts will understand that biological and chemical phenomena rarely, if ever, go to completion and/or proceed to completeness or achieve or avoid an absolute result. The term "substantially" is therefore used herein to capture the potential lack of completeness inherent in many biological and chemical phenomena.

[0107] *Suffering from:* An individual who is "suffering from" a disease, disorder, or condition has been diagnosed with and/or exhibits or has exhibited one or more symptoms or characteristics of the disease, disorder, or condition.

[0108] *Susceptible to:* An individual who is "susceptible to" a disease, disorder, or condition is at risk for developing the disease, disorder, or condition. In some embodiments, an individual who is susceptible to a disease, disorder, or condition does not display any symptoms of the disease, disorder, or condition. In some embodiments, an individual who is susceptible to a disease, disorder, or condition has not been diagnosed with the disease, disorder, and/or condition. In some embodiments, an individual who is susceptible to a disease, disorder, or condition is an individual who has been exposed to conditions associated with development of the disease, disorder, or condition. In some embodiments, a risk of developing a disease, disorder, and/or condition is a population-based risk (*e.g.*, family members of individuals suffering from allergy, *etc.*).

[0109] *Symptoms are reduced*: According to the present disclosure, "symptoms are reduced" when one or more symptoms of a particular disease, disorder or condition is reduced in magnitude (*e.g.*, intensity, severity, *etc.*) and/or frequency. For purposes of clarity, a delay in the onset of a particular symptom is considered one form of reducing the frequency of that symptom.

[0110] *Therapeutic agent:* As used herein, the phrase "therapeutic agent" refers to any agent that has a therapeutic effect and/or elicits a desired biological and/or pharmacological effect,

when administered to a subject. In some embodiments, an agent is considered to be a therapeutic agent if its administration to a relevant population is statistically correlated with a desired or beneficial therapeutic outcome in the population, whether or not a particular subject to whom the agent is administered experiences the desired or beneficial therapeutic outcome.

[0111] *Treatment:* As used herein, the term "treatment" (also "treat" or "treating") refers to any administration of a substance that partially or completely alleviates, ameliorates, relives, inhibits, delays onset of, reduces severity of, and/or reduces frequency, incidence or severity of one or more symptoms, features, and/or causes of a particular disease, disorder, and/or condition. Such treatment may be of a subject who does not exhibit signs of the relevant disease, disorder and/or condition and/or of a subject who exhibits only early signs of the disease, disorder, and/or condition. Alternatively or additionally, such treatment may be of a subject who exhibits one or more established signs of the relevant disease, disorder and/or condition. In some embodiments, treatment may be of a subject who has been diagnosed as suffering from the relevant disease, disorder, and/or condition. In some embodiments, treatment may be of a subject known to have one or more susceptibility factors that are statistically correlated with increased risk of development of the relevant disease, disorder, and/or condition.

[0112] *Uniform*: The term "uniform," when used herein in reference to a nanoparticle composition, refers to a nanoparticle composition in which individual nanoparticles have at least one dimension (*e.g.*, dimension of nanoparticle's cross-section, *e.g.*, diameter) within a specified range. For example, in some embodiments, a uniform nanoparticle composition is one in which the difference between the minimum dimension of the smallest nanoparticle and maximum dimension of the biggest nanoparticle. In some embodiments, a uniform nanoparticle composition contains nanoparticles with at least one dimension (*e.g.*, diameter) within the range of about 100 nm to about 300 nm. In some embodiments, a uniform nanoparticle composition contains nanoparticles with a mean particle size that is under about 500 nm. In some embodiments, a uniform nanoparticle composition contains nanoparticles with a mean particle size that is within a range of about 100 nm to about 500 nm. In some embodiments, a uniform nanoparticle composition is one in which a majority of the particles within the composition have at least one dimension below a specified size or within a specified range. In some embodiments, the majority is more than 50%, 60%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, 99.5%, 99.6%, 99.7%, 99.8%, 99.9% or more of the particles in the composition. In some

embodiments, a mean dimension or mean cross-section of nanoparticles is measured by dynamic light scattering (DLS), for example based on the scattering intensity distribution measured by photon correlation spectroscopy.

BRIEF DESCRIPTION OF THE DRAWING

[0113] The Drawing, which is comprised of at least the following Figures, is for illustration purposes only, not for limitation.

[0114] FIG. 1A is a schematic showing the structure of an exemplary nanoparticle.

[0115] FIG. 1B is a schematic showing an exemplary process to produce nanoparticles containing a polymer and payload(s).

[0116] FIG. 1C is an exemplary image of the fluid bilayer produced by some exemplary methods.

[0117] FIG. 1D is a graph of the size distribution of nanoparticles.

[0118] FIG. 1E is an exemplary image of nanoparticles.

[0119] FIG. 1F is a graph of TLR expression.

[0120] FIGs. 2A- 2C demonstrates activation of basophils from peanut allergic individuals. **2A**, Representative dose-response curve of basophil activation obtained with one of the study subject samples. Basophil activation is shown as percentage of CD63+ basophils in response to increasing concentration of the peanut extract protein. The blue dotted line is unmasked peanut extract (PNE) compared with encapsulated peanut PNs (PN+PNE) (green) and empty NPs (Empty NP) (dotted green). The concentration of the empty NPs used for the stimulation was calculated based on the ratio of PNE to the nanoparticle's material in the PN+PNE formulation. "0" on the X-axis corresponds to the negative (no stimulant) control condition. **2B**, The percentage of the CD63+ basophils when stimulated with formyl-methionyl-leucylphenylalanine (fMLP, gray Bar) or anti-human IgE (red Bar) as positive controls for basophil activation. 2c, EC50 values (Y-axis, logarithmic scale) obtained with the 7 study samples stimulated with PNE (blue bars) or with PN+PNE (green bars).

[0121] FIGs. 3A-3N show comparison of T cell activation (proliferation and cytokine production) induced by bone marrow derived dendritic cells (BMDC), pulsed with OVA PLG NPs and unencapsulated OVA in vitro. Bone marrow derived FLT3L dendritic cells (BMDCs) were pulsed with OVA PLG NPs including incorporated adjuvants (NP+OVA+adjuvant),

unencapsulated OVA alone (OVA), and no antigen control (Ctr) at various doses of antigen for 1 hour. The adjuvant includes both E. coli lipid extract that coats the NPs and sheared DNA embedded within the NPs. CD4 (OT2) and CD8 (OT1) T cells derived from spleens of OT 2 and OT1 transgenic mice and were labelled with CFSE. The BMDCs and T cells were cocultured for 3 days, and the proliferative response of the CD4 (OT2) and CD8 (OT1) T cells were analyzed 3 days later. **3b-3d**, Bar graphs show CD4T cell proliferation (%CFSE CD4 T cells) in response to BMDCs, pulsed with (100, 10, 1 μg/mL OVA or no OVA). **3a**, 3D Summary of CD4 T cell proliferation shown in 3**b-3d**. 3**e-3g**, CD8 T cell proliferation (%CFSE CD8 T cells) shown in bar graphs as described above. **3h**. 3D summary of CD8 T cell proliferation shown in **e-g**. Data are representative of three independent experiments. 3i-3l, CD4 T cell cytokines production (IFNγ, IL-10, IL17, IL4). 3**m-3n**, CD8 T cell cytokines production (IFNγ, and granzyme B (Gzb)). 3**h**, 3D summary of CD4 and CD8 T cell cytokine production. BMDC were pulsed with NP+OVA+adjuvant, NP+adjuvant, unencapsulated OVA along with adjuvants (OVA&adjuvant), and unencapsulated OVA alone (OVA). Data are representative of three independent experiments. P-values were calculated by an unpaired t test.

[0122] FIGs. 4a-4b shows the proliferative response of CD4 (OT2) and CD8 (OT1) T cells to OVA nanoparticles in vivo. **4a**, Isolated CD8 (OT1) and CD4 (OT2) T cells were adoptively transferred into WT mice. 18 hours later the mice were immunized (oral gavage) with Enano+OVA+adjuvant or Enano+adjuvant. The proliferation of T cells from mesenteric lymph nodes was measured at day 3 in 4 independent experiments. **4a**, 3D summary of the 4 experiments shown in **4b**. P-values were calculated by an unpaired t test.

[0123] FIGs. 5a-5b show T cell responses to OVA NP are OVA specific. BMDCs pulsed with either NP+OVA+adjuvant or NP+adjuvant were tested for their ability to induce CD4 (OT2) and CD8 (OT1) T cell proliferation. The NP+adjuvant pulsed BMDCs were pulsed with an equivalent amount of adjuvant as used in the NP+OVA+adjuvant group. **5b**. The proliferation of CD4 (OT2) and CD8 (OT1) T cells was analyzed 3 days later (bar graphs). **5a**, 3D summary of data shown in **5b**. Data are representative of three independent experiments. P-values were calculated by an unpaired t test.

[0124] FIG. 6 is a schematic showing an exemplary process to produce a nanoparticle.

[0125] FIG. 7 is a representative scanning electron microscope (SEM) image of an exemplary protein loaded nanoparticle sample (e.g., NP-PN1). Distance between white bars in the bottom right of the figure correspond to 2 μm.

[0126] FIG. 8 shows comparison of total protein content in μg/mL of exemplary protein loaded nanoparticle batches 1-11 between Bicinchoninic Acid (BCA) assay measurements made in Laboratory 1 (left bar for each batch) and Laboratory 2 right bar for each batch). Batch 1 contained exemplary protein loaded nanoparticles at a concentration of 0.125 mg/mL, Batch 2 contained exemplary protein loaded nanoparticles at a concentration of 0.5 mg/mL, and batches 3-11 contained exemplary protein loaded nanoparticles at a concentration of 2.0 mg/mL.

[0127] FIG. 9 comparison of free protein content in μg/mL of exemplary protein loaded nanoparticle batches 1-11 between BCA assay measurements made in Laboratory 1 (left bar for each batch) and Laboratory 2 right bar for each batch). Batch 1 contained exemplary protein loaded nanoparticles at a concentration of 0.125 mg/mL, Batch 2 contained exemplary protein loaded nanoparticles at a concentration of 0.5 mg/mL, and batches 3-11 contained exemplary protein loaded nanoparticles at a concentration of 2.0 mg/mL.

[0128] FIG. 10 shows total protein content of exemplary protein loaded nanoparticle batches 12-20 obtained from a BCA assay. Region delineated with dashed lines represents a target total protein concentration between 1800 and 3500 μg/mL.

[0129] FIG. 11 shows free protein content of exemplary protein loaded nanoparticle batches 12-20 obtained from a BCA assay.

[0130] FIG. 12 shows safety factor calculated for exemplary protein loaded nanoparticle batches 12-20 from total protein content and free protein content obtained from a BCA assay. A target safety factor of 10 or greater, as delineated with a dashed line, is desired.

[0131] FIG. 13 shows Z-average diameter of exemplary protein loaded nanoparticles from batches 12-20. Z-average diameter was measured with a dynamic light scattering (DLS) instrument. Region delineated with dashed lines represents a target z-average diameter between 225 and 450 nm.

[0132] FIG. 14 shows polydispersity index (PDI) of exemplary protein loaded nanoparticles from batches 12-20. PDI was calculated based on repeated diameter measurements obtained with a DLS instrument. Region delineated with dashed lines represents a target PDI between 0.1 and 0.4.

[0133] FIG. 15 shows Z-average diameter of exemplary protein loaded nanoparticles from batches 1-11. Batch 1 contained exemplary protein loaded nanoparticles at a concentration of 0.125 mg/mL, Batch 2 contained exemplary protein loaded nanoparticles at a concentration of 0.5 mg/mL, and batches 3-11 contained exemplary protein loaded nanoparticles at a concentration of 2.0 mg/mL. Z-average diameter was measured with a DLS instrument.

[0134] FIG. 16 shows poly(lactic-co-glycolic) acid (PLGA) concentration in exemplary protein loaded nanoparticles from batches 1-11. Batch 1 contained exemplary protein loaded nanoparticles at a concentration of 0.125 mg/mL, Batch 2 contained exemplary protein loaded nanoparticles at a concentration of 0.5 mg/mL, and batches 3-11 contained exemplary protein loaded nanoparticles at a concentration of 2.0 mg/mL. PLGA concentration was measured via mass spectrometry.

[0135] FIG. 17 shows percent measured free protein content relative to measured total protein content of exemplary protein loaded nanoparticles from batches 1-11. Batch 1 contained exemplary protein loaded nanoparticles at a concentration of 0.125 mg/mL, Batch 2 contained exemplary protein loaded nanoparticles at a concentration of 0.5 mg/mL, and batches 3-11 contained exemplary protein loaded nanoparticles at a concentration of 2.0 mg/mL. Shaded region (e.g., 0-20%) shows a range of desired values. Free protein content and total protein content were measured with a BCA assay.

[0136] FIG. 18 shows percent measured total protein content relative to a target total protein content of exemplary protein loaded nanoparticles from batches 1-11. Batch 1 contained exemplary protein loaded nanoparticles at a concentration of 0.125 mg/mL, Batch 2 contained exemplary protein loaded nanoparticles at a concentration of 0.5 mg/mL, and batches 3-11 contained exemplary protein loaded nanoparticles at a concentration of 2.0 mg/mL. Shaded region (e.g., 70-130%) shows a range of desired values. Total protein content was measured with a BCA assay.

[0137] FIG. 19 shows in vitro protein release from exemplary protein loaded nanoparticles from batches 1-10 at three timepoints at 0 hours, 8 hours, and 24 hours after beginning of experiment. Batch 1 contained exemplary protein loaded nanoparticles at a concentration of 0.125 mg/mL, Batch 2 contained exemplary protein loaded nanoparticles at a concentration of 0.5 mg/mL, and batches 3-10 contained exemplary protein loaded nanoparticles at a concentration of 2.0 mg/mL.

Each timepoint measurement is represented with a black circle, and dashed line connects each timepoint with a subsequent timepoint. In vitro protein release was measured with a BCA assay.

[0138] FIG. 20 shows measured concentration on E. Coli DNA in exemplary protein loaded nanoparticles from batches 1-11. Batch 1 contained exemplary protein loaded nanoparticles at a concentration of 0.125 mg/mL, Batch 2 contained exemplary protein loaded nanoparticles at a concentration of 0.5 mg/mL, and batches 3-11 contained exemplary protein loaded nanoparticles at a concentration of 2.0 mg/mL. For each batch, a left bar represents total E. Coli DNA, and a right bar represents free E. Coli DNA. E. Coli DNA concentration was measured via polymerase chain reaction (PCR).

DETAILED DESCRIPTION OF CERTAIN EMBODIMENTS

[0139] The following description is for illustration and exemplification of the present disclosure only and is not intended to limit the present disclosure to the specific embodiments described herein. Unless defined otherwise, technical and scientific terms have the same meaning as commonly understood by one of ordinary skill in the art to which this disclosure belongs. All references cited herein, including patent applications and publications, are incorporated by reference in their entirety.

[0140] In accordance with one or more embodiments, the present disclosure provides technologies for preparation of certain particle compositions, particularly including particles comprised of a polymer (*e.g.,* PLG). Provided technologies achieve production of desirable particle compositions, including, for example, compositions characterized by one or more of particular particle size (*e.g.,* average size, size range, size distribution, *etc.)*, zeta potential, payload, ratio of polymer:payload, concentration of unit of payload per unit of polymer, safety factor, outer surface decoration (*e.g.,* type of decoration, density of decoration, complexity of decoration, *etc.*), and/or immunological properties, *etc.* Moreover, provided technologies are particularly useful for preparation of nanoparticle compositions in which nanoparticles comprise one or more payloads and/or coating agents.

[0141] In some embodiments, certain aspects of provided technologies make them particularly useful and/or effective for production of nanoparticle compositions comprising fragile payload(s). Alternatively or additionally, in some embodiments, certain aspects of provided technologies make them particularly useful and/or effective for production of nanoparticle

compositions comprising complex payload(s) and/or complex coating agent(s). Polymer nanoparticle systems, and particularly PLG nanoparticle systems, have proven to be highly useful in the delivery of a variety of payload agents, including various therapeutic payloads. *See*, for example review article by Danheir *et al., J Controlled Release* 161, available online February 4, 2012. Danaheir *et al.* list among the properties for which PLG nanoparticles are celebrated "(i) biodegradability and biocompatibility, (ii) FDA and European Medicine Agency approval in drug delivery systems for parenteral administration, (iii) well described formulations and methods of production adapted to various types of drugs *e.g.*, hydrophilic or hydrophobic small molecules or macromolecules, (iv) protection of drug from degradation, (v) possibility of sustained release, (vi) possibility to modify surface properties to provide stealthiness and/or better interaction with biological materials and (vii) possibility to target nanoparticles to specific organs or cells". *See* Abstract of Danheir *et al., J Controlled Release* 161, available online February 4, 2012.

[0142] In some embodiments, the present disclosure provides technologies that may offer an enhanced synthesis process, and/or consistent product quality as compared with other nanoparticle preparations (*e.g.,* prepared by other technologies and/or including other component(s) and/or not sharing one or more characteristics as described herein). In some embodiments, disclosed preparations may offer different or unique properties that, for example, may address previously unmet requirements associated with production yield (*e.g.*, amount of waste), and/or fragile/complex material. In some embodiments, provided preparations are characterized by more stable formations (*e.g.*, can be stored longer), and/or other attributes relative to a standard preparation (*e.g.*, using emulsions), as described herein.

Components of Nanoparticle Compositions

[0143] In many embodiments, nanoparticle compositions to which the present disclosure relates are formed from:

a) A polymer component;

b) A payload component;

c) An optional coating component; and

d) One or more optional additional components.

[0144] In some embodiments, a payload component is incorporated into, or otherwise combined with, a polymer component so that the payload component is protected from one or more aspects of an external environment. For example, in some embodiments, a payload component is protected from degrading or otherwise damaging aspect(s) of an external environment (e.g., enzymes, temperature, immune system components, etc.)

[0145] In some embodiments, a payload is incorporated into, or otherwise combined with, a polymer component, and/or is otherwise incorporated into nanoparticles of the preparation (e.g., which may be coated), so that, when such nanoparticles are administered, e.g., orally, to a subject, such payload component is protected from exposure, for example to such subject's immune system (e.g., so that such subject does not experience a systemic allergic reaction to such payload component).

[0146] In some embodiments of nanoparticle composition(s) to which the present disclosure relates, one or more payload components is/are homogeneously or substantially homogenously distributed in a polymer matrix.

[0147] In some embodiments, provided nanoparticle compositions are useful, for example, in desensitizing a subject to a payload component.

Polymer component

[0148] In some embodiments, a polymer component of nanoparticles to which the present disclosure relates is or comprises a homopolymer, a diblock polymer, a triblock polymer, a multiblock copolymer, a linear polymer, a dendritic polymer, a branched polymer, a random block, *etc.*, or combinations thereof. In some embodiments, nanoparticles are comprised of a blend and/or mixture of polymers.

[0149] In some embodiments, nanoparticles are comprised of one or more biocompatible polymers and/or one or more biodegradable polymers. In some embodiments, nanoparticles are comprised of one or more synthetic polymers, or derivatives thereof. In some embodiments, nanoparticles are comprised of one or more natural polymers, or derivatives thereof. In some embodiments, nanoparticles are comprised of combinations of synthetic and natural polymers, or derivatives thereof.

[0150] In some embodiments, nanoparticles are comprised of one or more polymers selected from the group consisting of poly(hydroxy acids) such as poly(lactic acid), poly(glycolic acid),

poly(lactic acid-co-glycolic acid), poly(lactic-co-glycolic acid), and derivatives of poly(lactic-co-glycolic acid), PEGylated poly(lactic-co-glycolic acid), poly(lactide), poly(glycolide), poly(lactide-co-glycolide), poly(anhydrides), PEGylated poly(anhydrides), poly (ortho esters), derivatives of poly(ortho esters), PEGylated poly(ortho esters), poly(caprolactones), derivatives of poly(caprolactone), PEGylated poly(caprolactones), polyamines (*e.g.*, spermine, spermidine, polylysine, and derivatives thereof), PEGylated polylysine, polyamides, polycarbonates, poly(propylene fumarates), polyamides, polyphosphazenes, polyamino acids, polyethers, polyacetals, polylactides, polyhydroxyalkanoates, polyglycolides, polyketals, polyesteramides, poly(dioxanones), polyhydroxybutyrates, polyhydroxyvalyrates, polycarbonates, polyorthocarbonates, poly(vinyl pyrrolidone), polycyanoacrylates, polyalkylene oxalates, polyalkylene succinates, poly(malic acid), poly(methyl vinyl ether), poly(ethylene imine), poly(acrylic acid), poly(maleic anhydride), poly(ethylene imine), derivatives of poly(ethylene imine), PEGylated poly(ethylene imine), poly(acrylic acid), derivatives of poly(acrylic acid), PEGylated poly(acrylic acid), poly(urethane), PEGylated poly(urethane), derivatives of poly(urethane), poly(lactide), poly(glycolide), poly(hydroxy acids), polyesters, poly(acrylates), polyalkylenes such as polyethylene and polypropylene, polyalkylene glycols such as poly(ethylene glycol), polyalkylene oxides such as poly(ethylene oxide), polyalkylene terepthalates such as poly(ethylene terephthalate), polyvinyl alcohols, polyvinyl ethers, polyvinyl esters, polyvinyl halides such as poly(vinyl chloride), polyvinylpyrrolidone, polysiloxanes, poly(vinyl alcohols), poly(vinyl acetate), polystyrene, polyurethanes and co-polymers thereof, derivativized celluloses such as alkyl cellulose, hydroxyalkyl celluloses, cellulose ethers, cellulose esters, nitro celluloses, methyl cellulose, ethyl cellulose, hydroxypropyl cellulose, hydroxy-propyl methyl cellulose, hydroxybutyl methyl cellulose, cellulose acetate, cellulose propionate, cellulose acetate butyrate, cellulose acetate phthalate, carboxylethyl cellulose, cellulose triacetate, and cellulose sulfate sodium salt (jointly referred to herein as "synthetic celluloses"), polymers of acrylic acid, methacrylic acid or copolymers or derivatives thereof including esters, poly(methyl methacrylate), poly(ethyl methacrylate), poly(butylmethacrylate), poly(isobutyl methacrylate), poly(hexylmethacrylate), poly(isodecyl methacrylate), poly(lauryl methacrylate), poly(phenyl methacrylate), poly(methyl acrylate), poly(isopropyl acrylate), poly(isobutyl acrylate), and poly(octadecyl acrylate) (jointly referred to herein as "polyacrylic

acids"), poly(butyric acid), poly(valeric acid), and poly(lactide-co-caprolactone) and/or derivatives thereof.

[0151] In some embodiments, nanoparticles are comprised of one or more natural polymers. Exemplary natural polymers include, but are not limited to, proteins (such as albumin, collagen, gelatin), prolamines (for example, zein), carbohydrates (*e.g.*, polysaccharides)s (such as alginate), cellulose derivatives (such as hydroxypropyl cellulose, sodium carboxymethyl cellulose, ethyl cellulose and cellulose acetate), polyhydroxyalkanoates (for example, polyhydroxybutyrate), and/or combinations thereof. In some embodiments, a natural polymer may comprise or consist of chitosan.

[0152] In some embodiments, nanoparticles are comprised of one or more polymers such as poly(lactide-co-glycolide) copolymerized with polyethylene glycol (PEG). Without wishing to be held to a particular theory, it is proposed that arrangement of a nanoparticle so that PEG is exposed on the external surface, may increase stability of the nanoparticle in blood, perhaps at least in part due to the hydrophilicity of PEG.

[0153] In many embodiments, a polymer component comprises or consists of poly(lactic-co-glycolic acid)("PLGA" or "PLG").

[0154] In some embodiments, the present disclosure encompasses the recognition that viscosity of polymer preparation may impact its usefulness in producing nanoparticles as described herein. As those skilled in the art are aware, viscosity of a polymer solution is a function of the molecular weight of the polymer and operating temperature. In some embodiments, a polymer with a high molecular weight requires high operation temperature to have low enough viscosity to be processed as described herein.

Payload components

[0155] In some embodiments, provided nanoparticles and/or nanoparticle compositions include and/or deliver at least one payload (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more). In some embodiments, a payload may be or comprise a polypeptide agent. In some embodiments, a composition may deliver such payload by including a nucleic acid that encodes a polypeptide, where in the nucleic acid is expressed upon/after administration. In some embodiments, a payload may be or comprise a particular form of an agent or entity – *e.g.,* a glycosylated or phosphorylated form or a truncated form or a complexed form or an otherwise modified form. In

some embodiments, a composition may deliver such payload if it comprises the precursor (e.g., unmodified, uncomplexed etc) form, and conversion to the particular form occurs upon/after administration of the composition.

[0156] In some embodiments, a payload may be or comprise an agent or entity that elicits a particular biological response when delivered to an appropriate subject. Alternatively or additionally, in some embodiments, a payload may be or comprise an agent or entity that modulates a particular biological response to another, different, agent or entity. In some embodiments, a payload may be, comprise, and/or deliver (*e.g.,* encode) an agent or entity with respect to which a particular biological response is desired.

[0157] In some embodiments, a biological response elicited by or desired with respect to a particular payload may be or comprise an immune response. In some embodiments, a payload that modifies a biological response is or comprises an antigen to which an immune response in generated. In some embodiments, a payload that modifies a biological response is or comprises an immune adjuvant. In some embodiments, presence of an immune adjuvant may modify (*e.g.*, amplify, bias, or alter) an immune response to another entity (*e.g.*, to an antigen).

[0158] One feature of certain embodiments of the present disclosure is that it permits delivery of an antigen to a subject in a context that both (a) minimizes exposure of the antigen to immune system component(s) that might induce or mediate an undesirable reaction or response to the antigen while (b) achieving its exposure to immune system component(s) that might induce or mediate a beneficial response. For instance, in some embodiments, an antigen may be or comprise an allergic antigen and provided systems may minimize its exposure during delivery to mast cells, basophil cells, IgE and/or other immune system components that might mediate an anaphylactic response (and might be present, for example, in blood), while permitting its exposure to immune components (*e.g.*, Th1 and/or Treg cells) that might mediate an allergy-suppressing (*e.g.*, Th1 or Treg) response.

[0159] In some embodiments, a payload comprises one or more carbohydrates, lipids, metals, nucleic acids, polypeptides, small molecules and/or combinations thereof.

[0160] Those skilled in the art will appreciate that a variety of technologies may be utilized to provide payload(s) useful in accordance with the present disclosure. For example, in some embodiments, a payload that is or comprises a polypeptide may be produced recombinantly (*e.g.*, by expressing DNA encoding all or part of the polypeptide antigen in an appropriate expression

system. In some such embodiments, DNA may be in the form of vector DNA such as plasmid DNA. Alternatively or additionally, in some embodiments, a payload that is or comprises a polypeptide may be prepared by isolation from another source (e.g., a natural source).

[0161] In some embodiments, a payload may be provided in combination with another substance. In some embodiments, a payload may be provided as a complex mixture (*e.g.*, including different classes of compounds – *e.g.*, both polypeptides and nucleic acids, *etc.*) (*e.g.*, protein, carbohydrate, lipid and/or nucleic acid mixtures, which in some embodiments may represent or include one or more crude samples, cellular extracts, *etc.*). In some embodiments, nucleic acids (and in some embodiments, particularly RNA, which in some embodiments may specifically be or comprise longer RNAs such as mRNAs), polypeptides, and/or saccharides (which may, in some embodiments, be incorporated into other entities, such as glyopeptides) may be considered to be "fragile" payloads.

[0162] Indeed, one feature of certain embodiments of the present disclosure is that it permits utilization of relatively complex payloads, specifically including payloads that are or comprise relatively crude preparations (*e.g.,* only modestly processed samples). In some embodiments, a payload may be or comprise a crude preparation and/or other complex material (*e.g.*, an extract, *etc.*).

[0163] In some embodiments, provided nanoparticles comprise microbial and/or cellular components (*e.g.*, that are or comprise a microbial or other cellular extract). Without wishing to be bound by a particular theory, some embodiments of the present disclosure including one or more microbial cellular component(s) may permit development and/or production of useful immunomodulatory nanoparticle compositions at least in part because they utilize various evolved attributes of microbial cells relating to their ability to modulate or evade human or animal immune reactions.

[0164] The present disclosure also provides an insight that combining such evolved attributes with various features of certain nanoparticle systems such as, for example, ability to sequester antigens and/or cellular hydrophilic components from immune system elements, tunable degradation rates and/or locations, and/or modular association with targeting, immune adjuvant, or other surface entities, permits development and/or production of particularly useful immunomodulatory compositions.

[0165] In some embodiments, provided nanoparticles comprise microbial or other cellular extracts – *e.g.*, hydrophilic or hydrophobic extracts of cells (e.g., microbial) for use in or with nanoparticle compositions. In some embodiments, such microbial extracts may contain a collection of microbial components that share a chemical feature, so that they associate with other included components and not with excluded components during production of the extract. In some embodiments, extracts may contain at least some cellular components at relative levels comparable to those at which they are present in the cells. Those skilled in the art will be aware of a variety of techniques available to determine presence and/or level of particular components, and to compare such determined level(s) with those observed in intact cells. Moreover, those of ordinary skill in the art will readily appreciate reasonable and expected experimental variation and therefore will be able to determine whether components are present in absolute or relative levels or concentrations in an extract that are reasonably comparable to those at which they are present in cells.

[0166] In many embodiments, cellular (e.g., microbial) extracts are prepared from cell (e.g., microbial cell) preparations such as cell cultures. Those skilled in the art will appreciate that, in some embodiments, cell preparations (e.g., cell cultures) may be prepared by culturing microbial cells for a period of time and under conditions sufficient to achieve cell growth to a desirable level (*e.g.*, optical density, concentration, colony size, total protein, total DNA, and colony forming units). In some embodiments, cell (e.g., microbial cell) preparations contain intact cells, and optionally are substantially free of lysed cells. In some embodiments, microbial cell preparations contain lysed cells, and optionally are substantially free of intact cells.

[0167] In some embodiments, the present disclosure provides and/or utilizes (e.g., as payload components) hydrophilic cell (e.g., microbial cell) extracts, for example extracts prepared by contacting a preparation with a hydrophilic solvent so that hydrophilic cellular components partition into solution in the hydrophilic solvent. A hydrophilic solvent can then be separated from non-solubilized components which may, for example, be precipitated, solubilized in a hydrophobic solvent (optionally not miscible with the hydrophilic solvent), or otherwise separable from the hydrophilic solvent. In some embodiments, hydrophilic cellular components that partition into a hydrophilic solvent include, for example, components that are miscible and/or soluble in such solvent.

[0168] In some embodiments, provided nanoparticle compositions include a payload selected from the group consisting of polypeptides, nucleic acids, carbohydrates (*e.g.*, polysaccharides), and combinations thereof. In many embodiments, a payload is or comprises a polypeptide. In many embodiments, a payload is or comprises a nucleic acid; in some such embodiments, a payload is or comprises a long nucleic acid (e.g., a gene therapy vector, an mRNA, etc); in some embodiments, a payload is or comprises a partly or wholly single stranded nucleic acid).

[0169] In some embodiments, a payload is or comprises a RNA. In some embodiments, and RNA payload is an mRNA. In some embodiments, an RNA payload has a length within a range of about 15 to about 3,000,000 residues. In some embodiments, an RNA payload has a length within a range of about 500 to 50000 residues. In some embodiments, an RNA payload has a length within a range of about 1000 to about 10000 residues. In some embodiments, an RNA payload is an mRNA encoding a polypeptide having a length within a range of about 50 to about 5000 amino acids; in some embodiments, such encoded polypeptide has a length within a range of about 100 to about 3000 amino acids; in some embodiments, such encoded polypeptide has a length within a range of about 200 to about 1500 amino acids.

[0170] In some embodiments, a provided nanoparticle preparation is manufactured using one or more relatively complex components (e.g., a payload that is a relatively crude extract or combination of components).

[0171] In some embodiments, a payload is or comprises one or more antigens. In some embodiments, an antigen is an allergic antigen, an infectious antigen, and/or a disease-associated (e.g., a cancer-associated) antigen.

[0172] In some embodiments, a provided nanoparticle composition includes one or more payloads on (e.g., attached) nanoparticle surface(s).

Fragile payloads

[0173] In some embodiments, a payload may be fragile (*e.g.*, susceptible to damage from energy input (*e.g.*, increased temperature, pressure, applied shear force, *etc.*)) or other operation of an element of an environment to which the payload is or might be exposed (*e.g.,* but for its incorporation within a nanoparticle composition as described herein). In some embodiments, a fragile payload may be decomposed or (partly or fully) inactivated when exposed to energy input or certain environmental conditions (*e.g.*, high temperature, high or low pH, high pressure, high

shear force, high ionic strength, *etc.*). In some embodiments, one or more biological or pharmaceutical activities of a fragile payload may be decreased when exposed to energy input or such environmental condition(s) (*e.g.*, high temperature, high or low pH, high pressure, high shear force, high ionic strength, *etc.*).

[0174] In some embodiments, a fragile payload may be or comprise a polypeptide, a nucleic acid, or a combination thereof. In some embodiments, a fragile payload may be or comprise a DNA, a RNA, or a combination thereof. In some embodiments, a fragile payload comprises anti-micro RNA, antisense RNA (asRNA), circular RNA (circRNA), enhancer RNA (eRNA), long non-coding RNA (lncRNA), messenger RNA (mRNA), micro RNA (miRNA), Piwi-interacting RNA (piRNA), ribosomal RNA (rRNA), small interfering RNA (siRNA), small nuclear RNA (snRNA), small nucleolar RNA (snoRNAs), small rDNA-derived RNA (srRNA), transfer RNA (tRNA), tRNA-derived small RNA (tsRNA), or a combination thereof.

[0175] In some embodiments, a fragile payload comprises a mRNA encoding a polypeptide, *e.g.*, a polypeptide that elicits (or is intended to elicit) a particular immune response (*i.e.*, that is an immunologically relevant polypeptide).

[0176] In some embodiments, a fragile payload is or comprises a gene therapy vector. In some embodiments a gene therapy vector is a viral vector. In some embodiments, a viral vector is an adenoviral vector, an adenoviral associated viral (AAV) vector; or a lentiviral vector. In some embodiments, a gene therapy vector encodes a therapeutic agent. In some embodiments, a gene therapy vector encodes an immunologically relevant polypeptide. In some embodiments, a gene therapy vector targets antigen presenting cells (APCs). In some embodiments an immunologically relevant polypeptide encoded by a gene therapy vector is presented on an APC.

[0177] In some embodiments, an immunologically relevant polypeptide is one with respect to which a particular immune response (*e.g.*, a protective immune response such a Th1-type immune response, a tolerized immune response, *etc.*) is elicited or desired.

[0178] To give but a few examples, in some embodiments, an immunologically relevant polypeptide may be or comprise at least one (and, in many embodiments, a plurality) of epitopes associated with or characteristic of a pathogen or disease state. In some embodiments, such epitope(s) may be or comprise tumor-associated and/or tumor-specific epitope(s). Alternatively or additionally, in some embodiments, such epitope(s) may be or comprise epitope(s) of an infectious agent (*e.g.*, a microbe or virus). In some embodiments, immunologically relevant

polypeptide may be or comprise one or more infectious agent antigens, cancer antigens, alloantigens, or other antigens as described hereinbelow.

[0179] In some embodiments, a fragile payload comprises an RNA (*e.g.,* an mRNA or other RNA that encodes a polypeptide) having 200 to 100,000 residues, 200 to 50,000 residues, 200 to 10,000 residues, 500 to 100,000 residues, 500 to 50,000 residues, or 500 to 10,000 residues in length. In some embodiments, a fragile payload comprises an RNA (*e.g.,* an mRNA or other RNA that encodes a polypeptide) having 200 to 100,000 residues, 200 to 50,000 residues, 200 to 10,000 residues, 500 to 100,000 residues, 500 to 50,000 residues, or 500 to 10,000 residues length.

[0180] In some embodiments, a payload is or comprises (or otherwise delivers, *e.g.*, by expression of a payload construct) an siRNA. In some embodiments, an siRNA targets a disease-associated gene. To give but a few examples, an siRNA may target genes selected from the group consisting of Eg5/KSP, PCSK9, Serpina1, TTR, VEGF, XBP-1, and combinations thereof.

[0181] In some embodiments, a fragile payload comprises a RNA pre-complexed with chaperone proteins.

[0182] In some embodiments, a nanoparticle composition comprising an RNA payload further comprises one or more RNAse inhibitors.

[0183] In some embodiments, a fragile payload is or comprises one or more carbohydrates or carbohydrate structures (e.g., a glycosylated polypeptide), or a construct that delivers such carbohydrate or an agent (e.g., a polypeptide) including such carbohydrate structure (e.g., a glycosylated polypeptide, which may be delivered, for example, by administering a nucleic acid encoding the polypeptide to a system that will glycosylate it).

Antigens

[0184] In some embodiments, a payload is or comprises an antigen, or a construct that delivers (*e.g.,* encodes) an antigen. In some embodiments, an antigen may be or comprise a polypeptide (*e.g.*, a peptide, a protein, a glycoprotein, *etc.*), a carbohydrate (*e.g.*, polysaccharide), a lipid (*e.g.*, glycolipid) a nucleic acid, or combinations thereof.

[0185] In some embodiments, an antigen may be obtained from (or otherwise found in) a source such as, for example, a microbe (*e.g.*, a bacterium, fungus, protozoan, *etc.*), a virus, an organism

(*e.g.*, a plant, fish, mammal, reptile, *etc.*), or a cell or tissue thereof. In some embodiments, an antigen may be obtained from (or otherwise found in) a cell in culture (*e.g.*, a cancer cell, a cell of a graft to be transplanted, *etc.*). In some embodiments, an antigen may be or comprise whole cells and/or one or more intact cellular structures (*e.g.*, cell walls, organelles, and/or portions thereof).

[0186] Those skilled in the art, reading the present disclosure, will appreciate that, in some embodiments, antigen payload component(s), like other payload components may be utilized in a pure form and in other embodiments may be or comprise one or more crude (*e.g.*, unpurified or substantially unpurified) antigenic extracts. In some embodiments, crude extract can be a useful and inexpensive alternative to using individual antigens in provided nanoparticle compositions.

[0187] In some embodiments, suitable antigens are known in the art and are available from commercial, government, scientific or other sources. In some embodiments, antigens are provided as or obtained from whole, inactivated or attenuated organisms.

[0188] One of skill in the art will recognize that, in certain embodiments, multiple antigens may be delivered by nanoparticles simultaneously and/or sequentially in accordance with the present disclosure. Indeed, one feature of certain embodiments described herein is that they surprisingly achieve incorporation of multiple antigens (and/or other components) in nanoparticle compositions; in some such embodiments, some or all such antigens are provided together, e.g., as part of an extract, e.g., a relatively crude extract, from an appropriate source (e.g., an allergen or other antigen source).

[0189] In some embodiments, provided nanoparticle compositions incorporate multiple antigens (e.g., multiple antigen polypeptides) from a single source. In some embodiments, provided nanoparticle compositions incorporate multiple epitopes of a single antigen, and/or different epitopes of different antigens, in some embodiments as part of one or more natural antigens (e.g., one or more antigen proteins) and in some embodiments as part of one or more engineered antigen(s) (e.g., a polypeptide that may be engineered to includes epitopes from one than one antigen and/or to link together two or more epitopes from a single antigen but in a different arrangement than that which they have in the antigen.

[0190] Alternatively or additionally, in some embodiments, provided nanoparticle compositions incorporate one or more antigens together with one or more other agents (e.g., cytokines or adjuvants or other immune modulators or biologically active agents). For example, including as

exemplified herein, in some embodiments, provided nanoparticle compositions include (e.g., enfold and/or are coated with) one or more immunomodulatory agents such as, for example, one or more adjuvants, one or more interleukins, one or more TLR receptor agonists, etc.

[0191] In some embodiments, a particular provided composition may contain a combination of antigens. For example, in some embodiments, a particular provided composition may contain a combination of antigens (*e.g.*, at least two antigens) associated with a particular disease, disorder or condition (*e.g.*, with a particular cancer, a particular infectious disease, a particular graft v host or host v graft syndrome, *etc.*).

[0192] Those of skill in the art will recognize a wide variety of potential applications utilizing combinations of antigens; each of these is contemplated as within the scope of the present disclosure.

[0193] In some embodiments, a payload component is or comprises an antigen selected from the group consisting of an allergen, an infectious antigen, a disease-associated antigen (*e.g.*, a cancer antigen), an autoantigen, or combinations thereof.

a. Allergens

[0194] In some embodiments, an antigen is or comprises an allergen. In some embodiments, an allergen may be or comprise an environmental allergen. For example, in some embodiments, an environmental antigen may be or comprise one or more pollen allergens (grass-, tree-, and weed-pollen allergens), insect allergens (inhalant, saliva and venom allergens), animal hair and/or dander allergens.

[0195] In some embodiments, an allergen for use in accordance with the present disclosure may, for example, be an allergen found in certain foods, venom, drugs or rubber that elicits an allergic response; in some embodiments, an allergen is one that elicits an anaphylactic allergic response.

[0196] Those skilled in the art will be aware of various allergens that may induce anaphylaxis, including, for example, various allergens found in food (*e.g.,* egg, meat, milk, peanut, tree nuts, wheat), insect venom (*e.g.*, bees, reptiles), drugs, and latex.

[0197] In some embodiments, an allergen may be found in one or more venoms. Stings from organisms that inject venoms, such as insect stings are known to cause anaphylaxis in individuals with allergies to the venom. In some embodiments, an allergen may be from venom allergens including such originating from stinging or biting insects such as those from the taxonomic order

of Hymenoptera including and ants (superfamily Formicoidae; *e.g*, fire ants, velvet ants), bees (superfamily Apidae; *e.g.*, honey bees), and wasps (superfamily Vespidea; *e.g*, hornets, wasps, yellow jackets). For example, venom from honeybees of the genus *Apis* can cause anaphylaxis in stung victims who are allergic (Weber *et al. Allergy* 42:464-470). The venom from honeybees contains numerous compounds which have been extensively studied and characterized (*see* for a reference, Banks and Shipolini. *Chemistry and Pharmacology of Honey-bee Venom.* Chapter 7 of Venoms of the Hymenoptera. Ed. T. Piek. Academic Press. London. 1986). The two main components of bee venom are phospholipase A2 and melittin and may be used in some embodiments for treating and preventing allergies to bee venom.

[0198] Non-limiting examples of allergens found in food include proteins found in dairy products (*e.g.*, egg, milk), fish (*e.g.*, cod, salmon, tuna), fruit (*e.g.*, nectarines, peaches, plums; *Ann Allergy Asthma Immunol* 7(6):504-8 (1996); cherries, *Allergy* 51(10):756-7 (1996)), legume (*e.g.,* lentil, lupine, pea, peanut, soy), nuts (*e.g.*, almond, Brazil nut, cashew, hazelnut, macadamia, peanut, pecan, pine nut, pistachio, walnut), seafood (*e.g.*, clams, crab, lobster, shrimp), seeds (*e.g.,* mustard, poppy, sesame, sunflower), and Alpha-gal (galactose-a-1,3-galactose) found in certain meats (*e.g.,* beef, lamb, port, rabbit, venison, *etc.*) can also trigger allergic reactions in certain individuals.

[0199] In some embodiments an allergen may be in meat. In some embodiments an allergen may be in meat from mammals. In some embodiments an allergen may be a protein found in meat. In some embodiments an allergen may be a sugar group found in meat In some embodiments, an allergen may be a sugar group on a protein found in meat. In some embodiments, a sugar group that is an allergen comprises a hexose. In some embodiments, a sugar group that is an allergen comprises galactose. In some embodiments, a sugar group that is an allergen is a disaccharide. In some embodiments, a sugar group that is an allergen is galactose-alpha-1,3-galactose.

[0200] In some embodiments, an allergen is found in pollen-related food allergies (*e.g.*, birch pollen related to apple allergies) may be utilized in the practice of the present invention. In some embodiments, an allergen is a pollen allergens from grasses, herbs, and trees such as pollens of the taxonomic orders of Asterales (*e.g.*, *Ambrosia* and *Artemisia*), Cupressales (*e.g.*, cedar (*Cryptomeria* and *Juniperus*), Fagales (*e.g.*, alder (*Alnus*), birch (*Betula*), hazel (*Corylus*), and hornbeam (*Carpinus*)), Lamiales (*e.g.*, olive (*Olea*)), Pinales, Poales (*e.g.*, grasses (*Cynodon*,

Dactylis, *Holcus Lolium*, *Phalaris*, *Phleum*, *Poa*, *Secale,* and *Sorghum*), Proteales (*e.g.*, Platanaccae, Plane tree (*Platanus*)), and Urticales (*e.g.*, *Parietaria*).

[0201] In some embodiments, an allergen may be from cockroaches, fleas (*e.g.*, *Blatella*, *Periplaneta*, *Chironomus and Ctenocepphalides*), house dust mites (*e.g.*, *Dermatophagoides* and *Euroglyphus*), midges, and storage mite (*e.g.*, *Lepidoglyphys*, *Glycyphagus*, and *Tyrophagus*).

[0202] In some embodiments, an allergen may be from mammals such as cat, dog and horse. In some embodiments, an allergen may be from birds.

[0203] Still other allergens that may be used include inhalation allergens from fungi such as from the genera *Alternaria* and *Cladosporium.*

[0204] In some embodiments, it may be desirable to work in systems in which a single compound (*e.g.*, a single protein) is responsible for an observed allergy. In some embodiments, an antigen may comprise more complex allergens and/or crude allergenic extracts. Therefore, collections of more than one antigen may be used so that immune responses to multiple antigens may be modulated with a single embodiment.

[0205] In some embodiments, provided nanoparticles and/or nanoparticle compositions may include one or more allergens listed in **Table 4**. Exemplary crude extracts include, but are not limited to, to extracts derived from the Allergen Source listed in **Table 4**.

Table 4. Exemplary Allergic Antigens

ALLERGEN SOURCE	SYSTEMATIC AND ORIGINAL NAMES	MW kDa	SEQ	ACCESSION NO.	PMID (CITATION)
WEED POLLENS					
Asterales					
Ambrosia artemisiifolia (short ragweed)	Amb a 1; antigen E			P27759	1702434
				P27760	1809687
				P27761	1809687
				P28744	1809687
	Amb a 2; Amb a II; antigen K; AgK			P27762	1809687
	Amb a 3; Amb a III; Ra3			P00304	7459340
	Amb a 5; Allergen Amb a V; Ra5			P02878	1390654
	Amb a 6; Amb a VI; Allergen Ra6			O04004	6863927
				AAB51146	9714407
	Amb a 8			Q64LH1	15237444
	Major allergen			AAA32670	1809687

ALLERGEN SOURCE	SYSTEMATIC AND ORIGINAL NAMES	MW KDA	SEQ	ACCESSION NO.	PMID (CITATION)
Ambrosia trifida (giant ragweed)	Amb t 5; Ra5G	4.4	C	P10414	1711499 3862954 1606135
Artemisia vulgaris (mugwort)	Art v 1	27-29	C	Q84ZX5	12475905 14510717
	Art v 2	35	P	Q7M1G9	1703533 2233755
	Art v 3			P0C088	10998016
Helianthus annuus (sunflower)	Hel a 1	34	-		7920032
	Hel a 2; profilin	15.7	C	O81982	9798651
				CAA75506	9798651
				Y15210	9798651
Mercurialis annua	Mer a 1; profilin	14-15	C	O49894	9525453
				Y13271	n/a
GRASS POLLENS					
Poales					
Cynodon dactylon (Bermuda grass)	Cyn d 1	32	C	O04701	8757211
				AAB50734	8757211
	Cyn d 7		C	P94092	9037188 9363908
				X91256	9037188
	Cyn d 12; profilin	14	C	O04725	9420135
	Profilin			Y08390	9420135
	Profilin 1			CAA69670	9420135
	Profilin 2			CAA69669	9420135
	Cyn d 15			AAP80171	n/a
Dactylis glomerata (orchard grass)	Dac g 2			Q41183	1526648
	Dac g II			2103117A	8116860
	Dac g 3			P93124	8811075
	Dac g 4			P82946	8977507
Holcus lanatus (velvet grass)	Hol l 1		C	P43216	9215246 8768803
Lolium perenne (rye grass)	Lol p 1			P14946	1697854 2001733 3718469
	Lol p 2-A			P14947	2472390
	Lol p 3; Lol p III			P14948	2605214
	Lol p 4			CAH92637	16198308

ALLERGEN SOURCE	SYSTEMATIC AND ORIGINAL NAMES	MW KDA	SEQ	ACCESSION NO.	PMID (CITATION)
	Lol p 5a			Q40240	1671715
	Lol p 5b			Q40237	8262382
	Lol p 11			Q7M1X5	7751518
Phalaris aquatica (canary grass)	Pha a 1		C	Q41260	8564724 7687099
	Pha a 5			P56166	8564724
Phleum pratense (timothy grass)	Phl pI			CAA81613	7751520
	Phl p 4			CAD54670	16198308
	Phl p6			CAA76556	n/a
	Phl p11			AAN32987	12220472
Phleum pratense (timothy grass)	Phl p 1 Phl p 2 Phl p 4 Phl p 5; Ag25 Phl p 6 Phl p 12; profilin Phl p 13; polygalacturonase	27 32 55-60	C C P C C C C	X78813 41,X75925 41A 42 43,Z27082 44,X77583 AJ238848	
Poa pratensis (Kentucky bluegrass)	Poa p 1; group I Poa p 5	33 31/34	P C	46 34,47	
Sorghum halepense (Johnson grass)	Sor h 1		C	48	
TREE POLLENS					
Fagales					
Alnus glutinosa (alder)	Aln g 1	17	C	S50892	
Betula verrucosa (birch)	Bet v 1 Bet v 2; profilin Bet v 3 Bet v 4 Bet v 5; isoflavone reductase homologue Bet v 7; cyclophilin	17 15 8 33.5 18	C C C C C C	see list of isoallergens M65179 X79267 X87153/S54819 AF135127 P P81531	
Carpinus betulus (hornbeam)	Car b 1	17	C	51	
Castanea sativa (chestnut)	Cas s 1; Bet v 1 homologue Cas s5; chitinase	22	P	52	
Corylus avelana (hazel)	Cor a 1	17	C	53	

ALLERGEN SOURCE	SYSTEMATIC AND ORIGINAL NAMES	MW KDA	SEQ	ACCESSION NO.	PMID (CITATION)
Quercus alba (white oak)	Que a 1	17	P	54	
Cryptomeria japonica (sugi)	Cry j 1 Cry j 2	41-45	C C	55,56 57,D29772	
Juniperus ashei (mountain cedar)	Jun a 1 Jun a 3	43 30	P P	P81294 P81295	
Juniperus oxycedrus (prickly juniper)	Jun o 2; calmodulin-like	29	C	AF031471	
Juniperus sabinoides (mountain cedar)	Jun s 1	50	P	58	
Juniperus virginiana (eastern red cedar)	Jun v 1	43	P	P81825	
Oleales					
Fraxinus excelsior (ash)	Fra e 1	20	P	58A	
Ligustrum vulgare (privet)	Lig v 1	20	P	58A	
Olea europea (olive)	Ole e 1; Ole e 2; profilin Ole e 3; Ole e 4; Ole e 5; superoxide dismutase Ole e 6;	16 15-18 8 9.2 32 16 10	C C P P C	59,60 60A 60B P80741 P80740 U86342	
Syringa vulgaris (lilac)	Syr v 1	20	P	58A	
MITES					
Acarus siro (mite)	Aca s 13; fatty acid-bind.prot.	14*	C	AJ006774	
Blomia tropicalis (mite)	Blo t 5; Blo t 12; Bt11a Blo t 13; Bt6 fatty acid-binding prot		C C C	U59102 U27479 U58106	
Dermatophagoides pteronyssinus (mite)	Der p 1; antigen P1 Der p 2; Der p 3; trypsin Der p 4; amylase Der p 5; Der p 6; chymotrypsin	25 14 28/31 0 60 14	C C C C P C	61 62 63 64 65 66	

ALLERGEN SOURCE	SYSTEMATIC AND ORIGINAL NAMES	MW KDA	SEQ	ACCESSION NO.	PMID (CITATION)
	Der p 7; Der p 8; glutathione transferase Der p 9; collagenolytic serine prot. Der p 10; tropomyosin Der p 14; apolipophorin like p	25 22-2 8 36	C P C C	67 67A 67B Y14906 Epton p.c.	
Dermatophagoides microceras (mite)	Der m 1;	25	P	68	
Dermatophagoides farinae (mite)	Der f 1; Der f 2; Der f 3; Der f 10; tropomyosin Der f 11; paramyosin Der f 14; Mag3, apolipophorin	25 14 30 98 	C C C C C C	69 70,71 63 72 72a D17686	
Euroglyphus maynei (mite)	Eur m 14; apolipophorin	177	C	AF149827	
Lepidoglyphus destructor (storage mite)	Lep d 2.0101; Lep d 2.0102;	15 15	C C	73,74,75 75	
ANIMALS					
Bos (e.g., B.taurus, B. domesticus, etc.) (domestic cattle) (see also foods)	Bos d 2; Ag3,lipocalin Bos d 4; alpha-lactalbumin Bos d 5; beta-lactoglobulin Bos d 6; serum albumin Bos d 7; immunoglobulin Bos d 8; caseins	20 14.2 18.3 67 160 20-30	C C C C 	76,L42867 M18780 X14712 M73993 77 77	
Canis familiaris Canis domesticus (dog)	Can f 1; Can f 2; Can f ?; albumin	25 27 	C C C	78,79 78,79 S72946	
Equus caballus (domestic horse)	Equ c 1; lipocalin Equ c 2; lipocali	25 18.5	C P	U70823 79A,79B	
Felis domesticus (cat)	Fel d 1; cat-1	38	C	15	
Mus musculus (mouse)	Mus m 1; MUP	19	C	80,81	
Rattus norvegius (rat)	Rat n 1	17	C	82,83	
FUNGI					
Ascomycota					
Dothidiales					

ALLERGEN SOURCE	SYSTEMATIC AND ORIGINAL NAMES	MW KDA	SEQ	ACCESSION NO.	PMID (CITATION)
Alternaria alternata	Alt a 1; Alt a 2; Alt a 3; heat shock protein Alt a 6; ribosomal protein Alt a 7; YCP4 protein Alt a 10; aldehyde dehydrogenase Alt a 11; enolase Alt a 12;acid.ribosomal prot P1	28 25 70 11 22 53 45 11	C C C C C C C C	U82633 U87807,U87808 X78222, U87806 X78225 X78227, P42041 U82437 X84216	
Cladosporium herbarum	Cla h 1; Cla h 2; Cla h 3; aldehyde dehydrogenase Cla h 4; ribosomal protein Cla h 5; YCP4 protein Cla h 6; enolase Cla h 12;acid.ribosomal prot P1	13 23 53 11 22 46 11	 C C C C C	83a,83b 83a,83b X78228 X78223 X78224 X78226 X85180	
Eurotiales					
	Asp fl 13; alkaline serine proteinase	34		84	
Aspergillus fumigatus	Asp f 1; Asp f 2; Asp f 3; peroxisomal protein Asp f 4; Asp f 5; metalloprotease Asp f 6; Mn superoxide dismutase Asp f 7; Asp f 8; ribosomal protein P2 Asp f 9; Asp f 10; aspartic protease Asp f 11; peptidyl-prolyl isom Asp f 12; heat shock prot. P70 Asp f 13; alkaline serine proteinase Asp f 15; Asp f 16; Asp f 17; Asp f 18; vacuolar serine Asp f ?; Asp f ?;	18 37 19 30 42 26.5 12 11 34 34 24 65 34 16 43 34 90 55	C C C C C C C C C C C C C P P	83781,S39 330 U56938 U20722 AJ001732 Z30424 U53561 AJ223315 AJ224333 AJ223327 X85092 84a U92465 84b AJ002026 g3643813 AJ224865 84c 85 86	
Aspergillus niger	Asp n 14; beta-xylosidase Asp n 18; vacuolar serine	105 34	C C	AF108944 84b	

ALLERGEN SOURCE	SYSTEMATIC AND ORIGINAL NAMES	MW KDA	SEQ	ACCESSION NO.	PMID (CITATION)
	proteinase Asp n ?;	 85	 C	 Z84377	
Aspergillus oryzae	Asp o 2; TAKA-amylase A Asp o 13; alkaline serine proteinase	53 34	C C	D00434,M33218 X17561	
Penicillium brevicompactum	Pen b 13; alkaline serine Proteinase	33		86a	
Penicillium citrinum	Pen c 1; heat shock protein P70 Pen c 3; peroxisomal membrane protein Pen c 13; alkaline serine proteinase	70 33	C 	U64207 86b 86a	
Penicillium notatum	Pen n 1; N-acetyl glucosaminidase Pen n 13; alkaline serine proteinase Pen n 18; vacuolar serine proteinase	68 34 32	 	87 89 89	
Penicillium oxalicum	Pen o 18; vacuolar serine proteinase	34		89	
Onygenales					
Trichophyton rubrum	Tri r 2; Tri r 4; serine protease		C C	90 90	
Trichophyton tonsurans	Tri t 1; Tri t 4; serine protease	30 83	P C	91 90	
Saccharomycetales					
Candida albicans	Cand a 1	40	C	88	
Candida boidinii	Cand b 2	20	C	J04984,J04985	
Basidiomycota					
Basidiolelastomycetes					
Malassezia furfur	Mal f 1; Mal f 2; MF1 peroxisomal membrane protein Mal f 3; MF2 peroxisomal membrane protein Mal f 4, Mal f 5; Mal f 6; cyclophilin homologue	 21 20 35 18* 17*	 C C C C C	91a AB011804 AB011805 Takesako, p.c. AJ011955 AJ011956	
Basidiomycetes					
Psilocybe cubensis	Psi c 1; Psi c 2; cyclophilin	16		91b	
Coprinus comatus	Cop c 1;	11	C	AJ132235	

ALLERGEN SOURCE	SYSTEMATIC AND ORIGINAL NAMES	MW KDA	SEQ	ACCESSION NO.	PMID (CITATION)
(shaggy cap)	Cop c 2; Cop c 3; Cop c 5; Cop c 7;			Brander, p.c. Brander, p.c. Brander, p.c.	
INSECTS					
Aedes aegyptii (Yellow fever mosquito)	Aed a 1; apyrase Aed a 2;	68 37	C C	L12389 M33157	
Apis mellifera (honeybee)	Api m 1; phospholipase A2 Api m 2; hyaluronidase Api m 4; melittin Api m 6;	16 44 3 7-8	C C C P	92 93 94 Kettner,p.c.	
Bombus pennsylvanicus (bumblebee)	Bom p 1; phospholipase Bom p 4; protease	16	P P	95 95	
Blattella germanica (German cockroach)	Bla g 1; Bd90k Bla g 2; aspartic protease Bla g 4; calycin Bla g 5; glutathione transf. Bla g 6; troponin C	 36 21 22 27	C C C C C	96 97 98 98	
Periplaneta americana (American cockroach)	Per a 1; Cr-PII Per a 3; Cr-PI Per a 7; tropomyosin	72-78 37	C C C	98A Y14854	
Chironomus thummi (midges)	Chi t 1-9; hemoglobin Chi t 1.01; component III Chi t 1.02; component IV Chi t 2.0101; component I Chi t 2.0102; component IA Chi t 3; component II-beta Chi t 4; component IIIA Chi t 5; component VI Chi t 6.01; component VIIA Chi t 6.02; component IX Chi t 7; component VIIB Chi t 8; component VIII Chi t 9; component X	16 16 16 16 16 16 16 16 16 16 16 16 16 16	C C C C C C C C C C C C C C	99 P02229 P02230 P02221 P02221 P02222 P02231 P02224 P02226 P02223 P02225 P02227 P02228	

ALLERGEN SOURCE	SYSTEMATIC AND ORIGINAL NAMES	MW KDA	SEQ	ACCESSION NO.	PMID (CITATION)
Dolichovespula maculata (white face hornet)	Dol m 1; phospholipase A1 Dol m 2; hyaluronidase Dol m 5; antigen 5	35 44 23	C C C	100 101 102,103	
Dolichovespula arenaria (yellow hornet)	Dol a 5; antigen 5	23	C	104	
Polistes annularies (wasp)	Pol a 1; phospholipase A1 Pol a 2; hyaluronidase Pol a 5; antigen 5	35 44 23	P P C	105 105 104	
Polistes dominulus (Mediterranean paper wasp)	Pol d 1; Pol d 4; serine protease Pol d 5;	32-34	C	DR Hoffman DR Hoffman P81656	
Polistes exclamans (wasp)	Pol e 1; phospholipase A1 Pol e 5; antigen 5	34 23	P C	107 104	
Polistes fuscatus (wasp)	Pol f 5; antigen 5	23	C	106	
Polistes metricus (wasp)	Pol m 5; antigen 5	23	P	106	
Vespa crabo (European hornet)	Vesp c 1; phospholipase Vesp c 5.0101; antigen 5 Vesp c 5.0102; antigen 5	34 23 23	P C C	107 106 106	
Vespa mandarina (giant asian hornet)	Vesp m 1.01; Vesp m 1.02; Vesp m 5;			DR Hoffman DR Hoffman P81657	
Vespula flavopilosa (yellowjacket)	Ves f 5; antigen 5	23	C	106	
Vespula germanica (yellowjacket)	Ves g 5; antigen 5	23	C	106	
Vespula maculifrons (yellowjacket)	Ves m 1; phospholipase A1 Ves m 2; hyaluronidase Ves m 5; antigen 5	33.5 44 23	C P 23	108 109 104	
Vespula pennsylvanica (yellowjacket)	Ves p 5; antigen 5	23	C	106	
Vespula squamosa	Ves s 5; antigen 5	23	C	106	

ALLERGEN SOURCE	SYSTEMATIC AND ORIGINAL NAMES	MW KDA	SEQ	ACCESSION NO.	PMID (CITATION)
(yellowjacket)					
Vespula vidua (wasp)	Ves vi 5;	23	C	106	
Vespula vulgaris (yellowjacket)	Ves v 1; phopholipase A1 Ves v 2; hyaluronidase Ves v 5; antigen 5	35 44 23	C P C	105A 105A 104	
Myrmecia pilosula (Australian jumper ant)	Myr p 1, Myr p 2;		C C	X70256 S81785	
Solenopsis geminata (tropical fire ant)	Sol g 2; Sol g 4			DR Hoffman DR Hoffman	
Solenopsis invicta (fire ant)	Sol i 2; Sol i 3; Soli 4;	13 24 13	C C C	110,111 110 110	
Solenopsis saevissima (brazilian fire ant)	Sols 2;			DR Hoffman	
FOODS					
Gadus callarias (cod)	Gad c 1; allergen M	12	C	112,113	
Salmo salar (Atlantic salmon)	Sals 1; parvalbumin	12	C	X97824,X97825	
Bos (e.g., B.taurus, B. domesticus, etc.) (domestic cattle)	Bos d 4; alpha-lactalbumin Bos d 5; beta-lactoglobulin Bos d 6; serum albumin Bos d 7; immunoglobulin Bos d 8; caseins	14.2 18.3 67 160 20-30	C C C	M18780 X14712 M73993 77 77	
Gallus domesticus (chicken)	Gal d 1; ovomucoid Gald 2; ovalbumin Gald 3; conalbumin (Ag22) Gald 4; lysozyme Gal d 5; serum albumin	28 44 78 14 69	C C C C C	114,115 114,115 114,115 114,115 X60688	
Metapenaeus ensis (shrimp)	Met e 1; tropomyosin		C	U08008	
Penaeus aztecus (shrimp)	Pen a 1; tropomyosin	36	P	116	
Penaeus indicus (shrimp)	Pen i 1; tropomyosin	34	C	117	

ALLERGEN SOURCE	SYSTEMATIC AND ORIGINAL NAMES	MW KDA	SEQ	ACCESSION NO.	PMID (CITATION)
Todarodes pacificus (squid)	Tod p 1; tropomyosin	38	P	117A	
Haliotis midae (abalone)	Hal m 1	49	-	117B	
Apium graveolens (celery)	Api g 1; Bet v 1 homologue Api g 4; profilin Api g 5;	16* 55/58	C P	Z48967 AF129423 P81943	
Brassica juncea (oriental mustard)	Bra j 1; 2S albumin	14	C	118	
Brassica rapa (turnip)	Bra r 2; prohevein-like protein	25	?	P81729	
Hordeum vulgare (barley)	Hor v 1; BMAI-1	15	C	119	
Zea mays (maize, corn)	Zea m 14; lipid transfer prot.	9	P	P19656	
Corylus avellana (hazelnut)	Cor a 1.0401; Bet v 1 homologue	17	C	AF136945	
Malus domestica (apple)	Mal d 1; Bet v 1 homologue Mal d 3; lipid transfer protein	9	C C	X83672 Pastorello	
Pyrus communis (pear)	Pyr c 1; Bet v 1 homologue Pyr c 4; profilin Pyr c 5; isoflavone reductase homologue	18 14 33.5	C C C	AF05730 AF129424 AF071477	
Oryza sativa (rice)	Ory s 1;		C	U31771	
Persea americana (avocado)	Pers a 1; endochitinase	32	C	Z78202	
Prunus armeniaca (apricot)	Pru ar 1; Bet v 1 homologue Pru ar 3; lipid transfer protein	9	C P	U93165	
Prunus avium (sweet cherry)	Pru av 1; Bet v 1 homologue Pru av 2; thaumatin homologue Pru av 4; profilin	15	C C C	U66076 U32440 AF129425	
Prunus persica (peach)	Pru p 3;lipid transfer protein	10	P	P81402	
Sinapis alba (yellow mustard)	Sin a 1; 2S albumin	14	C	120	

ALLERGEN SOURCE	SYSTEMATIC AND ORIGINAL NAMES	MW KDA	SEQ	ACCESSION NO.	PMID (CITATION)
Glycine max (soybean)	Gly m 1.0101; HPS Gly m 1.0102; HPS Gly m 2 Gly m 3; profilin	7.5 7 8 14	P P P C	121 121 A57106 AJ223982	
Arachis hypogaea (peanut)	Ara h 1; vicilin Ara h 2; conglutin Ara h 3; glycinin Ara h 4; glycinin Ara h 5; profilin Ara h 6; conglutin homolog Ara h 7; conglutin homolog	63.5 17 14 37 15 15 15	C C C C C C C	L34402 L77197 AF093541 AF086821 AF059616 AF092846 AF091737	
Actinidia chinensis (kiwi)	Act c 1; cysteine protease	30	P	P00785	
Solanum tuberosum (potato)	Sol t 1; patatin	43	P	P15476	
Bertholletia excelsa (Brazil nut)	Ber e 1; 2S albumin	9	C	P04403,M17146	
Juglans regia (English walnut)	Jug r 1; 2S albumin Jug r 2; vicilin	44	C C	U66866 AF066055	
Ricinus communis (Castor bean)	Ric c 1; 2S albumin		C	P01089	
OTHERS					
Anisakis simplex (nematode)	Ani s 1 Ani s 2; paramyosin	24 97	P C	A59069 AF173004	
Ascaris suum (worm)	Asc s 1;	10	P	122	
Aedes aegyptii (Yellow fever mosquito)	Aed a 1; apyrase Aed a 2;	68 37	C C	L12389 M33157	
Hevea brasiliensis (rubber)	Hev b 1; elongation factor Hev b 2; (1,3-glucanase Hev b 2; (1,3-glucanase Hev b 3 Hev b 4; component of microhelix protein complex Hev b 5 Hev b 6.01 hevein precursor	58 58 34/36 24 100/110/115 16 20 5	P P C P P C C C C	123,124 123,124 125 126,127 128 U42640 M36986/p02877 M36986/p02877 M36986/p02877 U80598	

ALLERGEN SOURCE	SYSTEMATIC AND ORIGINAL NAMES	MW KDA	SEQ	ACCESSION NO.	PMID (CITATION)
	Hev b 6.02 hevein	14			
	Hev b 6.03 C-terminal		C	Y15042	
	fragment	46	C	AJ132580/	
	Hev b 7; patatin	14	C	AJ132581	
	homologue	51		AJ249148	
	Hev b 8; profilin		C		
	Hev b 9; enolase	26			
	Hev b 10; Mn-superoxide dismut				
Ctenocephalides felis (cat flea)	Cte f 1;	-	-	-	
	Cte f 2; M1b	27	C	AF231352	
Homo sapiens (human autoallergens)	Hom s 1;	73*	C	Y14314	
	Hom s 2;	10.3*	C	X80909	
	Hom s 3;	20.1*	C	X89985	
	Hom s 4;	36*	C	Y17711	
	Hom s 5;	42.6*	C	P02538	

[0206] The present disclosure encompasses the recognition that a particular subject may benefit from being exposed to a combination of antigens, such as multiple allergens. In some embodiments, it may be desirable to provide a nanoparticle composition comprising multiple antigens relevant to a specific subject, and/or to a population of subjects. For example, in some embodiments, a particular provided composition will contain a combination of allergens to address some or all of a particular subject's allergies and/or a combination of allergens to address some or all allergies commonly present within a population. For example, if a particular subject is allergic to peanuts and to dust mites, a nanoparticle composition may be designed and manufactured to address both allergies. Alternatively or additionally, in some embodiments it may be desirable to prepare nanoparticle compositions including antigens from a plurality of allergens (i) to which members of a particular community are commonly exposed (*e.g.*, by virtue of geographic location); (ii) to which subjects are exposed by a common route (*e.g.*, inhalation, injection, contact, *etc.*); (iii) to which incidence of allergy within a relevant population (*e.g.*, a geographic population, an age population, an ethnic population, *etc.*) is above a designated threshold; (iv) to which subjects allergic to one allergen also tend to have allergy to, for example, subjects allergic to tree nuts tend to also be allergic to pecans, walnuts, and pistachios, subjects

with allergy to crustaceans (*e.g.*, crab, crayfish, lobster, or shrimp) or mollusks (*e.g.*, clams, mussels, oysters, or scallops) tend to have allergy to various types, not just a single crustacean or mollusk.

[0207] In an effort to better exemplify some embodiments, an exemplary list of antigens and/or antigenic extracts (such as one or more allergens and/or allergenic extracts) that may be used in some embodiments include, but are not limited to, *Acarus siro* (mite) fatty acid-binding protein (Aca s 13); *Actinidia chinensis* (kiwi) cysteine protease (Act c 1); *Aedes aegyptii* (mosquito) antigen (Aed a 2); *Aedes aegyptii* (mosquito) antigen (Aed a 2); *Aedes aegyptii* (mosquito) apyrase (Aed a 1); *Aedes aegyptii* (mosquito) apyrase (Aed a 1); *Alnus glutinosa* (alder) antigen (Aln g 1); *Alternaria alternata* (fungus) acid. ribosomal protein P1 (Alt a 12); *Alternaria alternata* (fungus) aldehyde dehydrogenase (Alt a 10); *Alternaria alternata* (fungus) antigen (Alt a 1); *Alternaria alternata* (fungus) antigen (Alt a 2); *Alternaria alternata* (fungus) enloase (Alt a 11); *Alternaria alternata* (fungus) heat shock protein (Alt a 3); *Alternaria alternata* (fungus) ribosomal protein (Alt a 6); *Alternaria alternata* (fungus) YCP4 protein (Alt a 7); *Ambrosia artemisiifolia* (short ragweed) antigen E (Amb a 1); *Ambrosia artemisiifolia* (short ragweed) antigen K (Amb a 2); *Ambrosia artemisiifolia* (short ragweed) Ra3 antigen (Amb a 3); *Ambrosia artemisiifolia* (short ragweed) Ra5 antigen (Amb a 5); *Ambrosia artemisiifolia* (short ragweed) Ra6 antigen (Amb a 6); *Ambrosia artemisiifolia* (short ragweed) Ra7 antigen (Amb a 7); *Ambrosia trifida* (giant ragweed) Ra5G antigen (Amb t 5); *Anisakis simplex* (nematode) antigen (Ani s 1); *Anisakis simplex* (nematode) paramyosin (Ani s 2); *Apis mellifera* (honey bee) antigen (Api m 6); *Apis mellifera* (honey bee) hyaluronidase (Api m 2); *Apis mellifera* (honey bee) melittin (Api m 4); *Apis mellifera* (honey bee) phospholipase A2 (Api m 1); *Apium graveolens* (celery) antigen (Api g 5); *Apium graveolens* (celery) Bet v 1 homologue (Api g 1); *Apium graveolens* (celery) profilin (Api g 4); *Arachis hypogaea* (peanut) (conglutin Ar a h 2); *Arachis hypogaea* (peanut) (profilin Ar a h 5); *Arachis hypogaea* (peanut) conglutin homologue (Ar a h 6); *Arachis hypogaea* (peanut) conglutin homologue (Ar a h 7); *Arachis hypogaea* (peanut) glycinin (Ar a h 3); *Arachis hypogaea* (peanut) glycinin (Ar a h 4); *Arachis hypogaea* (peanut) vicilin (Ar a h 1); *Artemisia vulgaris* (mugwort) antigen (Art v 1); *Artemisia vulgaris* (mugwort) antigen (Art v 2); *Ascaris suum* (worm) antigen (Asc s 1); *Aspergillus flavus* (fungus) alkaline serine proteinase (Asp fl 13); *Aspergillus fumigatus* (fungus) alkaline serine proteinase (Asp f 13); *Aspergillus fumigatus* (fungus) antigen (Asp f 1); *Aspergillus fumigatus* (fungus) antigen

(Asp f 15); *Aspergillus fumigatus* (fungus) antigen (Asp f 16); *Aspergillus fumigatus* (fungus) antigen (Asp f 17); *Aspergillus fumigatus* (fungus) antigen (Asp f 2); *Aspergillus fumigatus* (fungus) antigen (Asp f 4); *Aspergillus fumigatus* (fungus) antigen (Asp f 7); *Aspergillus fumigatus* (fungus) antigen (Asp f 9); *Aspergillus fumigatus* (fungus) aspartis protease (Asp f 10); *Aspergillus fumigatus* (fungus) heat shock protein P70 (Asp f 12); *Aspergillus fumigatus* (fungus) metalloprotease (Asp f 5); *Aspergillus fumigatus* (fungus) Mn superoxide dismutase (Asp f 6); *Aspergillus fumigatus* (fungus) peptidyl-prolyl isomerase (Asp f 11); *Aspergillus fumigatus* (fungus) peroxisomal protein (Asp f 3); *Aspergillus fumigatus* (fungus) ribosomal protein P2 (Asp f 8); *Aspergillus fumigatus* (fungus) vacuolar serine (Asp f 18); *Aspergillus niger* (fungus) antigen (Asp n 18); *Aspergillus niger* (fungus) beta-xylosidase (Asp n 14); *Aspergillus niger* (fungus) vacuolar serine proteinase; *Aspergillus oryzae* (fungus) alkaline serine proteinase (Asp o 13); *Aspergillus oryzae* (fungus) TAKA-amylase A (Asp o 2); *Bertholletia excelsa* (Brazil nut) 2S albumin (Ber e 1); *Betula verrucosa* (birch) antigen (Bet v 1); *Betula verrucosa* (birch) antigen (Bet v 3); *Betula verrucosa* (birch) antigen (Bet v 4); *Betula verrucosa* (birch) cyclophilin (Bet v 7); *Betula verrucosa* (birch) isoflavone reductase homologue (Bet v 5); *Betula verrucosa* (birch) profilin (Bet v 2); *Blattella germanica* (German cockroach) aspartic protease (Bla g 2); *Blattella germanica* (German cockroach) Bd90k (Bla g 1); *Blattella germanica* (German cockroach) calycin (Bla g 4); *Blattella germanica* (German cockroach) glutathione transferase (Bla g 5); *Blattella germanica* (German cockroach) troponin C (Bla g 6); *Blomia tropicalis* (mite) antigen (Blo t 5); *Blomia tropicalis* (mite) Bt11a antigen (Blo t 12); *Blomia tropicalis* (mite) Bt6 fatty acid-binding protein (Blo t); *Bombus pennsylvanicus* (bumble bee) phospholipase (Bom p 1); *Bombus pennsylvanicus* (bumble bee) protease (Bom p 4); *Bos domesticus* (cow) Ag3, lipocalin (Bos d 2); *Bos domesticus* (cow) alpha-lactalbumin (Bos d 4); *Bos domesticus* (cow) beta-lactalbumin (Bos d 5); *Bos domesticus* (cow) casein (Bos d 8); *Bos domesticus* (cow) immunoglobulin (Bos d 7); *Bos domesticus* (cow) serum albumin (Bos d 6); *Brassica juncea* (oriental mustard) 2S albumin (Bra j 1); *Brassica rapa* (turnip) prohevein-like protein (Bar r 2); *Candida albicans* (fungus) antigen (Cand a 1); *Candida boidinii* (fungus) antigen (Cand b 2); *Canis familiaris* (dog) albumin (Can f ?); *Canis familiaris* (dog) antigen (Can f 1); *Canis familiaris* (dog) antigen (Can f 2); *Carpinus betulus* (hornbeam) antigen (Car b 1); *Castanea sativa* (chestnut) Bet v 1 homologue (Cas s 1); *Castanea sativa* (chestnut) chitinase (Cas s 5); *Chironomus thummi* (midge) component I (Chi t 2.0101); *Chironomus thummi thummi*

(midge) component IA (Chi t 2.0102); *Chironomus thummi thummi* (midge) component II-beta (Chi t 3); *Chironomus thummi thummi* (midge) component III (Chi t 1.01); *Chironomus thummi thummi* (midge) component IIIA (Chi t 4); *Chironomus thummi thummi* (midge) component IV (Chi t 1.02); *Chironomus thummi thummi* (midge) component IX (Chi t 6.02); *Chironomus thummi thummi* (midge) component VI (Chi t 5); *Chironomus thummi thummi* (midge) component VIIA (Chi t 6.01); *Chironomus thummi thummi* (midge) component VIIB (Chi t 7); *Chironomus thummi thummi* (midge) component VIII (Chi t 8); *Chironomus thummi thummi* (midge) component X (Chi t 9); *Chironomus thummi thummi* (midge) hemoglobin (Chi t 1-9); *Cladosporium herbarum* (fungus) acid. ribosomal protein P1 (Cla h 12); *Cladosporium herbarum* (fungus) aldehyde dehydrogenase (Cla h 3); *Cladosporium herbarum* (fungus) antigen (Cla h 1); *Cladosporium herbarum* (fungus) antigen (Cla h 2); *Cladosporium herbarum* (fungus) enolase (Cla h 6); *Cladosporium herbarum* (fungus) ribosomal protein); *Cladosporium herbarum* (fungus) YCP4 protein (Cla h 5); *Coprinus comatus* (shaggy cap) antigen (Cop c 1); *Coprinus comatus* (shaggy cap) antigen (Cop c 2); *Coprinus comatus* (shaggy cap) antigen (Cop c 3); *Coprinus comatus* (shaggy cap) antigen (Cop c 5); *Coprinus comatus* (shaggy cap) antigen (Cop c 7); *Corylus avellana* (hazel) antigen (Cor a 1); *Corylus avellana* (hazelnut) Bet v 1 homologue (Cor a 1.0401); *Cryptomeria japonica* (sugi) antigen (Cry j 1); *Cryptomeria japonica* (sugi) antigen (Cry j 2); *Ctenocephalides felis felis* (cat flea) antigen (Cte f 1); *Cynodon dactylon* (Bermuda grass) antigen (Cyn d 1); *Cynodon dactylon* (Bermuda grass) antigen (Cyn d 7); *Cynodon dactylon* (Bermuda grass) profilin (Cyn d 12); *Dactylis glomerata* (orchard grass) AgDg1 antigen (Dac g 1); *Dactylis glomerata* (orchard grass) antigen (Dac g 2); *Dactylis glomerata* (orchard grass) antigen (Dac g 3); *Dactylis glomerata* (orchard grass) antigen (Dac g 5); *Dermatophagoides farinae* (mite) antigen (Der f 1); *Dermatophagoides farinae* (mite) antigen (Der f 2); *Dermatophagoides farinae* (mite) antigen (Der f 3); *Dermatophagoides farinae* (mite) Mag 3, apolipophorin (Der f 14); *Dermatophagoides farinae* (mite) paramyosin (Der f 11); *Dermatophagoides farinae* (mite) tropomyosin (Der f 10); *Dermatophagoides microceras* (mite) antigen (Der m 1); *Dermatophagoides pteronyssinus* (mite) amylase (Der p 4); *Dermatophagoides pteronyssinus* (mite) antigen (Der p 2); *Dermatophagoides pteronyssinus* (mite) antigen (Der p 5); *Dermatophagoides pteronyssinus* (mite) antigen (Der p 7); *Dermatophagoides pteronyssinus* (mite) antigen P1 (Der p 1); *Dermatophagoides pteronyssinus* (mite) apolipophorin like p (Der p 14); *Dermatophagoides pteronyssinus* (mite) chymotrypsin

(Der p 6); *Dermatophagoides pteronyssinus* (mite) collagenolytic serine prot. (Der p 9); *Dermatophagoides pteronyssinus* (mite) glutathione transferase (Der p 8); *Dermatophagoides pteronyssinus* (mite) tropomyosin (Der p 10); *Dermatophagoides pteronyssinus* (mite) trypsin (Der p 3); *Dolichovespula arenaria* (yellow hornet) antigen 5 (Dol a 5); *Dolichovespula maculata* (white face hornet) antigen 5 (Dol m 5); *Dolichovespula maculata* (white face hornet) phospholipase (Dol m 1); *Dolichovespula maculate* (white face hornet) hyaluronidase (Dol m 2); *Equus caballus* (horse) lipocalin (Equ c 1); *Equus caballus* (horse) lipocalin (Equ c 2); *Euroglyphus maynei* (mite) apolipophorin (Eur m 14); *Felis domesticus* (cat) cat-1 antigen (Fel d 1); *Fraxinus excelsior* (ash) antigen (Fra e 1); *Gadus callarias* (cod) allergen M (Gad c 1); *Gallus domesticus* (chicken) conalbumin; A22 (Gal d 3); *Gallus domesticus* (chicken) lysozyme (Gal d 4); *Gallus domesticus* (chicken) ovalbumin (Gal d 2); *Gallus domesticus* (chicken) ovomucoid (Gal d 1); *Gallus domesticus* (chicken) serum albumin (Gal d 5); *Glycine max* (soybean) antigen (Gly m 2); *Glycine max* (soybean) HPS (Gly m 1.0101); *Glycine max* (soybean) HPS (Gly m 1.0102); *Glycine max* (soybean) profilin (Gly m 3); *Haliotis Midae* (abalone) antigen (Hal m 1); *Helianthus annuus* (sunflower) antigen (Hel a 1); *Helianthus annuus* (sunflower) profilin (Hel a 2); *Hevea brasiliensis* (rubber) 1,3-glucanase (Hev b 2); *Hevea brasiliensis* (rubber) antigen (Hev b 3); *Hevea brasiliensis* (rubber) antigen (Hev b 5); *Hevea brasiliensis* (rubber) component of microhelix protein complex (Hev b 4); *Hevea brasiliensis* (rubber) C-terminal fragment antigen (Hev b 6.03); *Hevea brasiliensis* (rubber) elongation factor (Hev b 1); *Hevea brasiliensis* (rubber) enolase (Hev b 9); *Hevea brasiliensis* (rubber) hevein (Hev b 6.02); *Hevea brasiliensis* (rubber) hevein precursor (Hev b 6.01); *Hevea brasiliensis* (rubber) Mn-superoxide dismut (Hev b 10); *Hevea brasiliensis* (rubber) patatin homologue (Hev b 7); *Hevea brasiliensis* (rubber) profilin (Hev b 8); *Holcus lanatus* (velvet grass) antigen (Hol l 1); *Homo sapiens* (human autoallergen) antigen (Hom s 1); *Homo sapiens* (human autoallergen) antigen (Hom s 2); *Homo sapiens* (human autoallergen) antigen (Hom s 3); *Homo sapiens* (human autoallergen) antigen (Hom s 4); *Homo sapiens* (human autoallergen) antigen (Hom s 5); *Hordeum vulgare* (barley) BMAI-1 (Hor v 1); *Juglans regia* (English walnut) 2S albumin (Jug r 1); *Juglans regia* (English walnut) vicilin (Jug r 2); *Juniperus ashei* (mountain cedar) antigen (Jun a 1); *Juniperus ashei* (mountain cedar) antigen (Jun a 3); *Juniperus oxycedrus* (prickly juniper) calmodulin-like antigen (Jun o 2); *Juniperus sabinoides* (mountain cedar) antigen (Jun s 1); *Juniperus virginiana* (eastern red cedar) antigen (Jun v 1);

Lepidoglyphus destructor (storage mite) antigen (Lep d 2.0101); L*epidoglyphus destructor* (storage mite) antigen (Lep d 2.0102); *Ligustrum vulgare* (privet) antigen (Lig v 1); *Lolium perenne* (rye grass) antigen (Lol p Ib); *Lolium perenne* (rye grass) group I antigen (Lol p 1); *Lolium perenne* (rye grass) group II antigen (Lol p 2); *Lolium perenne* (rye grass) group III antigen (Lol p 3); *Lolium perenne* (rye grass) group IX antigen (Lol p 5); *Lolium perenne* (rye grass) trypsin (Lol p 11); *Malassezia furfur* (fungus) antigen (Mal f 1); *Malassezia furfur* (fungus) antigen (Mal f 4); *Malassezia furfur* (fungus) antigen (Mal f 5); *Malassezia furfur* (fungus) cyclophilin homologue (Mal f 6); *Malassezia furfur* (fungus) MF1 peroxisomal membrane protein (Mal f 2); *Malassezia furfur* (fungus) MF2 peroxisomal membrane protein (Mal f 3); *Malus domestica* (apple) Bet v 1 homologue (Mal d 1); *Malus domestica* (apple) lipid transfer protein (Mal d 3); *Mercurialis annua* (annual mercury) profilin (Mer a 1); *Metapenaeus ensis* (shrimp) tropomyosin (Met e 1); *Mus musculus* (mouse) MUP antigen (Mus m 1); *Myrmecia pilosula* (Australian jumper ant) antigen (Myr p 1); *Myrmecia pilosula* (Australian jumper ant) antigen (Myr p 2); *Olea europea* (olive) antigen (Ole e 1); *Olea europea* (olive) antigen (Ole e 3); *Olea europea* (olive) antigen (Ole e 4); *Olea europea* (olive) antigen (Ole e 6); *Olea europea* (olive) profilin (Ole e 2); *Olea europea* (olive) superoxide dismutase (Ole e 5); *Oryza sativa* (rice) antigen (Ory s 1); *Penaeus aztecus* (shrimp) tropomyosin (Pen a 1); *Penaeus indicus* (shrimp) tropomyosin (Pen i 1); *Penicillium brevicompactum* (fungus) alkaline serine proteinase (Pen b 13); *Penicillium citrinum* (fungus) alkaline serine proteinase (Pen c 13); *Penicillium citrinum* (fungus) heat shock protein P70 (Pen c 1); *Penicillium citrinum* (fungus) peroxisomal membrane protein (Pen c 3); *Penicillium notatum* (fungus) alkaline serine proteinase (Pen n 13); *Penicillium notatum* (fungus) N-acetyl glucosaminidase (Pen n 1); *Penicillium notatum* (fungus) vacuolar serine proteinase (Pen n 18); *Penicillium oxalicum* (fungus) vacuolar serine proteinase (Pen o 18); *Periplaneta americana* (American cockroach) Cr-PI (Per a 3); *Periplaneta americana* (American cockroach) Cr-PII (Per a 1); *Periplaneta americana* (American cockroach) tropomyosin (Per a 7); *Persea americana* (avocado) endochitinase (Pers a 1); *Phalaris aquatica* (canary grass) antigen (Pha a 1); *Phleum pratense* (timothy grass) antigen (Phl p 1); *Phleum pratense* (timothy grass) antigen (Phl p 2); *Phleum pratense* (timothy grass) antigen (Phl p 4); *Phleum pratense* (timothy grass) antigen (Phl p 6); *Phleum pratense* (timothy grass) antigen Ag 25 (Phl p 5); *Phleum pratense* (timothy grass) polygalacturonase (Phl p 13); *Phleum pratense* (timothy grass) profilin (Phl p 12); *Poa pratensis*

(Kentucky blue grass) antigen (Poa p 5); *Poa pratensis* (Kentucky blue grass) group I antigen (Poa p 1); *Polistes annularies* (wasp) antigen 5 (Pol a 5); *Polistes annularies* (wasp) hyaluronidase (Pol a 2); *Polistes annularies* (wasp) phospholipase A1 (Pol a 1); *Polistes dominulus* (Mediterranean paper wasp) antigen (Pol d 1); *Polistes dominulus* (Mediterranean paper wasp) antigen (Pol d 5); *Polistes dominulus* (Mediterranean paper wasp) serine protease (Pol d 4); *Polistes exclamans* (wasp) antigen 5 (Pol e 5); *Polistes exclamans* (wasp) phospholipase A1 (Pol e 1); *Polistes fuscatus* (wasp) antigen 5 (Pol f 5); *Polistes metricus* (wasp) antigen 5 (Pol m 5); *Prunus armeniaca* (apricot) Bet v 1 homologue (Pru ar 1); *Prunus armeniaca* (apricot) lipid transfer protein (Pru ar 3); *Prunus avium* (sweet cherry) Bet v 1 homologue (Pru av 1); *Prunus avium* (sweet cherry) profilin (Pru av 4); *Prunus avium* (sweet cherry) thaumatin homologue (Pru av 2); *Prunus persica* (peach) lipid transfer protein (Pru p 3); *Psilocybe cubensis* (fungus) antigen (Psi c 1); *Psilocybe cubensis* (fungus) cyclophilin (Psi c 2); *Pyrus communis* (pear) Bet v 1 homologue (Pyr c 1); *Pyrus communis* (pear) isoflavone reductase homologue (Pyr c 5); *Pyrus communis* (pear) profilin (Pyr c 4); *Quercus alba* (white oak) antigen (Que a 1); *Rattus norvegius* (rat) antigen (Rat n 1); *Ricinus communis* (castor bean) 2S albumin (Ric c 1); *Salmo salar* (Atlantic salmon) parvalbumin (Sal s 1); *Sinapis alba* (yellow mustard) 2S albumin (Sin a 1); *Solanum tuberosum* (potato) patatin (Sol t 1); *Solenopsis geminata* (tropical fire ant) antigen (Sol g 2); *Solenopsis geminata* (tropical fire ant) antigen (Sol g 4); *Solenopsis invicta* (fire ant) antigen (Sol i 2); *Solenopsis invicta* (fire ant) antigen (Sol i 3); *Solenopsis invicta* (fire ant) antigen (Sol i 4); *Solenopsis saevissima* (Brazilian fire ant) antigen (Sol s 2); *Sorghum halepense* (Johnson grass) antigen (Sor h 1); *Syringa vulgaris* (lilac) antigen (Syr v 1); *Todarodes pacificus* (squid) tropomyosin (Tod p 1); *Trichophyton rubrum* (fungus) antigen (Tri r 2); *Trichophyton rubrum* (fungus) serine protease (Tri r 4); *Trichophyton tonsurans* (fungus) antigen (Tri t 1); *Trichophyton tonsurans* (fungus) serine protease (Tri t 4); *Vespa crabo* (European hornet) antigen 5 (Vesp c 5.0101); *Vespa crabo* (European hornet) antigen 5 (Vesp c 5.0102); *Vespa crabo* (European hornet) phospholipase (Vesp c 1); *Vespa mandarina* (giant Asian hornet) antigen (Vesp m 1.01); *Vespa mandarina* (giant Asian hornet) antigen (Vesp m 1.02); *Vespa mandarina* (giant Asian hornet) antigen (Vesp m 5); *Vespula flavopilosa* (yellowjacket) antigen 5 (Ves f 5); *Vespula germanica* (yellowjacket) antigen 5 (Ves g 5); *Vespula maculifrons* (yellowjacket) antigen 5 (Ves m 5); *Vespula maculifrons* (yellowjacket) hyaluronidase (Ves m 2); *Vespula maculifrons* (yellowjacket) phospholipase A1

(Ves m 1); *Vespula pennsylvanica* (yellowjacket) (antigen 5Ves p 5); *Vespula squamosa* (yellowjacket) antigen 5 (Ves s 5); *Vespula vidua* (wasp) antigen (Ves vi 5); *Vespula vulgaris* (yellowjacket) antigen 5 (Ves v 5); *Vespula vulgaris* (yellowjacket) hyaluronidase (Ves v 2); *Vespula vulgaris* (yellowjacket) phospholipase A1 (Ves v 1); *Zea mays* (maize, corn) lipid transfer protein (Zea m 14); and/or combinations thereof.

b. Infectious Antigens

[0208] In some embodiments, provided nanoparticles and/or nanoparticle compositions may include a payload that comprises or delivers (*e.g.,* encodes, releases, or otherwise becomes or provides) one or more epitopes of one or more antigens may be provided from an infectious agent or organisms, such as a virus, parasite and/or bacterium.

[0209] In some embodiments, an infectious antigen may be or comprise an epitope of one or more protein antigens derived from viral or bacterial sources.

[0210] Exemplary criteria for identifying and selecting effective antigenic sequences (*e.g.*, minimal peptide sequences capable of eliciting an immune response) may be found in the art. For example, Apostolopoulos, *et al.* (*Curr. Opin. Mol. Ther.*, 2:29-36 (2000)), discusses a strategy for identifying minimal antigenic peptide sequences based on an understanding of the three-dimensional structure of an antigen-presenting molecule and its interaction with both an antigenic peptide and T-cell receptor. Shastri, (*Curr. Opin. Immunol.*, 8:271-7 (1996)), discloses how to distinguish rare peptides that serve to activate T cells from the thousands of peptides normally bound to MHC molecules.

[0211] Generally, a virus consists of either two or three parts: 1) genetic material, which may be DNA or RNA, depending on the virus, 2) a protein coat that surrounds and protects the genetic material, and, in some viruses, 3) a lipid envelope that surrounds the protein coat. In some embodiments, a viral antigen may be provided from any component of a virus. In some embodiments, a viral antigen may be provided from the viral envelope. In some embodiments, a viral antigen may be provided from a glycoprotein of the viral envelope.

[0212] Representative viruses that may be the source of viral antigens relevant to the present disclosure may be from a viral family such as, for example: *Adenoviridae*, *Arenaviridae* (*e.g., Lymphocytic choriomeningitis mammarenavirus*), *Arteriviridae, Astroviridae, Baculoviridae, , Barnaviridae, Betaflexiviridae (e.g., Capillovirus* and *Carlavirus), Birnaviridae, Bromoviridae,*

Bunyaviridae, Caliciviridae, Caulimoviridae (*e.g., Badnavirus* and *Caulimovirus*), *Circoviridae, Closteroviridae* (*e.g., Closterovirus*), *Comoviridae, Coronaviridae* (*e.g., Coronavirus*, such as severe acute respiratory syndrome (SARS) virus, Severe Acute Respiratory Syndrome coronavirus 2 (SARS-CoV-2), Middle Eastern Respiratory Syndrome (MERS) coronavirus), *Corticoviridae*, *Cystoviridae*, , *Filoviridae* (*e.g.*, Marburg virus and Ebola virus (*e.g.*, Zaire, Reston, Ivory Coast, or Sudan strain)), *Flaviviridae* (*e.g.*, Dengue virus 1, Dengue virus 2, Dengue virus 3, and Dengue virus 4, Hepatitis C virus, hepatitis G virus (HGV), West Nile virus (WNV), yellow fever), *Hantaviridae*, *Hepadnaviridae* (*e.g.,* hepatitis B (HBV)), *Hepeviridae* (*e.g.*, Hepatitis E virus (HEV)), *Herpesviridae* (*e.g.*, Human herpesvirus 1, 2, 3, 4, 5, 6A, 6B, 7 and 8), *Hypoviridae*, *Iridoviridae*, *Kolmioviridae* (*e.g., Deltavirus* (*e.g.,* hepatitis D virus)), *Leviviridae*, *Lipothrixviridae*, *Matonaviridae* (*e.g.,* rubella virus*), Microviridae*, *Orthomyxoviridae* (*e.g.*, Influenza virus A and B and C), *Papillomaviridae* (*e.g., Papillomavirus*, human papillomavirus (HPV)), *Polyomaviridae*, *Paramyxoviridae* (*e.g.*, measles, mumps, hepatitis A virus (HAV), parainfluenza viruses), *Parvoviridae* (*e.g.,* parvovirus), *Phenuiviridae* (*e.g., Phlebovirus,* Rift Valley fever), *Picornaviridae* (*e.g.*, aphthovirus, coxsackievirus, hepatovirus, poliovirus, rhinovirus), *Pneumoviridae* (*e.g.,* human respiratory syncytial virus, human metapneumovirus), *Poxviridae* (*e.g.*, vaccinia virus and smallpox virus), *Reoviridae* (*e.g.*, rotavirus), *Retroviridae* (*e.g.*, lentivirus, such as human immunodeficiency virus (HIV) 1 and HIV 2, human T-lymphotrophic virus (HTLV)), *Rhabdoviridae* (*e.g.*, rabies virus, Indiana vesiculovirus (VSV)), *Solemoviridae* (*e.g.*, *Enamovirus*) *Togaviridae* (*e.g.*, eastern equine encephalitis virus, Japanese encephalitis virus), *Tombusviridae* (*e.g.*, *Dianthovirus*), and *Totiviridae.*

[0213] In some embodiments, a viral antigen may be or comprise epitopes of one or more viruses. In some embodiments, viral epitope may be comprised of one or more of 1) viral genetic material 2) a portion of a viral protein coat, and/or 3) a portion of a viral lipid envelope. In some embodiments, a payload for use in accordance with the present disclosure may comprise, consist of, or otherwise deliver one or more of 1) viral genetic material 2) a portion of a viral protein coat, and/or 3) a portion of a viral lipid envelope.

[0214] In some embodiments, provided nanoparticles and/or nanoparticle compositions may include one or more bacterial antigens. Bacterial antigens may originate from a pathogenic bacterium. Exemplary such pathogenic bacteria may include, but not be limited to, those of a

genus such as *Actinomyces*, *Aeromonas*, *Anabaena*, *Arthrobacter*, *Bacillus*, *Bacteroides*, *Bdellovibrio*, *Bordetella*, *Borrelia*, *Campylobacter*, *Caulobacter*, *Chlamydia*, *Chlorobium*, *Chromatium*, *Citrobacter*, *Clostridium*, *Corynebacterium*, *Cytophaga*, *Deinococcus*, *Enterobacter*, *Escherichia*, *Francisella*, *Haemophilus*, *Halobacterium*, *Heliobacter*, *Hemophilus (e.g., Haemophilus influenzae* type B(HIB)*)*, *Hyphomicrobium*, *Klebsiella*, *Lactococcus*, *Legionella*, *Leptospirosis*, *Listeria*, *Methanobacterium*, *Micrococcus*, *Morganella*, *Mycoplasma*, *Myobacterium (e.g., Mycoplasma pneumoniae)*, *Myxococcus*, *Neisseria (Neisseria meningitidis)*, *Nitrobacter*, *Norcardia (e.g., Nocardia asteroids)*, *Oscillatoria*, *Peptococcus*, *Phodospirillum*, *Plesiomonas*, *Prochloron*, *Proteus*, *Providencia*, *Pseudomonas*, *Rickettsia (e.g., Rickettsia ricketsii*, *Rickettsia typhi)*, *Salmonella*, *Serratia*, *Shigella*, *Spirillum*, *Spirochaeta*, *Sporolactobacillus*, *Staphylococcus*, *Streptococcus*, *Streptomyces*, *Sulfolobus*, *Thermoplasma*, *Thiobacillus*, *Treponema*, *Vibrio*, *Yersinia*, and combinations thereof.

[0215] In some embodiments, provided nanoparticles and/or nanoparticle compositions may include one or more parasite antigens. In some embodiments, a parasite is a fungus. In some embodiments, a parasite is a protozoan. In some embodiments, a parasite is a helminth. Parasite antigens can be obtained from parasites such as, but not limited to, an antigen derived from *Candida albicans*, *Candida tropicalis*, *Chlamydia trachomatis*, *Chlamydial psittaci*, *Cryptococcus neoformans*, *Entamoeba histolytica*, *Histoplasma capsulatum*, *Plasmodium falciparum*, *Schistosoma mansoni*, *Toxoplasma gondii*, *Trichomonas vaginalis, Trypanosoma brucei* and *Trypanosoma cruzi.* These include Sporozoan antigens, Plasmodian antigens, such as all or part of a Circumsporozoite protein, a Sporozoite surface protein, a liver stage antigen, an apical membrane associated protein, or a Merozoite surface protein.

c. Cancer Antigens

[0216] In some embodiments, provided nanoparticles and/or nanoparticle compositions may include a payload that comprises or delivers (*e.g.,* encodes, releases, or otherwise becomes or provides) one or more epitopes of one or more cancer antigens. In some embodiments, cancer antigens may be provided from tumor cells.

[0217] In some embodiments, a payload may be or comprise one or more polypeptides that is or comprises one or more tumor-associated or tumor-specific epitopes, or a construct that encodes

such polypeptide or another entity that delivers or becomes it. In some embodiments, a tumor-associated or tumor-specific epitope is a neoepitope.

[0218] In some embodiments, a payload may be or comprise a polypeptide (*e.g.*, that is recombinantly expressed or that is purified or partially purified from a tumor source. In some embodiments, a payload is or comprises a nucleic acid that encodes such polypeptide (*e.g.*, a DNA or RNA construct, *e.g.*, an mRNA).

[0219] In some embodiments, provided nanoparticles and/or nanoparticle compositions may include one or more crude (*i.e.*, unpurified or substantially unpurified) cancer antigenic extracts. In some embodiments, cancer antigens are provided in a crude form such as a cellular lysate or cellular fraction.

[0220] In some embodiments, an exemplary cancer antigen may be, for example, alpha-actinin-4, Bcr-Abl fusion protein, Casp-8, beta-catenin, cdc27, cdk4, cdkn2a, coa-l, dek-can fusion protein, EF2, ETV6-AML1 fusion protein, LDLR-fucosyltransferaseAS fusion protein, HLA-A2, HLA-All, hsp70-2, KIAAO205, Mart2, Mum-1, 2, and 3, neo-PAP, myosin class I, OS-9, pmlRARa fusion protein, PTPRK, K-ras, N-ras, Triosephosphate isomeras, Bage-l, Gage 3,4,5,6,7, GnTV, Herv-K-mel, Lage-1, MageAl, 2,3,4,6,10,12, Mage-C2, NA-88, NY-Eso-1/Lage-2, SP17, SSX-2, and TRP2-Int2, MelanA (MART-I), gplOO (Pmell7), tyrosinase, TRP-1, TRP-2, MAGE-l, MAGE-3, BAGE, GAGE-l, GAGE-2, p15(58), CEA, RAGE, NY-ESO (LAGE), SCP-1, Hom/Mel-40, PRAME, p53, H-Ras, HER-2/neu, BCR-ABL, E2A-PRL, H4-RET, IGH-IGK, MYL-RAR, Epstein Barr virus antigens, EBNA, human papillomavirus (HPV) antigens E6 and E7, TSP-180, MAGE-4, MAGE-5, MAGE-6, pl85erbB2, p180erbB-3, c-met, nm-25 23Hl, PSA, TAG-72-4, CA 19-9, CA 72-4, CAM 17.1, NuMa, K-ras, pCatenin, CDK4, Mum-1, pl6, TAGE, PSMA, PSCA, CT7, telomerase, 43-9F, 5T4, 791Tgp72, a-fetoprotein, 13HCG, BCA225, BTAA, CA 125, CA15-3 (CA 27.29\BCAA), CA 195, CA 242, CA-50, CAM43, CD68\KP1, C0-029, FGF-5, G250, Ga733 (EpCAM), HTgp-175, M344, MA-50, MG7-30 Ag, MOV18, NB\70K, NY-C0-1, RCASl, SDCCAG16, TA-90 (Mac-2 binding protein\cyclophilin C-associated protein), TAAL6, TAG72, TLP, and TPS.

[0221] Representative cancer cells that may be the source of cancer antigens relevant to the present disclosure may be from, for example, acute lymphoblastic leukemia (ALL); adrenocortical carcinoma; AIDS-related cancers including AIDS-related lymphoma; anal cancer; appendix cancer; astrocytomas; basal cell carcinoma; bile duct cancer; bladder cancer; bone

cancer (*e.g.*, osteosarcoma and malignant fibrous histiocytoma); brainstem glioma; brain cancer; brain tumors; breast cancer; bronchial adenomas/carcinoids; Burkitt lymphoma; carcinoid tumors (*e.g.*, childhood and gastrointestinal tumors); carcinoma (including carcinoma of unknown primary (CUP) whose origin or developmental lineage is unknown but that possess specific molecular, cellular, and histological characteristics of epithelial cells); central nervous system lymphoma; cerebellar astrocytoma; cervical cancer; childhood cancers; chronic lymphocytic leukemia; chronic myelogenous leukemia; chronic myeloproliferative disorders; colon cancer; cutaneous T-cell lymphoma; desmoplastic small round cell tumor; endometrial cancer; endometrial uterine cancer; ependymoma; esophageal cancer; Ewing's sarcoma in the Ewing family of tumors; extracranial germ cell tumor; extragonadal germ cell tumor; extrahepatic bile duct cancer; eye cancer; intraocular melanoma; gallbladder cancer; gastric carcinoid; gastric cancer; gastrointestinal carcinoid tumor; gastrointestinal stromal tumor (GIST); gestational trophoblastic tumor; hairy cell leukemia; head and neck cancer; heart cancer; hepatocellular (liver) cancer; Hodgkin lymphoma; hypopharyngeal cancer; hypothalamic and visual pathway glioma; intraocular Melanoma; Islet Cell Carcinoma (Endocrine Pancreas); Kaposi sarcoma; kidney cancer (renal cell carcinoma); laryngeal cancer; leiomyosarcoma; leukemias (including acute lymphoblastic or acute lymphocytic leukemia, acute myeloid or acute myelogenous leukemia, chronic lymphocytic or chronic lymphocytic leukemia, chronic myelogenous or chronic myeloid leukemia); Lip and Oral Cavity Cancer; liposarcoma; liver cancer; lung cancer (including non-small cell and small cell); lymphomas (*e.g.*, AIDS-related, Burkitt, cutaneous T-Cell, Hodgkin, non-Hodgkin, Primary Central Nervous System); macroglobulinemia; malignant glioma; medulloblastoma; melanoma; Merkel Cell Carcinoma; mesothelioma (*e.g.*, adult malignant mesothelioma, childhood mesothelioma); metastatic squamous neck cancer; mouth cancer; Multiple Endocrine Neoplasia Syndrome; Multiple Myeloma; Mycosis Fungoides; Myelodysplastic Syndromes; Myelodysplastic/Myeloproliferative Diseases; Myelogenous Leukemia; Myeloid Leukemia; nasal cavity and paranasal sinus cancer; nasopharyngeal carcinoma; neuroblastoma; oral cancer; oropharyngeal cancer; ovarian cancer; ovarian epithelial cancer (Surface epithelial-stromal tumor); ovarian germ cell tumor; ovarian low malignant potential tumor; pancreatic cancer; paranasal sinus and nasal cavity cancer; parathyroid cancer; penile cancer; pharyngeal cancer; pheochromocytoma; pineal astrocytoma; pineal germinoma; pineoblastoma and supratentorial primitive neuroectodermal tumors; pleuropulmonary blastoma;

prostate cancer; rectal cancer; renal pelvis and ureter and transitional cell cancer; retinoblastoma; rhabdomyosarcoma; Sézary syndrome; skin cancer (including melanoma and nonmelanoma); skin carcinoma; small intestine cancer; soft tissue sarcoma; squamous cell carcinoma; stomach cancer; testicular cancer; throat cancer; thymoma and thymic carcinoma; thyroid cancer; urethral cancer; uterine sarcoma; vaginal cancer; vulvar cancer; and/or combinations thereof.

d. Alloantigens

[0222] In some embodiments, provided nanoparticles and/or nanoparticle compositions may include a payload that comprises or delivers (*e.g.,* encodes, releases, or otherwise becomes or provides) one or more alloantigens. As described herein, an alloantigen refers to an antigen associated with allorecognition and/or graft rejection (*e.g.*, an antigen against which a rejection immune response is directed). Alloantigens are generally polypeptides expressed by an individual that are genetically different from another individual of the same species. The term "alloantigen polypeptide" refers to a polypeptide whose amino acid sequence includes at least one characteristic sequence of an alloantigen. A wide variety of alloantigen sequences are known in the art.

[0223] In some embodiments, an alloantigen for use in accordance with the present disclosure is a major histocompatibility complex (MHC) polypeptide. In some embodiments, an alloantigen for use in accordance with the present disclosure is a Class I MHC polypeptide. In some embodiments, an alloantigen for use in accordance with the present disclosure is a Class II MHC polypeptide. In some embodiments, an alloantigen for use in accordance with the present disclosure contains part of or all of an extracellular domain of an MHC polypeptide. In some embodiments, an alloantigen for use in accordance with the present disclosure is a minor histocompatibility complex polypeptide. In some embodiments, an alloantigen for use in accordance with the present disclosure is a co-stimulatory entity (*e.g.*, CD28, CD80, and CD86, among others). In some embodiments, an alloantigen for use in accordance with the present disclosure is a non-MHC protein produced by or present in graft tissue and not produced by or present in a host. One of ordinary skill in the art will recognize that alloantigens described herein are exemplary. Any polypeptide that is associated with an allorecognition and/or graft rejection can be classified as an alloantigen.

[0224] It will be appreciated that alloantigen polypeptides may have a complete sequence, or alternatively may be polypeptides that represent functional fragments (*i.e.*, fragments retaining at least one activity and/or one characteristic sequence or portion) of such complete polypeptides. Moreover, those of ordinary skill in the art understand that protein sequences generally tolerate some substitution without destroying activity. Thus, any polypeptide that retains activity and shares at least about 30-40% overall sequence identity, often greater than about 50%, 60%, 70%, or 80%, and further usually including at least one region of much higher identity, often greater than 90% or even 95%, 96%, 97%, 98%, or 99% in one or more highly conserved regions, usually encompassing at least 3-4 and often up to 20 or more amino acids, with another alloantigen polypeptide of the same class, is encompassed within the relevant term "alloantigen polypeptide" as used herein.

Coating agents

[0225] In some embodiments, nanoparticle compositions may be partially or wholly coated with a coating agent. In some embodiments, a coating agent may be or comprise one or more entities that target nanoparticles to a particular site (*e.g.*, to a specific cell, tissue, cell surface marker, *etc.*). Alternatively or additionally, in some embodiments, a coating agent may be or comprise a payload (*e.g.*, nanoparticles may be partially or wholly coated with a payload entity – *e.g.*, with an antigen and/or an immune adjuvant as described herein). In some embodiments, a coating agent may have adjuvant properties (*e.g.*, may be or act as an immune adjuvant). In some embodiments, a coating agent may differ depending upon payload and/or target and/or desired immune response (*e.g.*, Th1 vs Th2). For example, in some embodiments, if a nanoparticle composition is delivering a payload in order to desensitize a subject to an autoantigen, a coating agent may act to stimulate or assist in producing a desired response. In some embodiments, if a nanoparticle composition is delivering a payload to treat, *e.g.*, cancer or infectious disease, a different type of coating agent may be desirable.

[0226] One feature of certain embodiments of the present disclosure is that it permits delivery of an antigen to a subject in a context that minimizes exposure of the antigen to immune system component(s) that might induce or mediate an undesirable reaction or response to the antigen while achieving its exposure to immune system component(s) that might induce or mediate a beneficial response. For instance, in some embodiments including one or more coating agent(s),

an antigen may be or comprise an allergic antigen and provided systems may minimize its exposure during delivery to mast cells, IgE or other immune system components that might mediate an anaphylactic response (and might be present, for example, in blood), while permitting its exposure to immune components (*e.g.*, Th1 and/or Treg cells) that might mediate an allergy-suppressing (*e.g.*, Th1 or Treg) response.

[0227] In some embodiments, a coating agent comprises a hydrophobic component. For example, in some embodiments, a coating agent comprises a hydrophobic cellular component. In some embodiments, a hydrophobic component is or comprises a lipid component. In some embodiments, a hydrophobic component is or comprises LPS.

[0228] In some embodiments, a hydrophobic cellular component preparation may be provided from a cellular lysate (e.g., microbial lysate) or other extract.

[0229] Alternatively or additionally, one feature of certain embodiments of the present disclosure is that it permits utilization of relatively crude coating agents and/or coating agent preparations. In some embodiments, a coating agent may be or comprise a crude preparation and/or other complex material (*e.g.*, an extract, *etc.*).

[0230] In some embodiments, coating agents may comprise microbial hydrophobic and/or hydrophilic cellular components (*e.g.*, from a crude microbial extract, for example, an *E. coli* extract). Without wishing to be held by a particular theory, some embodiments of the present disclosure including a coating agent comprising one or more cellular components may permit development and/or production of useful immunomodulatory nanoparticle compositions at least in part because they utilize various evolved attributes of microbial cells relating to their ability to modulate or evade human or animal immune reactions. The present disclosure also captures an insight that combining such evolved attributes with various features of certain nanoparticle systems such as, for example, ability to sequester antigens and/or cellular components from immune system elements, tunable degradation rates and/or locations, and/or modular association with targeting, immune adjuvant, or other surface entities, permits development and/or production of particularly useful immunomodulatory compositions.

[0231] In some embodiments, coating agents may comprise microbial extracts – *e.g.*, hydrophilic or hydrophobic extracts of microbial cells (*e.g.*, *E. coli*) for use in or with provided nanoparticle compositions. In some embodiments, such microbial extracts may contain a collection of microbial components that share a chemical feature, so that they associate with other included

components and not with excluded components during production of the extract. In some embodiments, extracts may contain at least some cellular components at relative levels comparable to those at which they are present in the cells. Those skilled in the art will be aware of a variety of techniques available to determine presence and/or level of particular components, and to compare such determined level(s) with those observed in intact cells. Moreover, those of ordinary skill in the art will readily appreciate reasonable and expected experimental variation and therefore will be able to determine whether components are present in absolute or relative levels or concentrations in an extract that are reasonably comparable to those at which they are present in cells.

[0232] In general, microbial extracts are prepared from microbial cell preparations. Microbial cell preparations are prepared by culturing microbial cells for a period of time and under conditions sufficient to achieve cell growth to a desirable level (*e.g.*, optical density, concentration, colony size, total protein, total DNA, and colony forming units). In some embodiments, microbial cell preparations contain intact cells, and optionally are substantially free of lysed cells. In some embodiments, microbial cell preparations contain lysed cells, and optionally are substantially free of intact cells.

[0233] In some embodiments, one or more coating agents (*e.g.*, extracts, preparations and/or agents) is associated covalently with a nanoparticle surface. In some embodiments, one or more coating agents (*e.g.*, extracts, preparations and/or agents) is associated non-covalently with a nanoparticle surface. In some embodiments, non-covalent association involves incorporation of one or more components into the nanoparticle membrane. In some embodiments, non-covalent association involves specific binding with the nanoparticle membrane or an element incorporated therein. In some specific embodiments, one or more particular components of a coating agent (*e.g.*, an extract, preparation and/or agent) may be coupled with a ligand that specifically binds with a target in the nanoparticle membrane. In some embodiments, a ligand-target combination utilized in such an embodiment may be, for example, biotin-avidin, antibody-antigen, toll-like receptor 4 (TLR4) and lipopolysaccharide (LPS), GST-glutathione, mannose binding protein-mannose, Protein A-IgG, and/or S-tag, or components thereof.

[0234] In some embodiments, one or more coating agents is prepared using a process that involves mixture of a dry coating agent with water, followed by application of disruptive energy force (*e.g.*, sonication). For example, in some embodiments, organic *E. coli* extract (OEE)

powder is mixed with water. In some such embodiments, a combination of water and OEE powder is sonicated, producing OEE micelles in water.

[0235] In some embodiments, OEE micelles in water are coated onto nanoparticles of the present disclosure using a spray-drying method. In some embodiments, after spray drying of nanoparticles is completed a certain percentage of solid material (*e.g.*, coated nanoparticles), is recovered. In some such embodiments, approximately 50 to 95% of solids are recovered. In some such embodiments, approximately 60-85% of solids are recovered. In some such embodiments, approximately 65-80% of solids are recovered.

[0236] In some embodiments, OEE micelles in water are combined with a nanoparticle mixture, sonicated, and lyophilized. In some such embodiments, combining OEE micelles with a provided nanoparticle mixture, and lyophilizing results in an association of a coating (OEE) with a nanoparticle surface.

[0237] In some embodiments, concentration of coating agents is quantified and/or compared to one or more natural organisms. For example, in some embodiments, quantity of TLR4 ligand (LPS) present per nanoparticle as compared to LPS present in a given, wild-type *E. coli* cell may be calculated. In some embodiments, nanoparticles may have a lesser (*e.g.*, 10%, 25%, 50%, 75%), substantially equivalent, or greater (*e.g.*, 110%, 125%, 150%, 200%, 250%, 300% or more) amount of LPS than a given wild-type *E. coli*. In some such embodiments, it is contemplated that a coating applied using spray drying may be more concentrated than a coated applied using lyophilization procedures. For example, in some embodiments, nanoparticles coated with OEE using spray drying may have an LPS-equivalent of approximately 5-7 *E. coli* (*e.g.*, approximately 6.5-7 *E. coli*). In some embodiments, nanoparticles coated with OEE using a lyophilization procedure may have an LPS-equivalent of approximately 1-5 *E. coli* cells (*e.g.*, approximately 3-3.5 *E. coli*). Without being bound by any particular theory, it is contemplated that in some such embodiments, higher amount(s) of LPS relative to what is present on wild-type *E. coli* is/are favorable and will assist in function of a given nanoparticle composition. In some embodiments, higher amount(s) of LPS relative to wild-type *E. coli* may be desirable. In some embodiments, lower amounts of LPS than found on wild-type *E. coli* may be beneficial and/or desirable.

[0238] In some embodiments, nanoparticles of the present disclosure are coated with a "shell" (e.g., that is or comprises a lipid component such as an LPS component and, in some

embodiments, may be an organic cellular extract such as an organic microbial extract such as an organic *E.* coli extract) that is 5-7 nm thickness. In some such embodiments, a 5-7 nm thick shell approximates an amount of LPS on a single *E. coli*.

[0239] In some embodiments, nanoparticles of the present disclosure have a mass equivalent of OEE to that of LPS on *E. coli*. In some embodiments, nanoparticles of the present disclosure have a mass equivalent of OEE greater than that of LPS on *E. coli*. In some embodiments, nanoparticles of the present disclosure have a mass equivalent of OEE less than that of LPS on *E. coli*. For example, in some embodiments, mass of OEE on a population of particles approximates mass of OEE associated with an *E. coli*. By way of non-limiting example, in some embodiments, a mass/mass equivalent of 38 mg of OEE is used to approximate an amount of LPS on a single *E. coli*.

[0240] Additional methods and parameters suitable for the preparation of crude and/or microbial extract-based coating agents may be found in PCT. Application No. PCT/US14/32838, filed April 3, 2014.

Other agents

[0241] In some embodiments, provided nanoparticles and/or nanoparticle compositions may include one or more other agents (*e.g.*, agents which do not elicit a humoral immune response in a subject, and/or agents that may promote or sustain a particular immune response, *e.g.,* to an included antigen).

[0242] According to various embodiments, provided compositions comprising one or more other agents may comprise one or more other agents in any of a variety of forms. Exemplary forms include, without limitation, RNA, DNA, protein, and combinations thereof. In some embodiments, one or more other agents may be provided as a portion of a cell, tissue or extract thereof. For example, a nanoparticle comprising an RNA may further comprise RNAse inhibitors.

[0243] In some embodiments, one or more other agents may comprise immunomodulatory polypeptides or immunostimulatory factors to modulate an individual's immune response. In some embodiments, immunomodulatory polypeptides include cytokines which are small proteins or biological factors (in the range of 5-20 kD) that have specific effects on cell-cell interaction, communication and behavior of other cells. Cytokines are proteins that are secreted to T-cells to

induce a Th1 or Th2 response. In some embodiments, cytokine(s) may be selected to reduce production of a Th2 response to antigens associated with anaphylaxis. In some embodiments, cytokine(s) may be selected to reduce production of a Th1 response to antigens. Cytokines that, when presented during antigen delivery into cells, induce a Th1 response in T cells include IL-12, IL-2, I-18, IL-1 or fragments thereof, IFN, and/or IFNγ.

[0244] In some embodiments, one or more other agents may comprise immunological inducing agents. Inducing agents may prompt the expression of Th1 stimulating cytokines by T-cells and include factors such as, CD40, CD40 ligand, oligonucleotides containing CpG motifs, TNF, and microbial extracts such as preparations of Staphylococcus aureus, heat-killed Listeria, and modified cholera toxin, *etc*.

[0245] In some embodiments, one or more other agents may include preparations (including heat-killed samples, extracts, partially purified isolates, or any other preparation of a microorganism or macroorganism component sufficient to display immune adjuvant activity) of microorganisms such as Listeria monocytogenes or others (*e.g.*, Bacillus Calmette-Guérin[BCG], Corynebacterium species, Mycobacterium species, Rhodococcus species, Eubacte ria species, Bortadella species, and Nocardia species), and preparations of nucleic acids that include unmethylated CpG motifs. In some embodiments, one or more other agents (*e.g.*, immune adjuvant) include, for example, Aviridine (N,N-dioctadecyl-N′N′-bis(2-hydroxyethyl) propanediamine) and CRL 1005. In some embodiments, one or more other agents (*e.g.*, immune adjuvant) induce IL-12 production, including microbial extracts such as fixed Staphylococcus aureus, Streptococcal preparations, Mycobacterium tuberculosis, lipopolysaccharide (LPS), monophosphoryl lipid A (MPLA) from gram negative bacterial lipopolysaccharides (Richards *et al.* Infect Immun 1998 June; 66(6):2859-65), listeria monocytogenes, toxoplasma gondii, leishmania major.

[0246] In some embodiments, one or more other agents may be or comprise one or more immune adjuvants. In some embodiments, immune adjuvants may be provided from one or more bacterial sources, including, by way of non-limiting example, certain cellular lysate (e.g., microbial lysate (*e.g.*, bacterial lysate)), cellular lysate fractions, or specific components thereof. In some embodiments, cellular lysate fractions comprise entities known as pathogen-associated molecular patterns ("PAMPs"). In some embodiments, one or more of a hydrophobic bacterial lysate fraction and/or hydrophilic bacterial lysate fraction include one or more PAMPs as a

hydrophilic cellular component and/or hydrophobic cellular component. In some embodiments, a hydrophilic bacterial lysate fraction and/or hydrophilic cellular component may be encapsulated within or substantially encapsulated within provided nanoparticles. In some embodiments, an immune adjuvant is a mucosal immune adjuvant (*i.e.*, an immune adjuvant capable of eliciting or enhancing an immune response to a mucosally-administered antigen). Exemplary mucosal antigens include, but are not limited to, TLR4 ligands (*e.g.*, LPS, MPL), cytokines (*e.g.*, IL-1α), c48/80, R848, Pam3CSK4, CpG(ODN1826), lethal factor (LF), and cholera toxin. It will be recognized by those of skill in the art that particular mucosal immune adjuvants may induce different immune responses. The skilled artisan will understand and be aware of technologies that may be used to select particular immune adjuvant(s) for use in a particular product or products and such variation is specifically contemplated as within the scope of the present disclosure.

[0247] In some embodiments, PAMPs are entities associated with bacterial cells that are recognized by cells of the innate immune system. In some embodiments, PAMPs are recognized by Toll-like receptors (TLRs) and other pattern recognition receptors (PRRs) in both plants and animals. In some embodiments, PAMPs are recognized by C-type lectin receptors (CLRs). In some embodiments, a CLR is a type I or type II CLR. In some embodiments, PAMPs are recognized by RIG-I-like receptors (RLRs). In some embodiments, PAMPs are recognized by NOD-like receptors (NLRs). In some embodiments, PAMPs are or comprise entities associated with the outer surface of a bacterial cell, including, but not limited to, membrane-associated proteins and/or peptides, receptors embedded in bacterial membranes, *etc.* Exemplary PAMPs include, but are not limited to, bacterial lipopolysaccharide (LPS), bacterial flagellin, lipoteichoic acid from gram positive bacteria, peptidoglycan, double-stranded RNAs (dsRNAs), unmethylated CpG motifs, sheared *E. coli* genomic DNA, any of the TLR ligands presented in **Table 5**, characteristic portions thereof, and/or combinations thereof.

Table 5. Exemplary TLRs and TLR Ligands

TLR	TLR Ligand(s)
TLR1	Multiple triacyl lipopeptides (*e.g.*, from bacteria and mycobacteria), such as lipopeptide Pam3Cys-SK4 ("Pam")
TLR2	Multiple glycolipids, lipopeptides and lipoproteins, such as lipopeptide Pam3Cys-SK4 ("Pam") Lipoteichoic acid Peptidoglycan

TLR	TLR Ligand(s)
	HSP70 Zymosan Heat shock proteins, such as Hsp60
TLR3	Double-stranded RNA Single-stranded RNA Poly(I:C)
TLR4	lipopolysaccharide (LPS) Monophosphoryl lipid A (MPL) Several heat shock proteins Fibrinogen Heparin sulfate fragments Hyaluronic acid fragments
TLR5	Flagellin
TLR6	Multiple diacyl lipopeptides Lipoteichoic acid (LTA) Zymosan
TLR7	Imidazoquinolines (*e.g.*, imiquimod and resiquimod) Single-stranded RNA, such as GU-rich single-stranded RNA Loxoribine (a guanosine analog) Bropirimc
TLR8	Imidazoquinolines (*e.g.*, imiquimod and resiquimod) GU-rich single-stranded RNA Small synthetic compounds Single-stranded RNA
TLR9	Unmethylated CpG DNA Hemazoin crystals Double-stranded DNA
TLR10	HIV-gp41
TRL11	*Toxoplasma gondii* profilin Uropathogenic-bacteria-derived protein

[0248] In some embodiments, one or more other agents may comprise a pore forming toxin (PFT). In some embodiments, a PFT may be or comprise a bacterial cytotoxic protein for virulence. A PFT may disrupt host cell membranes. For example, in some embodiments, a nanoparticle preparation comprising a payload displayed by an MHC class I complex may comprise one or more other agents comprising a PFT. In some embodiments, an exemplary PFT may be, for example, α-pore-forming toxin, β-barrel pore-forming toxin, large β-barrel pore-forming toxin, binary toxin, small pore-forming toxin, *etc*.

[0249] In some embodiments, one or more other agents may be incorporated within nanoparticles. In some embodiments, one or more other agents may be coated on nanoparticles. Those skilled in the art will appreciate desirability of incorporating particular other agents within or on nanoparticles, or both. Without wishing to be bound by any particular theory, where it is

desirable to provide nanoparticle preparations that mimic one or more features of cells, e.g., of microbial cells, it may be desirable to incorporate other agents accordingly (for example, to include nucleic acids, and particularly nucleic acids containing unmethylated CpG motifs, within nanoparticles and/or lipids and/or other cell surface components on surface(s) thereof).

Nanoparticle compositions

[0250] In certain embodiments, provided nanoparticle compositions comprise nanoparticles (*e.g.*, comprised of polymer) combined with one or more payloads, one or more coating agents, and/or one or more other agents. In certain embodiments, certain combined elements are encapsulated within a polymer matrix.

[0251] In certain embodiments, provided nanoparticle compositions comprise nanoparticles combined with one or more payloads, one or more coating agents, and/or one or more other agents so that certain combined elements are distributed (*e.g.,* substantially homogenously) within a polymer matrix. For example, in some embodiments, one or more payloads are distributed substantially homogenously within a polymer matrix.

[0252] In some embodiments, provided nanoparticle compositions comprise nanoparticles combined with one or more payloads, one or more coating agents, and/or one or more other agents so that certain combined elements are associated with the external surface of nanoparticles.

[0253] In some embodiments, provided nanoparticle compositions comprise nanoparticles combined with one or more payloads, one or more coating agents, and/or one or more other agents so that certain combined elements are present both in and on nanoparticles.

[0254] In some embodiments, provided nanoparticle compositions comprise nanoparticles combined with one or more payloads, one or more coating agents, and/or one or more other agents so that certain combined elements are mixed with, but not specifically associated with any site on or in, nanoparticles.

[0255] In certain particular embodiments, the present disclosure provides nanoparticle compositions in which a coating agent is localized on the external surface of the nanoparticle; in some such embodiments, a coating agent is preferentially localized on the nanoparticle external surface; in some such embodiments, a coating agent is substantially exclusively localized on the external surface. In some embodiments, provided nanoparticle compositions comprise a

population of nanoparticles. In some embodiments, a population of nanoparticles comprises nanoparticles of a uniform size. In some embodiments, a population of nanoparticles comprises nanoparticles of different sizes; in some embodiments showing a particular size distribution. In many embodiments, provided nanoparticle compositions comprise nanoparticles having sizes (*e.g.*, average, or mean size) within a range defined by a lower limit and an upper limit. In some embodiments, the lower limit is 5 nm, 10 nm, 15 nm, 20 nm, 25 nm, 30 nm, 35 nm, 40 nm, 45 nm, 50 nm, 55 nm, 60 nm, 65 nm, 70 nm, 75 nm, 80 nm, 85 nm, 90 nm, 95 nm, 100 nm, 150 nm, 200 nm, or more. In some embodiments, the upper limit is 1000 nm, 950 nm, 900 nm, 850 nm, 800 nm, 750 nm, 700 nm, 650 nm, 600 nm, 550 nm, 500 nm, 450 nm, 400 nm, 350 nm, 300 nm, 250 nm or less. In some embodiments, provided nanoparticle compositions comprise nanoparticles having sizes (*e.g.*, average, or mean size) similar to the size of bacterial cells. For example, in some embodiments, provided nanoparticle compositions comprise nanoparticles having sizes (*e.g.*, average, or mean size) within a range of 100 nm to 2000 nm, 100 nm to 1000 nm, 100 nm to 500 nm, 100 nm to 400 nm, 100 nm to 300 nm, or 100 nm to 200 nm.

[0256] In some embodiments, provided nanoparticle compositions are substantially free of nanoparticles larger than about 2000 nm, about 1000 nm, about 900 nm, about 800 nm, about 700 nm, about 600 nm, about 500 nm, about 400 nm, or about 300 nm. In some embodiments, provided nanoparticle compositions comprise no more than about 50%, about 25%, about 10%, about 5%, or about 1% of nanoparticles larger than about 2000 nm, about 1000 nm, about 900 nm, about 800 nm, about 700 nm, about 600 nm, about 500 nm, about 400 nm, or about 300 nm. Without wishing to be held to a particular theory, it is contemplated that smaller nanoparticles contain more payloads than larger particles.

[0257] In some embodiments, a weight ratio of a payload to a polymer in a nanoparticle composition is within a range of about 0.001:1 to 1:1; 0.001 to 0.1:1, or 0.01:1 to 0.1:1.

[0258] In some embodiments, a weight ratio of a coating to a polymer in a nanoparticle composition is within a range of about 0.001:1 to 1:1; 0.001 to 0.1:1, or 0.01:1 to 0.1:1.

[0259] In some embodiments, a weight ratio of a payload to a polymer in a nanoparticle composition may be represented in, *e.g.*, μg (payload) / mg (polymer). For example, in some embodiments, a payload to polymer ratio is no less than 20 μg/mg and no greater than 250 μg/mg. In some embodiments, a ratio of payload to polymer is between 20 μg/mg and 200 μg/mg. In some embodiments, a ratio of payload to polymer is between 20 μg/mg and 150

μg/mg. In some embodiments, a ratio of payload to polymer is between 20 μg/mg and 100 μg/mg. In some embodiments, a ratio of payload to polymer is between 30 μg/mg and 150 μg/mg. In some embodiments, a ratio of payload to polymer is between 30 μg/mg and 100 μg/mg. In some embodiments, a ratio of payload to polymer is between 50 μg/mg and 100 μg/mg.

[0260] In some embodiments, a weight ratio of a payload on a surface of nanoparticles to a payload in nanoparticles (*e.g.*, encapsulated, mixed, associated within nanoparticles) is within a range of about 0.001:1 to 1:1; 0.001 to 0.1:1, or 0.01:1 to 0.1:1. Without wishing to be held to a particular theory, it is contemplated that nanoparticles with a low ratio of a payload on a surface to a payload in nanoparticles are beneficial, when a payload need to be protected from endogenous RNases (*e.g.*, a nanoparticle preparation is given by sublingual or oral administration). In some embodiments, a high ratio of a payload on a surface of to a payload in nanoparticles are beneficial when a fast release of a payload is required (*e.g.*, for uptake by skeletal muscle cells).

[0261] In some embodiments, provided compositions may also contain a certain amount (*e.g.*, relative to initial protein starting material input) of free (*e.g.*, unencapsulated) protein. For example, in some embodiments, an amount of unencapsulated protein is 5-30% of an originally input amount of protein. In some embodiments, a certain amount of free protein is allowed to remain in a given composition (*e.g.*, approximately 20% or less).

[0262] In some embodiments, free protein is removed from a preparation comprising nanoparticles using one or more separation methods as described herein. In some embodiments, free protein is reduced to approximately no greater than 1-5% of total protein relative to that originally put into an initial polymer/payload combination. In some embodiments, free protein is reduced to approximately no greater than 2.5-5%, 5-10%, 10-15%, 15-20%, or 20-25% of total protein relative to that originally put into an initial polymer/payload combination. In some such embodiments, an amount of free protein in a provided composition is not sufficient to trigger an allergic reaction when administered to a subject allergic to the protein. In some embodiments, an amount of free protein is not sufficient to increase risk of anaphylaxis when administered to a subject allergic to the protein. Without wishing to be bound by any particular theory, it is contemplated that a certain amount of free protein in a given composition as described herein may be desirable. For example, in some embodiments, a certain amount of free protein may act

synergistically with administered nanoparticles such that a desirable immune response is activated in an individual to whom the nanoparticles are administered.

[0263] The present disclosure provides an insight that manufacturing protocols as described herein may produce one or more populations of nanoparticles. As used herein, the term "population" refers to a group of nanoparticles sharing a particular characteristic (*e.g.*, size, payload, payload concentration, coating agent, amount of coating agent, *etc.*). For example, in some embodiments, a population of nanoparticles may have a mean size of between approximately 100-500 nm (*e.g.*, mean average size of, *e.g.*, 100 nm, 150 nm, 200 nm, 250 nm, 300 nm, 350 nm, 400 nm, 450 nm, 500 nm). In some embodiments, different populations of nanoparticles are represented by different sizes (*e.g.*, mean size, *e.g.*, mean range of approximately 100-200 nm in at least one dimension, 100-300 nm in at least one dimension, 100-400 nm in at least one dimension, 100-500 nm in at least one dimension, *etc.*).

[0264] In some embodiments, nanoparticles between 100-400 nm have higher ratios of payload: polymer than nanoparticles larger than 400 nm (*i.e.*, higher encapsulation percentage). In some embodiments, nanoparticles with the higher payload: polymer ratio are between 100-200 nm. In some embodiments, nanoparticles greater than approximately 400 nm have a lower payload: polymer ratios than nanoparticles smaller than 400 nm (*i.e.*, larger than 400 nm have a lower encapsulation percentage than smaller than 400 nm). In some embodiments, payloads are considered to be "encapsulated" when they are not detectable as "free" (e.g., when nanoparticles have not been disrupted); in some such embodiments, at least 80% or at least 85% or at least 90% of payload is encapsulated. For example, in some embodiments, assessment of total payload utilized in nanoparticle manufacturing, and of "free" payload detectable when nanoparticles have not been disrupted reveal that no more than about 10%, 15%, or 20% of the total payload is detected as "free" payload.

[0265] In some embodiments, a nanoparticle composition comprises at least one polymer having a concentration within a range of about 10 to 90 %, 20 to 80%, 25 to 70%, or 25 to 65% by weight. In some embodiments, a nanoparticle composition comprises a plurality of polymers with a total concentration of polymer within a range of about 10 to 90 %, 20 to 80%, 25 to 70%, or 25 to 65% by weight. In some embodiments, a nanoparticle composition comprises one or more payloads having a concentration within a range of, by way of non-limiting example, about 0.1 to 10 %, 0.1 to 5, 0.5 to 10%, 0.5 to 5%, or 1 to 3 % by weight. In some embodiments, a

nanoparticle composition comprises a coating agent having a concentration within a range of about 0.1 to 5 %, 0.1 to 3, 0.5 to 5, 0.5 to 3, or 1 to 3 % by weight.

[0266] In some embodiments, a nanoparticle composition is characterized with respect to the size of nanoparticles, uniformity of a payload within a nanoparticle, payload content, release rate of payload and/or surface exposure of payloads (*e.g.*, how much of the payload(s) are exposed at/accessible from the surface of the nanoparticle). Surface exposure of payloads may be assessed using a proteolysis assay (*e.g.*, surface exposed payloads are susceptible to protease added to the media, whereas materials encapsulated within particle are protected) or by an antibody binding assay.

[0267] In some embodiments, a nanoparticle composition is biodegradable. In some embodiments, a polymer of a nanoparticle composition is decomposed (*e.g.*, nanoparticles release payloads), when they are exposed to a physiological environment.

[0268] In some embodiments, a nanoparticle composition is capable of interacting with biological systems and/or of inducing one or more desired biological responses. For example, in some embodiments, a nanoparticle composition may be i) susceptible to uptake by macrophages and/or antigen presenting cells, ii) able to activate toll-like receptors, or iii) able to induce relevant responses *in vitro* or *in vivo* assays of immunological parameters (*e.g.*, cytokine release, proliferation, *etc.*).

[0269] In some embodiments, a provided preparation may include a plurality of nanoparticle populations, each of which shares one or more structural and/or functional characteristics. For example, in some embodiments, each nanoparticle population has substantially uniform size distribution, common payload(s), and/or the same amount of the common payload(s). In some embodiments, each of nanoparticle populations comprises one or more payloads. In some embodiments, nanoparticle populations comprise different payloads or different payload combinations from each other.

[0270] In some embodiments, each of nanoparticle populations may be manufactured separately. In some embodiments, nanoparticle populations may be manufactured together initially, and then divided for post-processing, coating, adding, drying, and/or freezing.

[0271] In some embodiments, each of nanoparticle populations may include a coating agent that localizes members of the set to a particular target site. Alternatively or additionally, in some embodiments, provided nanoparticle compositions may comprise a plurality of sets each of

which is designed to have and/or is characterized by a different half-life (*e.g.*, in a relevant tissue or organ of interest) and/or different components (*e.g.*, in the lumen or associated with external surface, different populations of antigens, *etc.*).

[0272] In some embodiments, provided nanoparticle compositions can achieve immune modulation. Those skilled in the art, reading the present disclosure, will appreciate that, in some embodiments, it may be desirable to prepare and/or utilize nanoparticle compositions that modulate an immune response to an antigen payload toward or away from a particular type of response. For example, in some embodiments, it may be desirable to stimulate or sustain a Th1 response (*e.g.*, when a payload is an allergen), a Th2 response (*e.g.*, when the payload is an antigen derived from an extracellular parasite or extracellular bacteria, a Treg response (*e.g.*, when the payload is an antigen derived from an autoantigen or alloantigen, such as antigens involved in autoimmune disease or graft-versus-host disease), *etc.*

[0273] In some embodiments, nanoparticle compositions with allergen payload modulate a recipient's immune response away from a Th2 response and/or toward a Th1 and/or Treg response to such allergen. In some embodiments, nanoparticle compositions with infectious antigens modulate a recipient's immune response toward immunity to the infectious agent from which the antigen(s) is/are derived. In some embodiments, nanoparticle compositions with cancer antigens modulate a recipient's immune response toward a T-cell response effective against cancer cells displaying or releasing such cancer antigen(s). In some embodiments, nanoparticle compositions containing alloantigens modulate a recipient's immune response toward desensitization to such alloantigen(s).

[0274] Among other things, the present disclosure documents stimulation of Th1-type immune reactions with nanoparticle compositions containing lipids (*e.g.*, an *E. coli* lipid extract and/or lipopolysaccharide) on their surfaces. Without wishing to be bound by any particular theory, we propose that such nanoparticle compositions may be viewed by a recipient's immune system as analogous to bacterial agents (e.g., to bacterial cells). The present disclosure proposes and demonstrates that this ability to direct a Th1-type immune response to an administered nanoparticle composition presents an opportunity to shift or otherwise bias a recipient's immune response to one or more antigens included in the nanoparticle composition toward such a Th1-type response; such an effect is particularly useful in the treatment of allergy in an encapsulated allergen.

[0275] In some embodiments, a particular subject may benefit from being exposed to a combination of antigens. For example, in some embodiments, a combination of antigens may promote an immune response to one agent (*e.g.*, infectious agent, tumor, *etc.*). In some embodiments, a combination of antigens may promote an immune response to two or more agents (*e.g.*, infectious agent, tumor, *etc.*). In some embodiments, a nanoparticle preparation may comprise a first nanoparticle population comprising a first payload, or precursor(s) thereof, that activate first antigen-specific T cells, and a second payload, or precursor(s) thereof, that activate second antigen-specific T cells. In some embodiments, the first payload is displayed by an MHC class I complex. In some embodiments, the second payload is displayed by an MHC class II complex. In some embodiments, a first nanoparticle population and a second nanoparticle population are included in a same composition (*e.g.*, capsules, tablets, pills, powders, and/or granules). In some embodiments, a first nanoparticle population and a second nanoparticle population are included in different compositions. In some embodiments, a multi-NP system comprises an immune adjuvant. In some embodiments, provided nanoparticle preparation comprises one or more immune adjuvants of each of one or more antigen.

[0276] In some embodiments, when a nanoparticle preparation is desired to comprise multiple combinations of payloads (*e.g.*, the first combination of the first antigen and the first adjuvant, the second combination of the second antigen and the second adjuvant) and the multiple combinations are chosen to be separated from each other, the nanoparticle preparation may include two or more nanoparticle populations for each combination, and two or more nanoparticle populations may be processed differently.

[0277] In some embodiments, manufacturing process of each nanoparticle population can be adapted to incorporate a particular payload effectively and/or accurately, maintaining biological activities of the payload. For example, in some embodiments, when a nanoparticle preparation is desired to have two or more payloads, and the payloads are chosen to be separated from each other or the payloads require different manufacturing processes, the nanoparticle preparation may include two or more nanoparticle populations for each payloads (*e.g.*, the first nanoparticle population for the first payload, and the second nanoparticle population for the second payload), and two or more nanoparticle populations may be processed separately and/or differently.

[0278] In some embodiments, when a nanoparticle preparation is desired to include a payload having multiple concentrations and/or amounts, a nanoparticle preparation may include two or

more nanoparticle populations for each concentration/amount, and two or more nanoparticle populations may be processed separately to facilitate multiple concentrations and/or amounts.

[0279] In some embodiments, a provided nanoparticle composition may be characterized by a safety factor (*e.g.*, when measured as described in Example 3, for instance). In some embodiments, a safety factor may be between 5-100 or more. In some embodiments, a safety factor is between approximately 5 and 20. In some embodiments, a safety factor is between approximately 25 and 100. In some embodiments, a safety factor is between a range of approximately 30-90. In some embodiments, a safety factor is between a range of approximately 40-80. In some embodiments, a target safety factor is greater than about 10. In some embodiments, a lower safety factor may be desirable. In some embodiments, a higher safety factor may be desirable. In some such embodiments, a particular safety factor indicates that a quantity of free protein is not great enough to result in risk of anaphylaxis, when administered to a subject with an allergy to the protein.

[0280] The present disclosure provides an insight that manufacturing protocols as described herein may produce one or more populations of nanoparticles. As used herein, the term "population" refers to a group of nanoparticles sharing a particular characteristic (*e.g.*, size, payload, payload concentration, coating agent, amount of coating agent, *etc.*). For example, in some embodiments, a population of nanoparticles may have a mean size of between approximately 100-500 nm (*e.g.*, mean average size of, *e.g.*, 100 nm, 150 nm, 200 nm, 250 nm, 300 nm, 350 nm, 400 nm, 450 nm, 500 nm). The mean size of nanoparticles may be measured using a Z-average. In some embodiments, different populations of nanoparticles are represented by different sizes (*e.g.*, mean size, *e.g.*, mean range of approximately 100-200 nm in at least one dimension, 100-300 nm in at least one dimension, 100-400 nm in at least one dimension, 100-500 nm in at least one dimension, *etc.*). In some preferred embodiments, a population of nanoparticles are about 225nm to about 450nm.

[0281] In some embodiments, a population of nanoparticles is represented by a particular mean size (*e.g.*, 150 nm), but is itself comprised of more than one population of nanoparticles.

[0282] In some embodiments, payload encapsulation results in one or more populations of nanoparticles, *e.g.*, one or more sets of sizes, *e.g.*, one or more of nanoparticles with higher encapsulation percentages than other sets of nanoparticles. In some embodiments, nanoparticles comprise approximately 20-90 μg, 30-90 μg, or 50-75 μg payload/mg polymer.

[0283] In some embodiments, a nanoparticle composition has a total protein concentration of 100-10,000 ug/mL. In some embodiments, a nanoparticle composition has a total protein concentration of 500-5,000 ug/mL. In some preferred embodiments, a nanoparticle composition has a total protein concentration of 1,800-3,500 ug/mL.

[0284] The present disclosure provides an insight that certain steps may be taken in order to improve encapsulation of payload in loaded nanoparticles. In some embodiments, encapsulation (relative to 100% of starting protein amount) is between approximately 10-95%. In some embodiments, encapsulation of protein is approximately 10-20%. In some embodiments, encapsulation of protein is approximately 20-30%. In some embodiments, encapsulation of protein is approximately 30-40%. In some embodiments, encapsulation of protein is approximately 40-50%. In some embodiments, encapsulation of protein is approximately 50-90%. In some embodiments, encapsulation of protein is approximately 60-90%. In some embodiments, encapsulation of protein is approximately 70-90%.

[0285] In some embodiments, purification procedures are altered to selectively eliminate and/or selectively enrich for a particular population of nanoparticles.

[0286] In some embodiments, a provided nanoparticle preparation may have a zeta potential range of about – 50 mV to about 0 mV, about – 40 mV to about 0 mV, about – 30 mV to about 0 mV, about – 20 mV to about 0 mV, or about – 15 mV to about 0 mV.

[0287] In some embodiments, a provided nanoparticle preparation may be suitable to be stored (*e.g.*, stable) at temperature at or above -80 °C, -70 °C, -60 °C, -50 °C, -40 °C, -30 °C, -20 °C, -4 °C, 0 °C, or room temperature.

[0288] In some embodiments, the present disclosure provides a nanoparticle preparation prepared by the methods provided herein, and the nanoparticle preparation comprises a plurality of nanoparticles, each of which comprises a payload (e.g., a hydrophilic payload) in a polymer.

[0289] In some embodiments, a separation step comprising centrifugation results in a composition comprising nanoparticles with improved PDI (*e.g.*, a lower PDI as compared to a population of nanoparticles produced via control or unoptimized methods) as compared to a separation step not comprising centrifugation. For example, in some embodiments, a composition comprising nanoparticles of the present disclosure has a PDI of less than about 0.5, 0.4, 0.3, 0.2, 0.1. In some preferred embodiments, a PDI is less than about 0.3, 0.2, 0.1. In some preferred embodiment, a PDI is from about 0.1 to about 0.4.

Pharmaceutical Compositions

[0290] In some embodiments, the present disclosure provides pharmaceutical compositions comprising one or more provided nanoparticle compositions together with one or more pharmaceutically acceptable excipients.

[0291] In some embodiments, provided pharmaceutical compositions may be prepared by any appropriate method, for example as known or hereafter developed in the art of pharmacology. In general, such preparatory methods include the step of bringing a provided nanoparticle composition into association with one or more pharmaceutically acceptable excipients, and then, if necessary and/or desirable, shaping and/or packaging the product into an appropriate form for administration, for example as or in a single- or multi-dose unit.

[0292] In some embodiments, compositions may be prepared, packaged, and/or sold in bulk, as a single unit dose, and/or as a plurality of single unit doses. As used herein, a "unit dose" is a discrete amount of the pharmaceutical composition comprising a predetermined amount of the provided nanoparticle composition. The amount of the provided nanoparticle composition is generally equal to the dosage of the provided nanoparticle which would be administered to a subject and/or a convenient fraction of such a dosage such as, for example, one-half or one-third of such a dosage.

[0293] In many embodiments, provided pharmaceutical compositions are specifically formulated for mucosal delivery (*e.g.*, oral, nasal, rectal or sublingual delivery).

[0294] In some embodiments, appropriate excipients for use in provided pharmaceutical compositions may, for example, include one or more pharmaceutically acceptable solvents, dispersion media, granulating media, diluents, or other liquid vehicles, dispersion or suspension aids, surface active agents and/or emulsifiers, isotonic agents, thickening or emulsifying agents, preservatives, solid binders, lubricants, disintegrating agents, binding agents, preservatives, buffering agents and the like, as suited to the particular dosage form desired. Alternatively or additionally, pharmaceutically acceptable excipients such as cocoa butter and/or suppository waxes, coloring agents, sweetening, flavoring, and/or perfuming agents can be utilized. Remington's *The Science and Practice of Pharmacy*, 21st Edition, A. R. Gennaro (Lippincott, Williams & Wilkins, Baltimore, MD, 2005; incorporated herein by reference) discloses various

excipients used in formulating pharmaceutical compositions and known techniques for the preparation thereof.

[0295] In some embodiments, an appropriate excipient is at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% pure. In some embodiments, an excipient is approved by United States Food and Drug Administration. In some embodiments, an excipient is pharmaceutical grade. In some embodiments, an excipient meets the standards of the United States Pharmacopoeia (USP), the European Pharmacopoeia (EP), the British Pharmacopoeia, and/or other International Pharmacopoeia.

[0296] In some embodiments, liquid dosage forms (*e.g.*, for oral and/or parenteral administration) include, but are not limited to, emulsions, microemulsions, solutions, suspensions, syrups, and/or elixirs. In addition to provided nanoparticle compositions, liquid dosage forms may comprise inert diluents commonly used in the art such as, for example, water or other solvents, emulsifiers such as ethyl alcohol, isopropyl alcohol, ethyl carbonate, ethyl acetate, benzyl alcohol, benzyl benzoate, propylene glycol, 1,3-butylene glycol, dimethylformamide, oils (in particular, cottonseed, groundnut, corn, germ, olive, castor, and sesame oils), glycerol, tetrahydrofurfuryl alcohol, polyethylene glycols and fatty acid esters of sorbitan, and mixtures thereof. Besides inert diluents, oral compositions can include adjuvants such as wetting agents, emulsifying and suspending agents, sweetening, flavoring, and/or perfuming agents. In certain embodiments for parenteral administration, compositions are mixed with solubilizing agents such a CREMOPHOR®, alcohols, oils, modified oils, glycols, polysorbates, cyclodextrins, polymers, and/or combinations thereof.

[0297] In some embodiments, injectable preparations, for example, sterile aqueous or oleaginous suspensions, may be formulated according to known methods using suitable dispersing agents, wetting agents, and/or suspending agents. In some embodiments, provided injectable preparations may be stored in a pre-filled syringe. Sterile liquid preparations may be, for example, solutions, suspensions, and/or emulsions in nontoxic parenterally acceptable diluents and/or solvents, for example, as a solution in 1,3-butanediol. Among the acceptable vehicles and solvents that may be employed, for example, are water, Ringer's solution, U.S.P., and isotonic sodium chloride solution. Sterile, fixed oils are conventionally employed as a solvent or suspending medium. For this purpose, any bland fixed oil can be employed including synthetic

mono- or diglycerides. Fatty acids such as oleic acid can be used in the preparation of liquid formulations.

[0298] Liquid formulations can be sterilized, for example, by filtration through a bacterial-retaining filter, and/or by incorporating sterilizing agents in the form of sterile solid compositions which can be dissolved or dispersed in sterile water or other sterile injectable medium prior to use.

[0299] In some embodiments, one or more strategies may be utilized prolong and/or delay the effect of a provided nanoparticle composition after delivery.

[0300] In some embodiments, provided pharmaceutical compositions may be formulated as suppositories, for example for rectal or vaginal delivery. In some embodiments, suppository formulations can be prepared by mixing utilizing suitable non-irritating excipients such as cocoa butter, polyethylene glycol or a suppository wax which are solid at ambient temperature but liquid at body temperature and therefore melt in the body (*e.g.*, in the rectum or vaginal cavity) and release the provided nanoparticle composition.

[0301] In some embodiments, solid dosage forms (*e.g.*, for oral administration) include one or more portions of a provided nanoparticle composition that may be or comprise capsules, tablets, pills, powders, and/or granules. In such solid dosage forms, the provided nanoparticle composition may be mixed with at least one inert, pharmaceutically acceptable excipient such as sodium citrate or dicalcium phosphate and/or fillers or extenders (*e.g.*, starches, lactose, sucrose, glucose, mannitol, and silicic acid), binders (*e.g.*, carboxymethylcellulose, alginates, gelatin, polyvinylpyrrolidinone, sucrose, and acacia), humectants (*e.g.*, glycerol), disintegrating agents (*e.g.*, agar, calcium carbonate, potato starch, tapioca starch, alginic acid, certain silicates, and sodium carbonate), solution retarding agents (*e.g.*, paraffin), absorption accelerators (*e.g.*, quaternary ammonium compounds), wetting agents (*e.g.*, acetyl alcohol and glycerol monostearate), absorbents (*e.g.*, kaolin and bentonite clay), and lubricants (*e.g.*, talc, calcium stearate, magnesium stearate, solid polyethylene glycols, sodium lauryl sulfate), and mixtures thereof. In the case of capsules, pills and tablets, the dosage form may comprise buffering agents.

[0302] In some embodiments, solid compositions of a similar type may be employed as fillers in soft and/or hard-filled gelatin capsules using such excipients as lactose or milk sugar as well as high molecular weight polyethylene glycols and the like. The solid dosage forms of capsules,

pills, and tablets, impregnated filter paper, and granules can be prepared with coatings and shells such as enteric coatings and other coatings well known in the pharmaceutical formulating art.

[0303] Exemplary enteric coatings include, but are not limited to, one or more of the following: cellulose acetate phthalate; methyl acrylate-methacrylic acid copolymers; cellulose acetate succinate; hydroxy propyl methyl cellulose phthalate; hydroxy propyl methyl cellulose acetate succinate (hypromellose acetate succinate); HP55; polyvinyl acetate phthalate (PVAlP); methyl methacrylate-methacrylic acid copolymers; methacrylic acid copolymers, cellulose acetate (and its succinate and phthalate version); styrol maleic acid co-polymers; polymethacrylic acid/acrylic acid copolymer; hydroxyethyl ethyl cellulose phthalate; hydroxypropyl methyl cellulose acetate succinate; cellulose acetate tetrahydrophtalate; acrylic resin; shellac, and combinations thereof.

[0304] In some embodiments, solid dosage forms may optionally comprise opacifying agents and can be of a composition that they release the provided nanoparticle composition(s) only, or preferentially, in a certain part of the intestinal tract, optionally, in a delayed manner. Examples of embedding compositions which can be used include polymeric substances and waxes. Solid compositions of a similar type may be employed as fillers in soft and hard-filled gelatin capsules using such excipients as lactose or milk sugar as well as high molecular weight polyethylene glycols and the like.

[0305] In some embodiments, the present disclosure provides compositions for topical and/or transdermal delivery, *e.g.*, as a cream, liniment, ointment, oil, foam, spray, lotion, liquid, powder, thickening lotion, or gel. Particular exemplary such formulations may be prepared, for example, as products such as skin softeners, nutritional lotion type emulsions, cleansing lotions, cleansing creams, skin milks, emollient lotions, massage creams, emollient creams, make-up bases, lipsticks, facial packs or facial gels, cleaner formulations such as shampoos, rinses, body cleansers, hair-tonics, or soaps, or dermatological compositions such as lotions, ointments, gels, creams, liniments, patches, deodorants, or sprays.

[0306] In some embodiments, an adjuvant is provided in the same formulation with provided nanoparticle composition(s) so that adjuvant and provided nanoparticle composition are delivered substantially simultaneously to the individual. In some embodiments, an adjuvant is provided in a separate formulation. Separate adjuvant may be administered prior to, simultaneously with, or subsequent to provided nanoparticle composition administration.

[0307] In some embodiments, provided compositions are stable for extended periods of time, such as 1 week, 2 weeks, 1 month, 2 months, 6 months, 1 year, 2 years, 3 years, or more. In some embodiments, provided compositions are easily transportable and may even be sent via traditional courier or other package delivery service. Accordingly, some embodiments may be useful in situations of disease outbreak, such as epidemics, or attacks with biological agents (*e.g.*, anthrax, smallpox, viral hemorrhagic fevers, plague, and others) at least in part due to their ability to be stored for long periods of time and transported quickly, easily, and safely. Such attributes may allow for rapid distribution of provided compositions to those in need.

[0308] In some embodiments, it may be advantageous to release a payload, for example, an antigen, at various locations along a subject's gastrointestinal (GI) tract. In some embodiments, it may be advantageous to release a payload, for example, an antigen, in a subject's mouth as well as one or more locations along the subject's GI tract. Accordingly, in some embodiments, a plurality of provided compositions (*e.g.*, two or more) may be administered to a single subject to facilitate release of a payload at multiple locations. In some embodiments, each of the plurality of compositions has a different release profile, such as provided by various enteric coatings, for example. In some embodiments, each of the plurality of compositions has a similar release profile. In some embodiments, the plurality of compositions comprises one or more antigens. In some embodiments, each of the plurality of administered compositions comprises a different antigen. In some embodiments, each of the plurality of compositions comprises the same antigen.

[0309] In some embodiments, a provided pharmaceutical composition is characterized in that the composition does not comprise an amount of free protein that is expected to and/or does increase risk of allergic reaction (*e.g.*, anaphylaxis) when administered to a subject allergic to the protein. In some such embodiments, a provided pharmaceutical composition is characterized by a particular safety factor as described herein, including, *e.g.*, in Example 7B (*e.g.*, 5-20, *e.g.*, 20-100, *e.g.*, 20-80, *etc.*).

[0310] In some embodiments, a pharmaceutical composition may include one or more stabilizing agents, such as one or more cryoprotectants. In some embodiments, a stabilizing agent may be or comprise a sugar such as sucrose and/or trehalose. The present disclosure teaches that such agent(s) may be particularly useful for emulsion formulations and/or liquid formulations, and particularly for liquid emulsion formulations.

[0311] Alternatively or additionally, in some embodiments, a pharmaceutical composition may include an agent such as TCA and/or PVA, one or both of which may be particularly useful for emulsion formulations and/or liquid formulations, and particularly for liquid emulsion formulations.

Characterization of compositions and components thereof

[0312] In some embodiments, provided compositions may be characterized in order to determine, for example, protein content per nanoparticle. Those skilled in the art will be aware of a variety of technologies available to characterize nanoparticle compositions provided in accordance with the present disclosure.

[0313] For example, in some embodiments, characterization may include, *e.g.*, one or more of assessing (e.g., identifying and/or quantifying) presence of polymer component, payload component(s), coating agent(s) and/or other agent(s), determining intactness of such polymer component, payload component(s), coating agent(s) and/or other agent(s), assessing degree of encapsulation of one or more payload component(s), determining extent of coating, assessing relative amount(s) (e.g., weight percent) of different components – e.g., payload to polymer, coating to polymer, coating to payload, etc., assessing one or more features of particle size and/or particle size distribution, determining microbial load, quantifying payload encapsulation efficiency, assessing content of payload (*e.g.*, determining if payload contains expected amounts and/or forms), evaluating a surface coating, *etc.*

[0314] In some embodiments, as described herein, a weight ratio of a payload to a polymer in a nanoparticle composition is within a range of about 0.001:1 to 1:1; 0.001 to 0.1:1, or 0.01:1 to 0.1:1. In some embodiments, a weight ratio of a payload to a polymer in a nanoparticle composition may be represented in, *e.g.*, μg (payload) / mg (polymer). For example, in some embodiments, a payload to polymer ratio is no less than 30 μg/mg and no greater than 250 μg/mg. In some embodiments, a ratio of payload to polymer is between 30 μg/mg and 150 μg/mg. In some embodiments, a ratio of payload to polymer is between 50 μg/mg and 100 μg/mg.

[0315] In some embodiments, as described herein, a weight of payload in an individual dose of the nanoparticles is within a range of about 1-10μg, 5-50μg, 10-100μg, 50-500μg, 100-1000μg, 500-5000μg, 1000-10,000μg.

[0316] In some embodiments, characterization includes an evaluation of encapsulation efficiency (*e.g.*, amount of payload provided during production of nanoparticles versus amount of payload encapsulated by polymer measured during or after nanoparticles are forming or formed). In some embodiments, encapsulation efficiency is no lower than 40%. In some embodiments, encapsulation efficiency is substantially 100%. In some embodiments, encapsulation efficiency is between 50% and 100%; 60% and 100%; 70% and 100%; 75% and 100%; 80% and 100%; 90% and 100%; and 95% and 100%. In some embodiments, encapsulation is between 75% and 95%; 80% and 90%; 85% and 95%.

[0317] In some embodiments, characterization includes analysis of certain properties or features of compositions as provided herein. Such characterization for, *e.g.*, nanoparticles or pharmaceutical compositions will be known to one of skill in the art. For example, in some embodiments, characterization includes visualization by microscopy (*e.g.*, fluorescent microscopy, scanning electron microscopy, *etc.*). In some embodiments, microscopic evaluation is performed after each of multiple steps (*e.g.*, to evaluate status of composition and any nanoparticles therein).

[0318] In some embodiments, characterization may include, *e.g.*, taking an aliquot from a composition during and/or at various points throughout the production process. In some embodiments, an aliquot of a nanoparticle composition, as described herein, is removed. By way of non-limiting example, when an aliquot of nanoparticle suspension solution is removed, the aliquot can be analyzed to determine, *e.g.*, free payload and/or payload encapsulation efficiency. For example, an aliquot of nanoparticle suspension may be analyzed in a method that comprises steps of removing an aliquot of nanoparticle suspension, centrifuging at low speed (*e.g.*, 1500-2500 rcf), hydrolyzing said suspension with NaOH, and then analyzing using an assay that measures payload content (*e.g.*, BCA, Bradford, *etc.* when a payload is or comprises protein). Without wishing to be bound by any theory, it is contemplated that in some embodiments, such a low-speed spin prior to hydrolysis accomplishes separation of nanoparticles from free payload without damaging any already formed nanoparticles. Once an assay has been performed, the resulting number(s) represent quantification of total payload per volume of suspension. Remaining suspension (*i.e.*, suspension that has not been analyzed via payload measurement assay) can then be spun down using an ultracentrifuge (*e.g.*, spinning at or about 350,000 rcf), and resulting supernatant analyzed for free payload, resulting in another measurement of total

payload per volume of solution (a method involving a high-speed spin as described herein may be referred to as "Method 1"). Results from the initial aliquot and the ultracentrifuged sample are then compared to determine encapsulation percentage. In some embodiments, a sample may be filtered through a 100 nm centrifuge filter, prior to ultracentrifugation. In some embodiments, a sample is not filtered through a centrifuge filter, prior to centrifugation.

[0319] In some embodiments, rather than spinning in an ultracentrifuge [*e.g.*, after initial aliquot removal, spinning, hydrolysis and protein analysis], an additional low speed spin (*e.g.*, spin at or about 1500-2500 rcf) may be performed (a method involving a second, low-speed spin as described herein may be referred to as "Method 2"). One of skill in the art, depending on context, will be able to determine when low and/or higher speed centrifugation steps will desirably to be performed.

[0320] In some embodiments, method 1 is a preferred method for characterizing quantity of free payload and/or encapsulation efficiency of payload in compositions as described herein. Without wishing to be bound by any theory, it is contemplated that a second, low speed spin may not recover all nanoparticles and/or protein in a given nanoparticle suspension or aliquot thereof.

[0321] In some embodiments, presence, amount, form and/or integrity of one or more non-payload components is determined (e.g., quantified). For example, in some embodiments, polymer component (e.g., PLG) is detected, quantified and/or assessed for integrity. Alternatively or additionally, presence, amount, form and/or integrity of one or more other components (e.g., solvent or medium, such as water; cryoprotectant such as trehalose, stabilizer such as PVA, etc., is quantified.

[0322] In certain particular embodiments, nanoparticle compositions may be or include nanoparticles that are substantially free of a payload component; in some such examples, presence, amount, form and/or integrity of one or more non-payload components can be determined.

[0323] Dynamic Light Scattering ("DLS") is a method that can be used to characterize nanoparticles of the present disclosure and can allow for measures such as polydispersity index ("PDI"), which is a measure of size distribution of a given population of, for example, nanoparticles. In some embodiments, a separation step comprising centrifugation results in a composition comprising nanoparticles with improved PDI (*e.g.*, a lower PDI as compared to a population of nanoparticles produced via control or unoptimized methods) as compared to a

separation step not comprising centrifugation. For example, in some embodiments, a composition comprising nanoparticles of the present disclosure has a PDI of less than about 0.5, 0.4, 0.3, 0.2, 0.1. In some preferred embodiments, a PDI is less than about 0.3, 0.2, 0.1.

[0324] In some embodiments, characterization of nanoparticles includes evaluation using dynamic light scattering ("DLS") and/or polydispersity index ("PDI"). For example, in some embodiments, dynamic light scattering may be used to evaluate one or more aliquots of solution from one or more stages of manufacturing processes as described herein. In some embodiments, dynamic light scattering may provide information that can be used to alter manufacturing protocols. For example, in some embodiments, if dynamic light scattering shows nanoparticles of particular sizes that are not found in later samples, additional or different steps may be inserted into manufacturing processes. In some embodiments, PDI may be used to evaluate the breadth of distribution of nanoparticle sizes in a given sample. In some embodiments, if PDI shows increased numbers (*e.g.*, greater than 0.5, 0.6, 0.7, 0.8), DLS data may not be able to accurately quantify the size distribution of a sample of nanoparticles.

[0325] In some embodiments, DLS data are expressed as z-averages. In some embodiments, when two populations are compared, a lower z-average in one population indicates a more uniform/homogenous set of nanoparticles as compared to the other population.

[0326] PDI measurements are unitless and, in some embodiments, when comparing two nanoparticle populations, a lower PDI in one population indicates a more uniform/homogenous sample as compared to the other.

[0327] In some embodiments, a z-average is lower than 500 nm. In some embodiments, a z-average is lower than 450, 400, 350, 300, 250 nm.

[0328] In some embodiments, a PDI measurement is lower than 0.7, 0.6, 0.55, 0.5, 0.45, 0.4, 0.35, 0.3, and lower.

Characterization of payload

[0329] In some embodiments, a payload of nanoparticle compositions is evaluated in one or more ways at one or more times.

[0330] For example, in some embodiments, payload encapsulated by a provided nanoparticle composition is evaluated both before and after incorporation into nanoparticles (and compared to protein not encapsulated by nanoparticles). In some such embodiments, evaluation is performed,

for example to ensure that processing into nanoparticles has not materially altered payload components.

[0331] Alternatively or additionally, in some embodiments, payload component(s) may be characterized while nanoparticles are still intact, for example to assess extent to which payload may be present on nanoparticle surface(s) and/or may have been released from nanoparticles.

[0332] Those skilled in the art will be aware of a variety of technologies available for evaluating payloads of different types (*e.g.,* proteins, nucleic acids, carbohydrates, *etc.*), and will be able to select appropriate such technologies depending on, for example, type and/or identity of payload component(s) to be assessed.

[0333] In some embodiments, it may be desirable to assess overall amount of one or more payload component(s). In some embodiments, it may be desirable to assess "intactness" of one or more payload components. In some embodiments, it may be desirable to assess specific identity of a payload component(s); in some embodiment(s) detection of payload type (e.g., protein, nucleic acid) may be sufficient. Again, those skilled in the art will be aware of appropriate analysis technologies (e.g., ELISA to assess identity of a payload component, particularly of a protein payload component, vs BCA for protein content generally).

Release Testing

[0334] In some embodiments, methods disclosed herein can be used to confirm the identity and/or quality of a given composition and/or its components protein, *e.g.*, nanoparticles and/or nanoparticle payload. In some embodiments, methods can include assessing preparations (*e.g.*, samples, lots, and/or batches) of a given composition, *e.g.*, to confirm whether a composition comprises all necessary components, and, optionally, qualifying a compoistion as acceptable for use in administration to subjects (e.g., human subjects) if qualifying criteria (*e.g.*, predefined qualifying criteria) are met; thereby evaluating, identifying, and/or producing (*e.g.*, manufacturing) a nanoparticle composition.

[0335] In some embodiments, methods as disclosed herein can have a variety of applications and can include, *e.g.*, quality control at different stages of manufacture (e.g., of a therapeutic drug substance or drug product), analysis of a nanoparticle preparation prior to and/or after completion of manufacture (*e.g.*, prior to or after distribution to a fill/finish environment or facility), and/or prior to and/or after release into commerce (*e.g.*, before distribution to a

pharmacy, a caregiver, a patient, or other end-user). In some embodiments, a nanoparticle preparation may be a drug substance (*i.e.*, an active pharmaceutical ingredient or "API") or a drug product (*i.e.*, an API formulated for use in a subject such as a human patient). In some embodiments, a given nanoparticle preparation may be from a stage of manufacture or use that is prior to release to end-users; prior to packaging into individual dosage forms, such as single portions of powder or tablets; prior to determination that a batch can be commercially released, prior to production of a Certificate of Testing, Material Safety Data Sheet (MSDS) or Certificate of Analysis (CofA) of a preparation. In some embodiments, a nanoparticle preparation may be from an intermediate step in production, *e.g.*, after formation of a nanoparticle comprising one or more payloads, but prior to further modification and/or purification of a drug substance.

[0336] In some embodiments, evaluations described in the present disclosure can be useful for guiding, controlling or implementing one or more of a number of activities or steps in a process of making, distributing, and monitoring and/or providing for a safe and efficacious use of a nanoparticle preparation. Accordingly, in some embodiments, *e.g.*, responsive to an evaluation, *e.g.*, depending on whether a criterion is met, a decision or step is taken. In some embodiments, methods can further include one or both of a decision to take a step and/or carrying out the step itself. For example, in some embodiments, a step can include one in which a preparation (or another preparation for which the preparation is representative, or an intermediate of a preparation) is: classified; selected; accepted or discarded; released or processed into a drug product; rendered unusable for commercial release, *e.g.*, by labeling it, sequestering it, or destroying it; passed on to a subsequent step in manufacture; reprocessed (*e.g.*, a preparation may undergo a repetition of a previous process step or subjected to a corrective process); formulated, *e.g.*, into drug substance or drug product; combined with another component, *e.g.*, an excipient, buffer or diluent; disposed into a container; divided into smaller aliquots, *e.g.*, unit doses, or multi-dose containers; combined with another nanoparticle preparation (*e.g.*, nanoparticles with the same or different payloads); packaged; shipped; moved to a different location; combined with another element to form a kit; combined, *e.g.*, placed into a package with a delivery device, diluent, or package insert; released into commerce; sold or offered for sale; delivered to an end-user; or administered to a subject. For example, in some embodiments, based on a result of a determination or whether one or more subject entities is present, or upon

comparison to a reference standard, a batch from which a preparation is taken can be processed, *e.g.*, as just described.

[0337] In some embodiments, methods disclosed herein may include making a decision: (a) as to whether a nanoparticle preparation may be formulated into drug substance or drug product; (b) as to whether a nanoparticle preparation may be reprocessed (*e.g.*, a preparation may undergo a repetition of a previous process step, *e.g.*, at any point in the manufacture process, *e.g.*, another homogenization pass during microfluidization and nanoparticle formation); and/or (c) that a nanoparticle preparation may not be suitable for formulation into drug substance or drug product. In some embodiments, methods can include: formulating as referred to in step (a), reprocessing as referred to in step (b), or rendering a preparation unusable for commercial release, *e.g.*, by labeling it or destroying it, as referred to in step (c).

[0338] In some embodiments, methods (*e.g.*,., evaluation, identification, and/or production methods) can further include, *e.g.*, one or more of: providing or obtaining a nanoparticle preparation (*e.g.*, such as a nanoparticle drug substance or a precursor thereof); memorializing confirmation or identification of the nanoparticle preparation as comprising expected and sufficient payload (*e.g.*, protein and DNA) using a recordable medium (*e.g.*, on paper or in a computer readable medium, *e.g.*, in a Certificate of Testing, Certificate of Analysis, Material Safety Data Sheet (MSDS), batch record, or Certificate of Analysis (CofA)); informing a party or entity (*e.g.*, a contractual or manufacturing partner, a care giver or other end-user, a regulatory entity, *e.g.*, the FDA or other U.S., European, Japanese, Chinese or other governmental agency, or another entity, *e.g.*, a compendial entity (*e.g.*, U.S. Pharmacopoeia (USP)) or insurance company) that a nanoparticle preparation contains the expected payload in the expected quantity; selecting the nanoparticle preparation for further processing (*e.g.*, processing (*e.g.*, formulating) the nanoparticle preparation as a drug product (*e.g.*, a pharmaceutical product) if the nanoparticle preparation is identified as containing the expected identiy and quantity of payload; reprocessing or disposing of the nanoparticle preparation if the nanoparticle preparation is not identified as containing the expected identity and/or quantity of payload and/or if the preparation contains something unexpected as detected through quality control analysis and release assays.

[0339] In some embodiments, methods (*e.g.*,., evaluation, identification, and/or production methods) include taking action (*e.g.*, physical action) in response to methods disclosed herein. For example, in some embodiments, a given nanoparticle preparation is classified, selected,

accepted or discarded, released or withheld, processed into a drug product, shipped, moved to a different location, formulated, labeled, packaged, released into commerce, or sold or offered for sale, depending on whether the preselected relationship is met.

[0340] In some embodiments, processing may include formulating, packaging (*e.g.*, in a vial or other container), labeling, or shipping at least a portion of the nanoparticle preparation. In some embodiments, processing may include formulating, packaging (*e.g.*, in a vial or other container), and labeling at least a portion of the nanoparticle as a particular drug product (*e.g.*, NP-PN1). In some embodiments, processing can include directing and/or contracting another party to process as described herein.

Protein Quantification

[0341] In many embodiments, provided nanoparticular compositions comprise a protein component (e.g., a protein payload). In some embodiments, it may be desirable to assess one or more features (e.g., extent of encapsulation, intactness and/or activity while encapsulated, degree and/or timing of release, intactness and/or activity when released, etc) of such protein.

[0342] Those skilled in the art will be aware of various technologies for assessing one or more aspects of a protein component, such as for example its level, form, degree of intactness, activity, extent of encapsulation, timing and/or extent of release, etc. Moreover, those skilled in the art reading the present disclosure will appreciate that it may be desirable to do such assessments at one or more times during manufacture, storage and/or use of provided nanoparticle compositions.

[0343] In some embodiments, protein is assessed using a BCA assay. For example, in some embodiments, a BCA assay may be used to quantify protein in a particular sample (e.g., in a nanoparticle preparation supernatant, and/or after disruption of nanoparticles in a preparation).The present disclosure appreciates that, in some embodiments, performance of one or more assays, specifically including BCA assays, can be impacted by one or more features of nanoparticle production. For example, the present disclosure identifies the source of a problem that can be encountered when PVA is utilized during manufacturing of nanoparticle compositions. PVA solubility is reduced in cold solutions. Without wishing to be bound by any particular theory, the present disclosure proposes that PVA, at least when present in amounts above a particular threshold, may have a tendency to gel under certain circumstances, for

example when made cold and/or when exposed to basic conditions (and/or specifically to NaOH). The present disclosure appreciates that such insolubility and/or gelling may interfere with TFF and/or with other aspect(s) of nanoparticle composition producing, processing and/or assessment. The present disclosure specifically teaches that, in some embodiments, it may be desirable or even necessary to reduce PVA in, or remove PVA from, a nanoparticle preparation prior to performance of a BCA assay thereon.

[0344] In some embodiments, PVA is present during protein measurement.

[0345] In some embodiments, PVA is at least partially removed prior to measuring protein quantity. In some embodiments, at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or 99% of PVA is removed prior to protein measurement.

[0346] In some embodiments, PVA is removed using TFF.

[0347] In some embodiments, if a TFF step is performed to remove PVA, the material is dried (*e.g.*, lyophilized) prior to performing the TFF step.

[0348] In some embodiments, PVA needs to be removed from a sample prior to dissolving the sample in cold buffer. In some such embodiments, if an amount of PVA is greater than 1%, a sample will not be likely to dissolve in cold buffer, leaving undissolved solid material.

[0349] In some embodiments, one or more lyophilization steps that may otherwise be performed, is/are not performed if the amount of PVA present in a particular sample or intermediate composition is above a particular threshold. For example, in some embodiments, if PVA is not removed, one or more lyophilization steps that may otherwise be performed is skipped. In some such embodiments, if one or more lyophilization steps is skipped, it occurs prior to performing filtration. In some embodiments, not completing one or more lyophilization steps occurs before performing a TFF step.

[0350] In some embodiments, a certain amount or concentration of PVA (*e.g.*, 0.5, 1, 2, 3, 4, 5%) may negatively impact accuracy of a BCA assay.

[0351] In some embodiments, concentrations of reagents (*e.g.*, NaOH) may be varied (*e.g.*, 0.5M, 1M, 1.5M, 2M, 2.5M) when performing a BCA assay or any steps (*e.g.*, preparation of sample) related thereto.

[0352] In some embodiments, a standard curve may need to be adjusted, for example to accommodate conditions employed in the production of a particular batch of composition (*e.g.*, of nanoparticles). For example, in some embodiments, one or more standard curves may be

generated using, *e.g.*, NaOH, Ammonium Bicarbonate buffer, or a mixture of NaOH and Ammonium Bicarbonate buffer. In some such embodiments, standard curve adjustments will be determined by measuring total and free protein concentrations (TPC and FPC) in different solvent mixtures. For example, in some embodiments, a standard curve for a BCA assay may be generated using 2/3 Ammonium Bicarbonate buffer and 1/3 NaOH. In some embodiments, these standard curves may reflect conditions used to process samples for BCA assay analyses (*e.g.*, samples comprising a certain ratio of NaOH and/or ammonium bicarbonate buffer). The present disclosure also provides the insight that BCA assay accuracy may be impacted (*e.g.*, improved or reduced) due to one or more components of a solvent. Preparation and testing of solvent mixtures for standard curves. Accordingly, in some embodiments, it is important that standard curves with components of solvent mixtures (*e.g.*, NaOH and ammonium bicarbonate) are prepared.

[0353] In some embodiments, more NaOH than is used in standard protocols may be used in samples as described herein. For example, the present disclosure provides the insight that, in some embodiments, higher concentrations of NaOH or ammonium bicarbonate may be required to process samples for BCA analysis. In some such embodiments, standard curves will be prepared and tested to reflect changes in processing steps (*e.g.*, changes in solvent mixtures used in a given assay and/or preparation).

Assessing Payload Exposure

[0354] In some embodiments, technologies provided by the present disclosure permit or otherwise include one or more assessments of payload exposure – i.e., the degree to which a subject receiving a nanoparticle composition becomes exposed to its payload while nanoparticles remain intact.

[0355] In some embodiments, a payload included in a nanoparticle composition and/or otherwise delivered through administration of such nanoparticle composition is or has the potential to be harmful to the recipient. For example, in some embodiments, a payload included in and/or otherwise delivered through administration of such nanoparticle composition is an allergen to which the subject is or may be allergic; in some such embodiments, the allergen is an anaphylactic allergen (e.g., a food allergen such as a peanut and/or milk allergen, a venom, etc).

Assessment of degree of payload encapsulation, and/or of subject exposure to payload upon administration may be particularly useful or important in such contexts.

[0356] In some embodiments, the present disclosure provides and/or utilizes one or more assessments of allergen encapsulation. For example, in some embodiments, a composition comprising nanoparticles encapsulating a payload is assayed to determine quantity of payload; in some embodiments, encapsulated payload is determined, for example, by quantifying "free" (outside of nanoparticles) payload and "total" payload (amount detectable after nanoparticles have been disrupted); the difference is encapsulated payload. Encapsulated payload as a percentage of total payload may be referred to as the "Encapsulation Ratio".

[0357] In some embodiments, a nanoparticle preparation in accordance with the present disclosure is substantially free of detectable unencapsulated payload (in a relevant assay – e.g., a detection assay such as an ELISA, or an activity assay such as an assessment of immune impact or toxicity).

[0358] In some embodiments, a nanoparticle preparation is characterized in that it includes less detectable "free" payload than does a comparable preparation of payload (e.g., containing an equivalent amount of payload to that used to generate the nanoparticle composition) that is not so encapsulated. In some such embodiments, amount of detectable "free" payload is assessed through binding detection of the payload. Alternatively or additionally, in some embodiments, amount of detectable "free" payload is determined by assessing impact of such payload on a relevant system. To give but one specific example, in some embodiments, where the payload is or comprises an antigen, amount of detectable "free" payload can be or comprise detection of an immune response in an appropriate system – e.g., an allergic response to an allergen in a system (e.g., cell, tissue, organism) reactive to such allergen.

[0359] In some embodiments, reactivity of a composition is determined using an assay in which cells (*e.g.*, immune cells, *e.g.*, basophils) are exposed to either encapsulated or free payload and reactivity of the cells is measured. In some embodiments, reactivity of a composition is determined using whole blood basophil activation test (BAT). In some embodiments, reactivity to encapsulated payload is reduced as compared to reactivity to an equivalent quantity of free payload (*e.g.*, weight/weight equivalents). That is, in some embodiments, a subject may consume a greater quantity of encapsulated payload without reaction as compared to the same or lesser amount of free payload (unencapsulated). In some embodiments, reactivity to

encapsulated payload is reduced by a certain amount relative to reactivity to an equivalent (ug) quantity of free payload. In some embodiments, the reduction of reactivity of encapsulated payload is 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 85, 100, 125, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, 500, 550, 600, 650, 700, 750, 800, 850, 900, 950, 1000, 1500, 2000, 2500, 3000, 3500, 4000, 4500, 5000, 5500, 6000, 6500, 7000, 7500, 8000, 8500, 9000, 9500, 10000-fold or greater, as compared to payload that is unencapsulated.

Manufacturing Nanoparticle Compositions

[0360] The present disclosure is based, in part, on a surprising insight that desirable nanoparticle compositions can be prepared by the manufacturing processes described herein. Among other things, the present disclosure identifies one or more problems (*e.g.,* one or more sources of problems) in prior nanoparticle manufacturing technologies.

[0361] Furthermore, in some embodiments, the present disclosure provides insights that permit preparation of nanoparticle compositions that comprise payloads and/or coating agents (*e.g.*, complex payloads and/or complex coating agents). Alternatively or additionally, in some embodiments, the present disclosure provides insights that permit preparation of nanoparticle compositions that incorporate two or more materials having different physicochemical properties (*e.g.*, hydrophobic polymer and hydrophilic payloads). Still further alternatively or additionally, in some embodiments, the present disclosure provides technologies that permit preparation of nanoparticle compositions incorporating one or more fragile payloads. Yet further alternatively or additionally, in some embodiments, the present disclosure provides scalable technologies, amenable to commercial scale production of nanoparticle preparations as described here.

[0362] As noted, the present disclosure provides an insight that nanoparticles comprising fragile (*e.g.*, susceptible to damage from energy input during manufacturing) and/or complex payloads can be prepared by manufacturing processes described herein. Among other things, the present disclosure identifies a problem with conventional nanoparticle manufacturing technologies in that they typically involve one or more steps that utilize harsh manufacturing conditions (*e.g.*, high temperature, pressure, shear force, *etc.*). The present disclosure appreciates that such conditions can degrade fragile payloads, and/or can decrease one or more biological or pharmaceutical activities of fragile and/or complex payloads. The present disclosure notes that,

as one skilled in the art will be aware, many such existing nanoparticle manufacturing technologies could only incorporate fragile payloads if such payloads could be introduced after nanoparticle manufacture were complete; the present disclosure appreciates that such strategies would be unlikely to achieve encapsulation of such fragile payloads and therefore would not achieve certain advantages provided by preparations as provided by the present disclosure.

[0363] Teachings provided by the present disclosure are particularly applicable to preparations of polymer nanoparticles comprising payload(s). As discussed herein, those skilled in the art are aware of a variety of polymers that can be utilized in the preparation of nanoparticles, and of solvent systems that can be utilized to prepare appropriate solutions of such polymers and/or payloads.

[0364] In many embodiments, methodologies provided by the present disclosure utilize an initial polymer/payload preparation, *e.g.*, solution that includes both polymer and payload. As described herein, a variety of strategies can be utilized to provide or prepare an initial polymer/payload preparation. For example, in some embodiments, an initial polymer/payload preparation is made from mixing a polymer solution and a payload solution. In some embodiments, an initial polymer/payload preparation is made by dissolving dry polymer and dry payload in a solvent system. In some embodiments, a polymer solution is made by dissolving polymer (*e.g.*, that is or comprises PLG) into organic liquid (*e.g.*, that is or comprises dimethyl sulfoxide (DMSO), acetone, acetonitrile, tetrahydrofuran, or a combination thereof). In some embodiments, a payload solution is made by dissolving payload into water to produce an aqueous solution. In some embodiments, preparation of an aqueous solution also involves pH adjustment (*e.g.*, using buffers, NaOH, *etc.*), and/or application of disruptive energy and/or force such as, *e.g.*, sonication, and/or homogenization. In some embodiments, an initial polymer/payload preparation is prepared by combining a payload (aqueous) and a polymer (organic) solution. In some embodiments, an initial polymer/payload preparation is prepared by adding a payload (aqueous) solution into a polymer (organic) solution. In some embodiments, an initial polymer/payload preparation is prepared by adding a polymer (organic) solution into a payload (aqueous) solution. In some embodiments, an initial polymer/payload preparation is prepared by solubilizing a dry material containing both polymer and payload. In some embodiments, dry material is added slowly or in steps; in some embodiments, added dry material

is permitted to solubilize substantially completely before a further addition of dry material is made.

[0365] As discussed herein above, provided technologies are amenable to encapsulation of a variety of different payloads. To reiterate just a few examples here, in some embodiments, provided nanoparticle compositions include a payload selected from the group consisting of polypeptides, nucleic acids, carbohydrates (*e.g.*, polysaccharides), and combinations thereof. In many embodiments, a payload is or comprises a polypeptide. In many embodiments, a payload is or comprises a nucleic acid; in some such embodiments, a payload is or comprises a long nucleic acid (e.g., a gene therapy vector, an mRNA, etc); in some embodiments, a payload is or comprises a partly or wholly single stranded nucleic acid).

[0366] In some embodiments, a payload is or comprises a RNA. In some embodiments, and RNA payload is an mRNA. In some embodiments, an RNA payload has a length within a range of about 15 to about 3,000,000 residues. In some embodiments, an RNA payload has a length within a range of about 500 to 50000 residues. In some embodiments, an RNA payload has a length within a range of about 1000 to about 10000 residues.

[0367] In some embodiments, a provided nanoparticle preparation is manufactured using one or more relatively complex components (e.g., a payload and/or a coating agent that is a relatively crude extract or combination of components).

[0368] In some embodiments, the present disclosure provides technologies in which a nanoparticle preparation is manufactured by (i) providing a first liquid preparation, which comprises a payload (e.g., a fragile payload and/or a complex payload, and/or a combination of payloads wherein one or more may be a fragile and/or complex payload) in a first aqueous solvent system and a second liquid preparation, which comprises a polymer (e.g., a hydrophobic polymer) in a second solvent system; (ii) combining the first and second preparations to form a mixture that comprises the payload and the polymer in a combined solvent system, and (iii) adding a liquid non-solvent system to the mixture, so that a population of nanoparticles comprising the payload and the polymer is formed (*e.g.*, wherein the method does not involve energy input such as, for example, input of heat) (*e.g.*, wherein the non-solvent system does not degrade the payload, or decrease one or more biological or pharmaceutical activities of the payload) (*e.g.*, wherein one or more biological or pharmaceutical activities of payload are substantially same before and after the step of adding).

[0369] Alternatively or additionally, in some embodiments, the present disclosure provides manufacturing technologies that include a heterogeneous, layered two-fluid process that achieves remarkable uniformity in nanoparticle production (e.g., in average size and/or in polydispersity); typically, the process utilizes mild mixing at the fluid interface. In some embodiments (see, for example, Example 10), this heterogeneous, layered two-fluid process is referred to as a "Tequila Sunrise" process. **FIG. 1B** provides a flow diagram for an exemplary Tequila Sunrise process as utilized in Example 10 to produce PLG nanoparticles including crude peanut extract and fragmented *E. coli* DNA payloads.

[0370] In some such Tequila Sunrise embodiments, including as described in Example 10 and depicted in **FIG. 1C**, an aqueous lipid preparation including payload(s) is combined with a hydrophobic (e.g., DMSO) liquid preparation including a hydrophobic polymer (e.g., PLG), and the combination is optionally concentrated (e.g., by rotovap) before Tequila Sunrise nanoprecipitation is performed.

[0371] In some embodiments, the present disclosure provides nanoparticle manufacturing technologies in which (i) payload materials and polymer materials are combined in the presence of a solvent/antisolvent system; typically at least the payload material(s) are sufficiently hydrophilic to be provided in water or other aqueous system (the present disclosure provides an insight that use of an organic antisolvent can reduce payload loss during the encapsulation process); and (ii) the combined materials are mixed in an intentionally heterogeneous, layered two-fluid process, that typically involves mild mixing (quite different from conventional teachings of desirability or even necessity of intense mixing to homogenize a solvent/antisolvent mixture) at the fluid interface. The present disclosure demonstrates that this approach achieves surprising and remarkable consistency in nanoparticle size (e.g., average size) and/or polydispersity. Furthermore, the approach is scalable and, thanks to its gentle conditions, is particularly useful for the incorporation of fragile payloads (e.g., nucleic acid, polypeptide and/or carbohydrate (*e.g.*, polysaccharide) payloads).

[0372] In some embodiments, provided manufacturing technologies utilize a solvent system that comprises water and DMSO. In some such embodiments, a volume ratio of water and DMSO is within a range of 1:99 to 20:80, or 1:99 to 10:90.

[0373] In some embodiments, provided technologies utilize an anti-solvent system (which may in some embodiments be referred to as a non-solvent system). In some embodiments, an anti-

solvent system is or comprises an alcohol. In some embodiments, an anti-solvent system is or comprises propanol, ethanol, methanol, or combination thereof. In some embodiments, an anti-solvent is or comprises IPA.

[0374] In certain embodiments, provided nanoparticle manufacturing technologies may include one or more homogenization steps.

[0375] In certain embodiments, provided nanoparticle manufacturing technologies may utilize one or more stabilizers. For example, in some embodiments, deoxycholate may be utilized (e.g., being included at least in a homogenization step).

[0376] In some embodiments, provided nanoparticle manufacturing technologies achieve a ratio of payload to polymer in the nanoparticles that is between about 0.1 to about 0.9 of the ratio of payload to polymer in the original mixture from which nanoparticles are precipitated.

[0377] Typically, at least one polymer is present in an initial polymer/payload preparation as described herein, at a concentration within a range of about 0.01 to 20 weight %, 0.1 to 20 weight %, 1.0 to 20 weight %, 0.01 to 15 weight %, 0.1 to 15 weight %, 1.0 to 15 weight%, 0.91 to 10 weight %, 0.1 to 10 weight%, 1.0 to 10 weight %, 0.01 to 1 weight %, 0.1 to 1 weight %, 1.0 to 5 weight %, 5 to 10 weight %, 5 to 15 weight %, or 5 to 20 weight % in an appropriate solvent system. Payloads will commonly be present in such a solution at a concentration within a range of about 0.01 to 20 weight %, 0.1 to 20 weight %, 1.0 to 20 weight %, 0.01 to 15 weight %, 0.1 to 15 weight %, 1.0 to 15 weight%, 0.91 to 10 weight %, 0.1 to 10 weight%, 1.0 to 10 weight %, 0.01 to 1 weight %, 0.1 to 1 weight %, 1.0 to 5 weight %, 5 to 10 weight %, 5 to 15 weight %, or 5 to 20 weight % in an appropriate solvent system.

[0378] In some embodiments, polymer and payload, and/or relative amounts thereof, are selected so that, when processed, a payload is encapsulated within polymer matrix, distributed throughout and/or coated by polymer.

[0379] In some embodiments, polymer and payload are present at a weight ratio within a range of 1:1 to 10^{20}:1 (*e.g.*, 1:99 to 20:80; 1:99 to 10:90) in an initial polymer/payload preparation. In some embodiments, polymer and payload are present at a weight ratio within a range of 50:1 to 10^{20}:1 in an initial polymer/payload preparation. In some embodiments, polymer and payload are present in an initial polymer/payload preparation in relative amounts such that, when the solution is processed as described herein, they are present in a nanoparticles as described herein

and/or in a processed material as described herein, at a weight ratio of polymer to payload within a range of 1:1 to 10^{20}:1 by weight (*e.g.*, 50:1 to 10^{20}:1 by weight).

[0380] Among other things, the present disclosure provides, in some embodiments, technologies that achieve sufficiently uniform combinations of polymer and payload in an initial polymer/payload preparation (*e.g.*, solution). In some embodiments, technologies provided by the present disclosure achieve such uniform combination with or without application of disruptive energy or force (*e.g.*, sonication).

[0381] In some embodiments, the present disclosure provides technologies that achieve a material comprising a combination of polymer and payload(s) that does not have a substantially homogenous distribution of payload with respect to polymer (*e.g.*, before and/or after one or more post-combining steps) in an initial polymer/payload preparation. In some such embodiments, additional steps as further described herein, may be employed to achieve a desirable distribution of payload with respect to polymer.

[0382] In some embodiments, provided technologies include one or more steps that remove solvent (e.g., the combined solvent/antisolvent system).

[0383] In some embodiments, a solvent system used to prepare an initial polymer/payload preparation as described herein utilizes only a single solvent (*e.g.*, when both polymer and payload are sufficiently soluble in the single solvent). In some embodiments, an initial polymer/payload preparation that utilizes only a single solvent, may include one or more additional components, for example, that may improve or facilitate solubilization of one or both of the polymer and the payload in the single solvent.

[0384] In some embodiments, a solvent system used to prepare an initial polymer/payload preparation utilizes two or more solvents. For example, a solvent system comprising two or more solvents may be particularly useful when polymer and payload do not readily dissolve together into a single solvent. In some particular embodiments, a solvent system comprising two or more solvents may be useful when either a polymer is substantially hydrophobic (*i.e.*, relatively insoluble in water or other aqueous media) and a payload is substantially hydrophilic, or vice versa. Many embodiments exemplified or otherwise described herein utilize a substantially hydrophobic polymer and one or more substantially hydrophilic payloads.

[0385] In some embodiments, an initial polymer/payload preparation may include one or more other components in addition to polymer and payload. To give but a few examples, in some

embodiments, an initial polymer/payload preparation may include one or more emulsifiers, preservatives, solubilizers, surfactants, viscosity modifiers, salt, buffers (*e.g.*, volatile buffers [*e.g.*, ammonium bicarbonate]) *etc*. Those skilled in the art will be aware of a variety of such agents that may be useful in the practice of certain embodiments as disclosed herein.

[0386] In some embodiments, during preparation of an initial polymer/payload preparation, stirring is performed (*e.g.*, during dissolution of a solid material in a solvent system and/or during combination of two or more solvents or solutions). Stirring rate and/or time can be controlled. For example, in some embodiments, stirring is conducted under conditions that do not exert a high shear force. In some embodiments, stirring is performed at room temperature. In some embodiment, stirring is performed below room temperature, but above a freezing temperature of any component present in a preparation being stirred.

[0387] In some embodiments, stirring is performed with a mixer. In some embodiments, a mixer may be or comprise a stir bar or other device that, for example, utilizes an axial or radial flow impeller (*e.g.*, a bar, paddle, or blade that may, for example, be magnetic), and/or any other impeller or propeller) to achieve mixing. In some embodiments, a mixer may be or comprise a magnetic stirrer, a turbine, or any electrical or mechanical impeller or propeller.

[0388] In some embodiments, mixing is performed for one or more time periods (which may be consecutive and/or may have gaps between them). In some embodiments, a time period maybe approximately 5, 10, 15, 20, 25, 30, 40, 45, 50, 55, or 60 minutes, or longer. In some embodiments, a time period may be approximately 1, 2, 3, 4, 5, 10, 12, 15, 20, or 24 hours, or longer.

[0389] In some embodiments, mixing is performed at a temperature within a range of about 15 °C to 30 °C (*e.g.*, 15-25°C, 15-20°C, or 20-30°C). In some embodiments, mixing is performed without application of heat from an external source. In some embodiments, mixing is performed without application of cooling from an external source. In some embodiments, mixing is performed under conditions in which temperature is controlled (*e.g.*, external heat and/or cooling may be applied).

[0390] In some embodiments, sedimentation (*e.g.*, centrifugation) is performed during preparation of an initial polymer/payload preparation. For example, after dissolution of a solid material (*e.g.*, payload or polymer) in a solvent system (*e.g.*, prior to mixing payload and

polymer), a solution may be centrifuged to remove aggregated, undissolved and/or partially dissolved solid material.

[0391] In some embodiments, an initial polymer/payload preparation is characterized by certain material properties. In some embodiments, an initial polymer/payload preparation is not turbid (*e.g.*, is substantially transparent).

[0392] The present disclosure identifies a source of a problem that may be encountered with certain technologies that involve combining organic and aqueous solutions to achieve a homogenous combination. In some embodiments, the present disclosure provides methodologies (*e.g.*, steps) that can mitigate one or more such identified sources of problem(s). Among other things, in some embodiments, the present disclosure provides technologies for preparing substantially homogenous combinations of organic and aqueous materials as described herein, as well as the substantially homogenous compositions generated thereby. In some such embodiments, resultant compositions are substantially homogenous even if combinations of one or more precursors/components of, or one or more precursors/components used in the making thereof is/are not homogenous.

Optional Concentration

[0393] In some embodiments, a polymer/payload combination (*e.g.*, an initial polymer/payload preparation) is concentrated. In some embodiments, concentration (*e.g.*, removal of a certain percentage of water and/or other solvent(s) or non-solvent(s) (*e.g.*, using, *e.g.*, evaporation, *e.g.*, rotary evaporation) from a polymer/payload combination (*e.g.*, an initial polymer/payload preparation)) may ultimately increase encapsulation of payload in polymer, in subsequent steps. In some embodiments, concentration improves one or more properties of a nanoparticle in subsequent steps (*e.g.*, shape, size, payload: polymer ratio, *etc.*).

[0394] Without wishing to be held to a particular theory, it is contemplated that excess water and/or other solvent(s) may interfere with obtaining homogenous nanoparticles. Accordingly, in some embodiments, concentration of an initial polymer/payload preparation (removal of a portion of water and/or other solvent(s)) or non-solvent(s) may be performed (*e.g.*, via evaporation (*e.g.*, rotary evaporation)) before an initial polymer/payload preparation is further manipulated (*e.g.*, addition of a non-solvent system) (*see*, *e.g.*, **FIG. 6**).

[0395] In some embodiments, polymer/payload combination (*e.g.*, solution) homogeneity may desirably be improved by concentration; the present disclosure encompasses the recognition that such improved homogeneity may facilitate and may even be required for reasonable performance of additional production steps. For example, without being bound to any particular theory, it is contemplated that a particular concentration of water and/or other solvent(s) in a polymer/payload combination (*e.g.*, solution) may result in a non-homogenous combination (*e.g.*, solution) during subsequent steps (*e.g.*, precipitation). Therefore, in some embodiments, the present disclosure provides technologies in which steps may be added (*e.g.*, solution concentration such as, *e.g.*, by water and/or other solvent(s) evaporation step(s)) such that homogeneity (*e.g.*, extent of mixing) is improved.

[0396] In some embodiments, a polymer/payload combination (*e.g.*, solution) is concentrated using evaporation methods. In some embodiments, an initial polymer/payload preparation (*e.g.*, solution) is concentrated using evaporation methods. In some embodiments, a utilized evaporation method is or comprises rotary evaporation.

[0397] In some embodiments, concentration of a polymer/payload combination (*e.g.*, solution) is concentrated for a time and under conditions sufficient to remove a certain percentage of water and/or other solvent(s) or non-solvent(s). In some embodiments, a percentage of water and/or other solvent(s) removed from a polymer/payload combination (*e.g.*, solution, *e.g.*, relative to amount present prior to concentration) is approximately 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, or 95%.

[0398] In some embodiments, time of concentration (*e.g.*, length of time of water and/or other solvent(s) evaporation process(es), such as by rotary evaporation) is within a range of about one hour to about 24 hours or more. In some embodiments, concentration is performed for a time period of at least or about one or more of 1 hour, 2 hours, 3 hours, 4, hours, 5 hours, 6 hours, 7 hours, 8 hours, 9 hours, 10 hours, 11 hours, 12 hours, 13 hours, 14 hours, 15 hours, 16 hours, 17 hours, 18 hours, 19 hours, 20 hours, 21 hours, 22 hours, 23 hours, 24 hours, or more.

[0399] In some embodiments, polymer and payload are present at a weight ratio within a range of 40:60 to 99:1 in a polymer/payload combination after concentration. In some embodiments, polymer and payload are present at a weight ratio within a range of 40:60 to 95:1 in a polymer/payload combination after concentration. In some embodiments, polymer and payload are present at a weight ratio within a range of 50:50 to 99:1 in a polymer/payload combination

after concentration. In some embodiments, polymer and payload are present at a weight ratio within a range of 50:50 to 95:1 in a polymer/payload combination after concentration. In some embodiments, polymer and payload are present at a weight ratio within a range of 60:40 to 99:1 in a polymer/payload combination after concentration. In some embodiments, polymer and payload are present at a weight ratio within a range of 60:40 to 99:1 in a polymer/payload combination after concentration. In some embodiments, polymer and payload are present at a weight ratio within a range of 60:40 to 95:1 in a polymer/payload combination after concentration. In some embodiments, polymer and payload are present at a weight ratio within a range of 70:30 to 99:1 in a polymer/payload combination after concentration. In some embodiments, polymer and payload are present at a weight ratio within a range of 70:30 to 95:1 in a polymer/payload combination after concentration. In some embodiments, polymer and payload are present at a weight ratio greater than about 40:60, greater than about 50:50, greater than about 60:40, greater than about 70:30, greater than about 80:20, greater than about 90:10, or greater than about 95:5 or more.

[0400] In some embodiments, conditions under which concentration (*e.g.*, water and/or other solvent or non-solvent evaporation, such as by rotary evaporation) is performed may be altered according to particular solvents or non-solvents and/or components in a combination. It will be understood to those of skill in the art that certain parameters used in concentration techniques (*e.g.*, pressure, temperature, time), will be altered to desirably achieve concentration of provided combinations. In some embodiments, concentration may be performed at variable temperatures. For example, in some embodiments, concentration (*e.g.*, evaporation, *e.g.*, rotary evaporation) may be performed at temperatures between 20 ° C and 120 ° C; in some embodiments, such temperature may be within a range of, for example, about 25 ° C and about 90 ° C, and/or within a range that does not exceed about 90 ° C, about 85 ° C, about 80 ° C, or about 78 ° C, and/or at a temperature of about 75 ° C.

[0401] In some embodiments, concentration (*e.g.*, evaporation, *e.g.*, rotary evaporation) may involve rotation at a particular speed or speeds. In some such embodiments, a plurality of distinct speeds (*e.g.*, variable speed) may be employed. In some embodiments, where rotation is used, speeds may vary between approximately 40 rpm and 100 rpm. In some embodiments, where rotation is used, speeds may vary between approximately 50 rpm and 90 rpm. In some embodiments, where rotation is used, speeds may vary between approximately 60 rpm and 80

rpm. In some embodiments, where rotation is used, a rotary evaporator may be set to achieve a speed of about 60 rpm.

[0402] In some embodiments, concentration (*e.g.*, evaporation, *e.g.*, rotary evaporation) may be performed at a particular pressure or pressures (*e.g.*, approximately 50 mbar – 250 mbar). In some such embodiments, pressure may vary between approximately 50 mbar and 175 mbar. In some embodiments, pressure may vary between approximately 50 mbar and 150 mbar. In some embodiments, pressure may vary between approximately 75 mbar and 150 mbar.

[0403] In some embodiments, concentration (*e.g.*, evaporation, *e.g.*, rotary evaporation) may be performed at a particular pressure or pressures (*e.g.*, approximately 1-5 torr), for example at about 1 torr or about 2 torr or about 3 torr.

[0404] In certain embodiments, concentration (*e.g.*, by water evaporation such as by rotary evaporation) of a polymer/payload combination (*e.g.*, solution) results in increased encapsulation of protein in the polymer, e.g., relative to otherwise comparable processes that do not include such concentration.

Nanoparticle Precipitation

[0405] The present disclosure provides the insight that a non-solvent system can initiate and/or allow for the precipitation of polymer and payload from a polymer/payload combination (*e.g.*, from a concentrated polymer/payload solution). In some embodiments, precipitation of polymer and payload in a polymer/payload combination creates a nanoparticle preparation (e.g., a nanoparticle suspension) comprising nanoparticles.

[0406] The present disclosure provides an insight that certain non-solvent system(s) may allow for the precipitation of nanoparticles in mild conditions (*e.g.*, conditions do not require energy input, such as increasing temperature and/or pressure, applying shear force). In some embodiments, provided methods allow for the precipitation of fragile (*e.g.*, susceptible to damage from energy input, such as increasing temperature, pressure, applying shear force, *etc.*) and/or complex payloads.

[0407] The present disclosure provides an insight that provided methods generate a uniform/homogenous set of nanoparticles (*e.g.*, with respect to size of nanoparticles, uniformity of a payload within a nanoparticle, payload content, release rate of payload and/or surface

exposure of payloads). In some embodiments, uniform/homogenous characteristics of provided nanoparticles allow for the removal of certain purification/sorting steps (*e.g.*, centrifugation).

[0408] Without wishing to be bound by any particular theory, addition of a non-solvent system may allow for the simultaneous precipitation of polymer and payload. In some embodiments, it is contemplated simultaneous precipitation (*e.g.*, co-precipitation) of polymer and payload generates nanoparticles comprising both polymer and payload. In some embodiments, it is contemplated polymer and payload nucleate and grow separately. In some embodiments, nucleated/grown polymer and payload aggregate into nanoparticles. In some embodiments, it is contemplated simultaneous precipitation (*e.g.*, co-precipitation) of polymer and payload results in nanoparticles having uniform and/or homogeneous distribution of polymer and payload therein.

[0409] In some embodiments, payload is substantially insoluble in a non-solvent system. In some embodiments, polymer is substantially insoluble in a non-solvent system. In some embodiments, payload and polymer are substantially insoluble in a non-solvent system.

[0410] In some embodiments, a volume ratio of a solvent system for a polymer/payload preparation to a non-solvent system is within a range of 1:0.1 to 1:1000, 1:0.1 to 1:100, 1:0.1 to 1:10, 1:0.1 to 1:1000, 1:1 to 1:1000, 1:1 to 1:100, 1:1 to 1:10, or 1:5 to 1:10 in a nanoparticle suspension.

[0411] In some embodiments, a non-solvent system comprises one solvent. In some embodiments, a non-solvent system comprise two or more solvents. In some embodiments, a non-solvent system comprises a solvent selected from the group consisting of propanol, ethanol, methanol, and combinations thereof.

[0412] In some embodiments, a non-solvent system provides mild precipitation conditions (*e.g.*, conditions do not require energy input, such as increasing temperature and/or pressure, applying shear force). In some embodiments, a non-solvent system allows for the precipitation of nanoparticles without requiring high temperature, high pressure, or high shear force. In some embodiments, a non-solvent system does not have high or low pH, and/or high ionic strength.

[0413] In some embodiments, nanoparticle precipitation is performed by combining a polymer/payload preparation and a non-solvent system. In some embodiments, a polymer/payload preparation is added (*e.g.*, poured, injected, dropped) into a non-solvent system.

In some embodiments, a non-solvent system is added (*e.g.*, poured, injected, dropped) into a polymer/payload preparation.

[0414] In some embodiments, nanoparticle precipitation is performed by adding (e.g., injecting, inserting) a polymer/payload preparation into a non-solvent system. In some embodiments, adding (e.g., injecting, inserting) a polymer/payload preparation into a non-solvent system results in at least two separate layers: a polymer/payload preparation and a non-solvent system. In some embodiments, adding (e.g., injecting, inserting) a payload/polymer preparation into a non-solvent system comprises adding the payload/polymer preparation under the non-solvent system. For example, in some embodiments, a non-solvent system is present in a container (e.g., a beaker) and a payload/polymer preparation is added to the non-solvent system by dispensing the payload/polymer preparation at the interface between the non-solvent system and the container (e.g., the bottom of the container). In some embodiments, where a payload/polymer preparation is added to a non-solvent system, the non-solvent system comprises one or more or isopropyl alcohol (IPA), dimethyl-sulfoxide (DMSO).

[0415] Without wishing to be bound by any particular theory, adding (e.g., injecting, inserting) a polymer/payload preparation into a non-solvent system enables formation of a population of nanoparticles in a nanoparticle suspension. For example, in some embodiments, adding a polymer/payload preparation into a non-solvent system where the payload/polymer preparation and the non-solvent system are in separate layers, a population of nanoparticles is formed in a nanoparticle suspension at an interface of the payload/polymer preparation layer and the non-solvent system layer.

[0416] Without wishing to be bound by any particular theory, the present disclosure teaches that, in some embodiments, rate of addition (*e.g.*, addition of a non-solvent system into a polymer/payload preparation, addition of a polymer/payload preparation into a non-solvent system) may affect characteristics of nanoparticles. For example, average size, size distribution, ratio of polymer and payload within nanoparticles, surface charge, and/or surface hydrophobicity may be affected by a rate of addition.

[0417] In some embodiments, conditions under which precipitation is performed may be altered according to particular solvents or non-solvents and/or components in a combination. It will be understood to those of skill in the art that certain parameters used in concentration techniques (*e.g.*, pressure, temperature, time), will be altered to desirably achieve concentration of provided

combinations. In some embodiments, precipitation may be performed at variable temperatures. For example, in some embodiments, precipitation may be performed at temperatures between 20 ℃ and 120 ℃. In some embodiments, precipitation may be performed for 5 mins to 120 mins, 5 mins to 100 mins, 5 mins to 80 mins, 5 mins to 60 mins, 5 mins to 40 mins, 5 mins to 20 mins, 20 mins to 120 mins, 40 mins to 120 mins, 60 mins to 120 mins, 80 mins to 120 mins, or 100 mins to 120 mins.

[0418] In certain embodiments, precipitation is performed according to a "Tequila Sunrise" process that involves non-homogenous, layered precipitation. Specifically, combined payload/polymer liquid preparation is carefully layered under liquid non-solvent system so that an interface is formed. A stirrer (e.g., stirring paddle) is placed at the interface and operated to perform gentle stirring so that the layering is eliminated (as can be detected, e.g., by presence of turbidity). Stirring speed can be adjusted – e.g., optionally being increased once turbidity has been established. The present disclosure teaches that, in some embodiments, adjustment of stirring speed and/or of component(s) of the utilized non-solved system may be adjusted, for example until a desired particle size range and/or distribution is achieved.

[0419] Note that a Tequila Sunrise process, as described herein, represents a departure from a conventional water-in-oil-in-water (W/O/W) emulsion precipitation process for nanoparticles and achieves very different results including, for example, improved payload loading (including specifically for protein payloads). In some embodiments, a provided Tequila Sunrise process achieves payload loading efficiency (e.g., for a protein payload) of at least about 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, or more. Alternatively or additionally, in many embodiments, a Tequila Sunrise process achieves a Z-average diameter within a range of about 50 nm to about 450 nm, or about 100 nm to about 400 nm, or about 100 nM to about 300 nm, or about 100 nM to about 200 nM, or about 120 nM to about 180 nM; in some embodiments nanoparticles in a preparation have a size (and/or a preparation has an average size) within about 15, about 14, about 13, about 12, about 11, about 10, about 9, about 8, about 7, about 6, about 5, about 4, about 3, about 2, or about 1. In some embodiments, a Tequila Sunrise process achieves a polydispersity of about 0.05 to about 0.3, or about 0.1 to about 0.3, or about 0.1 to about 0.2. In some embodiments, a Tequila Sunrise process produces a nanoparticle preparation characterized by a zeta potential below about -20, about -25, about -30, about -35, about -40, or less.

[0420] In some embodiments, precipitation may be stopped in order to obtain a desired nanoparticle population. In some embodiments, a solvent system of a polymer/payload precipitation is added to a nanoparticle suspension to terminate precipitation. In some embodiments, a solvent system comprising a stabilizing agent is added to a nanoparticle suspension to terminate precipitation. In some embodiments, stirring is stopped to terminate precipitation.

[0421] In some embodiments, provided nanoparticle manufacturing technologies may include one or more concentration and/or purification steps. In some embodiments, provided technologies utilize one or more tangential flow filtration (TFF) steps. Among other things, the present disclosure provides an insight that, particularly when TFF is utilized, if a stabilizing agent is desired, deoxycholate is a particularly useful stabilizing agent (and/or that other standard stabilizing agents, such as polyvinyl alcohol, PVA, may be less useful or not useful and, in fact, may damage a TFF membrane.

[0422] In some embodiments, provided manufacturing technologies utilize a stabilizing agent. In some embodiments, a stabilizing may be or comprise PVA. In some embodiments, however, particularly when one or more TFF steps is utilized, PVA is not used. In some embodiments, particularly in embodiments that utilize one or more TFF steps, deoxycholate is utilized as a stabilizing agent.

[0423] In some embodiments, provided nanoparticles include (e.g., are manufactured) from a polymer that is or comprises Poly (lactic-co-glycolic acid) (PLGA or PLG) .

[0424] In some embodiments, provided nanoparticles utilize (e.g., are manufactured from) a polymer preparation (e.g., a PLG preparation) where the polymer has a molecular weight within a range of 5,000 – 5,000,000 Daltons.

[0425] In some embodiments, during precipitation of nanoparticles, stirring is performed. Stirring rate and/or time can be controlled. In some embodiments, stirring includes placing a blade (e.g., a). A four-bladed stirring paddle with a diameter of 114 mm into the mixture. In some embodiments, mixing is performed for one or more time periods (which may be consecutive and/or may have gaps between them). In some embodiments, a time period maybe approximately 5, 10, 15, 20, 25, 30, 40, 45, 50, 55, or 60 minutes, or longer. In some embodiments, mixing is performed at a temperature within a range of about 0 °C to 30 °C (*e.g.*, 10 -25°C, 10-20°C, or 15-20°C). In some embodiments, stirring is performed by a magnetic

stirrer and/or mechanical stirrer. In some embodiments, stirring is conducted under conditions that do not exert a high shear force. In some embodiments, stirring is performed at approximately 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190 or 200 revolutions per minute (rpm), or higher. In some embodiments, stirring is performed for one or more time periods (which may be consecutive and/or may have gaps between them). In some embodiments, each of the one or more time periods were stirring is performed involves stirring at different speeds. For example, in some embodiments, stirring includes one time period where stirring is performed at 80 rpm and a second time period were stirring is performed at 150 rpm. In some embodiments, stirring is performed until nanoprecipitation is complete.

[0426] In some embodiments, where a population of nanoparticles is formed a nanoparticle suspension as a result of adding (*e.g.*, injecting, inserting) a payload/polymer preparation into a non-solvent system, stirring is performed before, concomitantly with, or after adding of the payload/polymer preparation into the non-solvent system. In some embodiments, stirring is performed using an overhead stirrer. For example, in some embodiments, stirring is performed using an overhead stirrer where the top edge of the fin is placed at the interface between the payload/polymer preparation layer and the non-solvent layer, the bottom edge of the fin is placed at the interface between the payload/polymer preparation layer and the non-solvent layer, or the midline of the fin is place at the interface between the payload/polymer preparation layer and the non-solvent layer.

[0427] Without being bound by any particular theory, the present disclosure contemplates that, some embodiments, after precipitation, nanoparticles may exist in more than one subtype. That is, for example, some nanoparticles may have a particular polymer:payload ratio while others may have a different polymer:payload ratio. For example, in some embodiments, after precipitation, a population of nanoparticles may be smaller than another population of nanoparticles. In some such embodiments, a population of nanoparticles with a small average size may contain more payload than a population of nanoparticles with a large average size. In some such embodiments, a population of nanoparticles with a small average size may have higher density than a population of nanoparticles with a large average size.

[0428] In some embodiments, nanoparticles may be dried prior to separation (*e.g.*, using TFF). In some embodiments, nanoparticles may be separated (*e.g.*, using TFF) directly after precipitation.

Stabilized Nanoparticles

[0429] In some embodiments, a solution/suspension of nanoparticles is stabilized using one or more additives (*e.g.*, one or more liquid or powder additives to, *e.g.*, stabilize a combination comprising nanoparticles. In some embodiments, a solution/suspension of nanoparticles is stabilized to prevent nanoparticles from agglomeration.

[0430] In some embodiments, a stabilizing agent may be used to reduce agglomeration of nanoparticles. Without wishing to be bound by any particular theory, surfaces of nanoparticles may be modified by a stabilizing agent.

[0431] In some embodiments, a stabilizing agent may be or comprise a surfactant based on sugar units, or polyethylene glycol units, or ionic units, or combinations thereof. The hydrophobic units of the surfactant will be alkane or alkene units. The surfactants may be biologically sourced or synthetic. An example of a biologically based surfactant would be tocopherol units derivatized with polyethylene oxide units. In some embodiments, amphiphilic copolymers may be used. Exemplary surfactants would include ionic surfactants (*e.g.*, sodium dodecyl sulfate, cetrimonium bromide, *etc.*), sugar-based surfactants such as TWEEN® or SPAN®, and combinations thereof.

[0432] In some embodiments, a stabilizing agent may be or comprise a lipid (e.g., a polar lipid). In some embodiments polar lipids may be ionizable – e.g., may be cationic or anionic under relevant conditions. Those skilled in the art will be aware of a wide variety of polar lipids that may be useful in accordance with the present disclosure.

[0433] In some embodiments, a stabilizing agent may be or comprise an amphiphilic copolymer (*i.e.*, a copolymer of a hydrophilic block coupled with a hydrophobic block). In some embodiments, nanoparticles formed by the process of the present disclosure can be stabilized with graft, block or random amphiphilic copolymers. These copolymers can have a molecular weight between 1,000 g/mole and 50,000 g/mole or more, or between about 3,000 g/mole to about 25,000 g/mole, or at least 2,000 g/mole.

[0434] Examples of suitable hydrophobic blocks in an amphiphilic copolymer include but are not limited to the following: acrylates including methyl acrylate, ethyl acrylate, propyl acrylate, n-butyl acrylate (BA), isobutyl acrylate, 2-ethyl acrylate, and t-butyl acrylate; methacrylates including ethyl methacrylate, n-butyl methacrylate, and isobutyl methacrylate; acrylonitriles;

methacrylonitrile; vinyls including vinyl acetate, vinylversatate, vinylpropionate, vinylformamide, vinylacetamide, vinylpyridines, and vinylimidazole; aminoalkyls including aminoalkylacrylates, aminoalkylmethacrylates, and aminoalkyl(meth)acrylamides; styrenes; cellulose acetate phthalate, cellulose acetate succinate, hydroxypropylmethylcellulose phthalate, poly(D,L lactide), poly (D,L-lactide-co-glycolide), poly(glycolide), poly(hydroxybutyrate), poly(alkylcarbonate) and poly(orthoesters), polyesters, poly(hydroxyvaleric acid), polydioxanone, poly(ethylene terephthalate), poly(malic acid), poly(tartronic acid), polyanhydrides, polyphosphazenes, poly(amino acids) and their copolymers (*see* generally, Illum, L., Davids, S. S. (eds.) Polymers in Controlled Drug Delivery, Wright, Bristol, 1987; Arshady, *J. Controlled Release* (1991) 17:1-22; Pitt, *Int. J. Phar.* (1990) 59:173-196; Holland, *et al., J. Controlled Release* (1986) 4:155-180); hydrophobic peptide-based polymers and copolymers based on poly(L-amino acids) (Lavasanifar, A., *et al., Advanced Drug Delivery Reviews* (2002) 54:169-190), poly(ethylene-vinyl acetate) ("EVA") copolymers, silicone rubber, polyethylene, polypropylene, polydienes (polybutadiene, polyisoprene and hydrogenated forms of these polymers), maleic anhydride copolymers of vinyl methylether and other vinyl ethers, polyamides (nylon 6,6), polyurethane, poly(ester urethanes), poly(ether urethanes), poly(ester-urea). Particularly preferred polymeric blocks include poly(ethylenevinyl acetate), poly (D,L-lactic acid) oligomers and polymers, poly (L-lactic acid) oligomers and polymers, poly (glycolic acid), copolymers of lactic acid and glycolic acid, poly (caprolactone), poly (valerolactone), polyanhydrides, copolymers of poly (caprolactone) or poly (lactic acid) In some embodiments, for non-biologically related applications particularly preferred polymeric blocks include polystyrene, polyacrylates, and butadienes.

[0435] Examples of suitable hydrophilic blocks in an amphiphilic copolymer include but are not limited to the following: carboxylic acids including acrylic acid, methacrylic acid, itaconic acid, and maleic acid; polyoxyethylenes or poly ethylene oxide; polyacrylamides and copolymers thereof with dimethylaminoethylmethacrylate, diallyldimethylammonium chloride, vinylbenzylthrimethylammonium chloride, acrylic acid, methacrylic acid, 2-acrylamido-2-methylpropane sulfonic acid and styrene sulfonate, polyvinyl pyrrolidone, starches and starch derivatives, dextran and dextran derivatives; polypeptides, such as polylysines, polyarginines, polyglutamic acids; poly hyaluronic acids, alginic acids, polylactides, polyethyleneimines,

polyionenes, polyacrylic acids, and polyiminocarboxylates, gelatin, and unsaturated ethylenic mono or dicarboxylic acids.

[0436] In some embodiments, blocks of a particular copolymer may be either diblock or triblock repeats. In some embodiments, block copolymers include blocks of polystyrene, polyethylene, polybutyl acrylate, polybutyl methacrylate, polylactic acid, polycaprolactone, polyacrylic acid, polyoxyethylene and polyacrylamide. A listing of suitable hydrophilic polymers compatible with some embodiments can be found in Handbook of Water-Soluble Gums and Resins, R. Davidson, McGraw-Hill (l980).

[0437] In some embodiments including one or more graft copolymers, the length of a grafted moiety can vary. In some embodiments, the grafted segments are alkyl chains of 12 to 32 carbons or equivalent to 6 to 16 ethylene units in length. In some embodiments, the grafting of the polymer backbone can be useful to enhance solvation or nanoparticle stabilization properties. In some embodiments, a grafted butyl group on the hydrophobic backbone of a diblock copolymer of a polyethylene and polyethylene glycol may increase the solubility of the polyethylene block. In some embodiments, suitable chemical moieties grafted to the block unit of the copolymer comprise alkyl chains containing species such as amides, imides, phenyl, carboxy, aldehyde or alcohol groups. One example of a commercially available stabilizer is the Hypermer family marketed by Uniqema Co. In some embodiments, an amphiphilic stabilizer could also be of the gelatin family such as the gelatins derived from animal or fish collagen.

[0438] In some embodiments, a stabilizing agent may be or comprise a poloxamer, or small ionic surfactant. In some embodiments, a stabilizing agent is selected from the group consisting of polyvinyl alcohol (PVA), ionic surfactants (*e.g.*, sodium dodecyl sulfate, cetrimonium bromide, *etc.*), and combinations thereof.

[0439] In some embodiments, a stabilizing agent is directly added to a solution/suspension of nanoparticles. In some embodiments, a stabilizing agent solution is added to a solution/suspension of nanoparticles.

[0440] In some embodiments, a solvent system of a stabilizing agent solution may comprise water. In some embodiments, a solvent system of a stabilizing agent solution is the same as a diluting solvent system.

[0441] In some embodiments, a stabilizing agent (*e.g.*, PVA) is present in a solution at mass ratio within a range of about 10:1 to 1:10, relative to the mass of one or more components of a

given nanoparticle composition (*e.g.*, polymer, payloads, *etc.*) in the solution. In some embodiments, a stabilizing agent (*e.g.*, PVA) is present in a solution at mass ratio within a range of about 10:1 to 1:10, relative to the mass of nanoparticles in the solution.

[0442] In some embodiments, a stabilizing solution is at a temperature within a range of 0°C to 40°C, 0°C to 30°C, 0°C to 35°C, 0°C to 30°C, 0°C to 25°C, 0°C to 20°C, 0°C to 15°C, 0°C to 10°C, 0°C to 5°C, 5°C to 40°C, 10°C to 40°C, 15°C to 40°C, 20°C to 40°C, 10°C to 30°C, 20°C to 30°C, or 15°C to 25°C, when it is added to a nanoparticle suspension (*e.g.*, a substantially homogenized nanoparticle suspension).

[0443] In some embodiments, an aqueous stabilizing solution is added to a nanoparticle suspension to reduce aggregation of nanoparticles. In some embodiments, no stabilizing agent is added.

[0444] In some embodiments, aqueous stabilizing solution and nanoparticle suspension are mixed for about 10 to 45 mins (*e.g.*, approximately 10, 20, 30, or 40 minutes). In some embodiments, aqueous stabilizing solution and nanoparticle suspension are mixed by a stirrer. In some embodiments, aqueous stabilizing solution and nanoparticle suspension are mixed in a homogenizer (*e.g.*, microfluidizer).

[0445] In some embodiments, one or more solutes or solvents is added to a combination (*e.g.*, solution, *e.g.*, suspension) comprising of homogenized nanoparticles; in some such embodiments, nanoparticles are stabilized when one or more such solutes or solvents is/are present.

[0446] In some embodiments, nanoparticles are stabilized by addition of a stabilizing agent (*e.g.*, PVA) in water to the solution of nanoparticles in propanol. In some such embodiments, a stabilizing agent in water is added to the nanoparticle containing solution, and the solution is cooled to room temperature before proceeding to any further steps.

[0447] As described throughout in the present disclosure, PVA is a commonly used material in manufacturing processes. However, an insight of the present disclosure is that, in some embodiments, the percentage of PVA in one or more intermediate steps of a procedure impacts materials throughout the procedure and/or the quality and quantity of a final product. For example, in some embodiments, the amount of PVA impacts certain properties of nanoparticles (*e.g.*, adherences to one another, Z-average, PDI, *etc.*) and/or intermediates and may produce outcomes such as particles that stick to one another and/or loss of product relative to input in a

manufacturing procedure as described herein. In some embodiments, the presence and/or amount of PVA may impact one or more later steps such as, *e.g.*, a separation step (*e.g.*, TFF).

[0448] In some embodiments, where a stabilizing agent has been included in a suspension solution, the present disclosure contemplates that the percentage of a stabilizing agent present in a sample at particular stages of nanoparticle production may impact overall process efficacy and quality of intermediates and/or final products.

[0449] In some embodiments, it is contemplated that dilution may stabilize nanoparticles. Without wishing to be held to a particular theory, it is contemplated that dilution (*e.g.*, through use of a diluting solvent or non-solvent system) would dilute a combination comprising nanoparticles, thereby reducing agglomeration.

[0450] In some embodiments, a diluting solvent system may be or comprise a non-solvent of polymer. In some embodiments, a diluting solvent system is miscible with at least one of water and DMSO. In some embodiments, a diluting solvent system is the same as or comprises the original solvent used for nanoparticle formulation. In some embodiments, a diluting solvent system is at a temperature within a range of 0 °C to 40 °C, 0 °C to 30 °C, 0 °C to 35 °C, 0 °C to 30 °C, 0 °C to 25 °C, 5 °C to 40 °C, 10 °C to 40 °C, 15 °C to 40 °C, 20 °C to 40 °C, 10 °C to 30 °C, 20 °C to 30 °C, or 15 °C to 25 °C, when it is added to a solution/suspension of homogenized nanoparticles.

Post-processing Nanoparticles

[0451] In some embodiments, provided methods further include a post-processing step applicable to provided nanoparticle preparations. As will be apparent to one of ordinary skill in the art, certain post-processing parameters and/or procedures may be altered in order to accommodate conditions such as, *e.g.*, materials used, and/or scale of processing (*e.g.*, in larger scale processing different parameters may be desirable). Among other things, the present disclosure provides an insight that post-processing may reduce a burst rate (*e.g.*, payload amount released in first 15 minutes, when nanoparticles are exposed to a physiological condition). The present disclosure also recognizes that certain processing steps may result in improved encapsulation and/or yield/retention of nanoparticles, as well as improved safety factor of solutions comprising loaded nanoparticles. Without wishing to be bound by any particular theory, a post-processing step may remove a payload that is weakly associated with a

nanoparticle, a payload associated with and/or exposed to an outer surface of a nanoparticle, and/or a payload that is not associated with a nanoparticle (*e.g.*, free payload). In some embodiments, post-processing steps may change (*e.g.*, increase or decrease) recovery of solids during a nanoparticle manufacturing procedure.

[0452] In some embodiments, post-processing may comprise one or more filtration steps. In some embodiments, two or more filtration steps may be performed (*e.g.*, in a serial manner). In some embodiments, two or more filtration steps are performed with one or more other steps (*e.g.*, centrifugation, dilution) between filtration. In some embodiments, when two or more filtration steps are performed, each filtration step may be at the same or different conditions. In some embodiments, one or more optional steps between filtration may include centrifugation (*e.g.*, low speed centrifugation) and/or dilution.

[0453] In some embodiments, provided manufacturing technologies include one or more steps of purifying nanoparticles (*e.g.*, by one or more of filtration, (*e.g.*, tangential flow filtration), sonication, dilution).

[0454] In some embodiments, filtration may be used to remove one or more materials from a particular preparation (*e.g.*, a sample comprising nanoparticles). In some embodiments, filtration may occur through a column comprising a medium (*e.g.*, a resin). In some embodiments, filtration may occur through a membrane In some such embodiments, tangential flow filtration may be performed by contacting a surface (*e.g.*, filter, membrane) with a composition.

[0455] For example, in some embodiments, tangential flow filtration is used to remove some or substantially all stabilizing agents from one or more preparations comprising nanoparticles as described herein. In some embodiments, a stabilizing agent is removed before one or more tangential flow filtration steps. In some embodiments, a stabilizing agent is removed by or during one or more tangential flow filtration steps. In some embodiments, tangential flow filtration is used to remove some or substantially all payloads that are not associated with or incorporated in nanoparticles from one or more samples. In some embodiments, tangential flow filtration is used to remove some or substantially all polymers that are not associated with or formed into nanoparticles from one or more samples.

[0456] In some embodiments, provided technologies include one or more steps of drying nanoparticles.

[0457] In some embodiments, a nanoparticle preparation in accordance with the present disclosure (e.g., manufactured as described herein) has a mean size within a range of approximately 100-500 nm. In some embodiments, mean size is within a range of about 225 nm to about 450 nm. In many embodiments, mean size is determined by dynamic light scattering.

[0458] In some embodiments, a provided nanoparticle preparation has a mean diameter within a range of about 50 nm to about 150 nm.

[0459] In some embodiments, two or more filtration steps may be performed. Without wishing to be held to a particular theory, it is contemplated that first tangential flow filtration may remove one or more of unformed polymer molecules, unassociated stabilizing agents, solvent system of nanoparticle suspension. Without wishing to be held to a particular theory, it is contemplated that second tangential flow filtration may remove one or more of free payloads and large nanoparticles (*e.g.*, average size larger than 200 nm, 300 nm, 400 nm or 500 nm).

[0460] The present disclosure recognizes that certain formats of samples may be difficult to process in one or more ways. For example, in some embodiments, samples with certain threshold amounts of a stabilizing agent are unable to be re-suspended in solution once those samples have been dried. In some embodiments, the present disclosure also overcomes such challenge, for example by providing one or more methods that solve the problem presented by drying a sample that then needs to be re-suspended for further filtration (*e.g.*, via tangential flow filtration) or processing/analysis (*e.g.*, BCA assay).

[0461] In some embodiments, adjustment of one or more conditions or parameters of a filtration step may be helpful in overcoming, *e.g.*, changes in flow rates due to sample format. For example, in some embodiments, insufficient flow rates may occur due to one or more components of a solution comprising nanoparticles as described herein.

[0462] Among other things, the present disclosure identifies the source of a problem that can be associated with production of certain nanoparticle preparations. For example, the present disclosure appreciates that, in some circumstances, tangential flow filtration may become problematic (*e.g.,* may proceed slowly and/or with the development of increasing pressure and/or decreasing flow rate). Without wishing to be bound by any particular theory, the present disclosure proposes that polymer component(s) (*e.g.,* nanoparticles, aggregates, or other sources – *e.g.,* unincorporated polymer) may form a gel or pseudogel in some circumstances that may interfere with efficient progress to and/or through a filter.

[0463] In certain embodiments, the present disclosure provides technologies that may be utilized in conjunction with and/or as part of tangential flow filtration and that modifying one or more fluid motion characteristics of a sample. Without wishing to be bound by any particular theory, the present disclosure proposes that, in some embodiments, such fluid motion altering technologies may improve tangential flow filtration, *e.g.,* by disrupting or reducing formation or stability of a gel or pseudogel that might otherwise interfere to some degree with such filtration). In some embodiments, turbulence is utilized to modify fluid motion characteristics. In some embodiments, turbulence is created at a membrane. In some embodiments, turbulence is created via cassette filters. Alternatively or additionally, in some embodiments, fluid motion characteristic(s) may be modified by vibration (*e.g.,* by vibrating a sample before and/or during filtration, either continuously or periodically). Without wishing to be held to a particular theory, turbulence may reduce gelation of polymer, payload and/or stabilizing agent on a membrane of TFF.

[0464] The present disclosure recognizes that one way to overcome a challenge of insufficient or reduced flow rates for the support of high throughput/high output processes (relative to those flow rates achieved during lower scale processes), such as those needed to support large clinical trials and/or commercial production is to modify filtration procedures and/or concentrations in solutions being filtered. For instance, in some embodiments, a tangential flow filtration step is performed in approximately six hours or less. In some embodiments, percentage of a stabilizing agent in a solution that is subjected to tangential flow filtration comprises 5% stabilizing agent or less. In some embodiments, percentage of a stabilizing agent in a solution that is subjected to tangential flow filtration comprises 1% stabilizing agent or less. In some embodiments, percentage of a stabilizing agent in a solution that is subjected to tangential flow filtration comprises 0.1% stabilizing agent or less. In some embodiments, percentage of a stabilizing agent in a solution that is subjected to tangential flow filtration comprises .01% stabilizing agent or less.

[0465] The present disclosure also recognizes that yield is impacted by various parameters of tangential flow filtration. For example, the present disclosure provides insight that overcomes the challenge of a low yield of nanoparticles after being subjected to a separation protocol comprising tangential flow filtration.

[0466] In some embodiments, nanoparticles of the present disclosure may adhere to a filter during a filtration step. In some such embodiments, overall yield, relative to nanoparticles produced, will be reduced.

[0467] In some such embodiments, a filter may be "primed" with one or more components of one or more solutions as described herein.

[0468] In some embodiments, "priming" or "pre-washing" of a filter may slow down one or more filtration steps or flow rates. Accordingly, in some embodiments, a filter is not pre-washed and flow does not show reduced rates. For example, in some embodiments, a tangential flow filtration procedure may take one-half to one hour longer (from 1 to 1.5 hours to 2 hours with a stabilizing agent prime), as compared to the same process without priming with a stabilizing agent, representing a 25% increase in processing time.

[0469] In some embodiments, a tangential flow filtration filter may be flushed and or cleaned with one or more solutions. For example, in some embodiments, a filter is cleaned with NaOH, rinsed with water, and then recirculated with 0.6 mg/mL stabilizing agent solution for 20 minutes. In some embodiments, a filter is flushed with a solution comprising a surfactant (*e.g.*, sodium dodecyl-sulfate (SDS)), a pH adjusting solution, a solution comprising chaotropic ion (*e.g.*, sodium iodide). Without wishing to be held to a particular theory, flushing a filter may reduce attractive interaction between the filter and nanoparticles (*e.g.*, nanoparticles' blocking the filter), so that it enhances filtration. In some such embodiments, a filtration system may also be flushed/recirculated with fresh buffer to recover any material that may be adhered to a filter membrane.

[0470] In some embodiments, changes to filtration and filter membrane handling, for example, as described above, may increase yield by 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 76, 80, 86, 90, 95, 100% or more.

[0471] In some embodiments, changes to filtration (*e.g.*, tangential flow filtration) do not have as great an impact on yield as compared to addition and/or manipulation of centrifugation post-processing steps. For example, in some embodiments, use of one or more centrifugation steps resulted in better yields than use of filtration alone.

[0472] In some embodiments, separation is performed using a filter. In some such embodiments, a filter is prewashed (*e.g.*, such as with a stabilizing agent [*e.g.*, PVA]) to, *e.g.*, reduce materials sticking to the filter. In some embodiments, a filter is prewashed and a pulse wash is conducted during microfluidization.

[0473] The present disclosure provides the insight that in certain situations of low yield during a process, a low yield at the end of a separation step may be due to one or more factors such as

temperatures that were too high to produce a particular yield of product, loss of product in one or more components of processing (*e.g.*, loss of product due to sticking to a membrane on a separation filter), and/or foaming of a solution during one or more steps of the process.

[0474] In some embodiments, when nanoparticles are filtered, a consistent mass of nanoparticles per filtration medium surface area is maintained. For example, in some embodiments, a particular concentration of nanoparticles per square centimeter of a membrane is maintained during filtration.

[0475] In some embodiments, a surface area of a membrane is relative to a volume of initial input solution to be filtered. In some such embodiments, a surface area of a membrane is between a range of approximately 0.01 – 0.1 m^2/L. In some such embodiments, a surface has a surface area of approximately 75 – 1000 cm^2. In some embodiments, tangential flow filtration may be performed using a filter with a surface area of approximately 1000-5000 cm^2. In some embodiments, a surface area of a filter may be between 100-750 cm^2. In some embodiments, a surface used in tangential flow filtration has a molecular weight cut-off (MWCO) of about approximately 100 kilodaltons to approximately 1000 kilodaltons. In some embodiments, a MWCO is approximately 200 kilodaltons to approximately 600 kilodaltons. In some embodiments, a MWCO is approximately 300 kilodaltons to approximately 500 kilodaltons. In some embodiments, the surface area and/or MWCO may be altered according to desired output (*e.g.*, higher recovery of a particular composition or portion thereof, recovery of a particular size range of materials of a composition or portion thereof, *etc.*) of filtering a provided composition. One of skill in the art will bring with them an understanding of surface area and MWCO sizes appropriate to filter a provided composition.

[0476] In some embodiments, the present disclosure contemplates that one or more challenges associated with low yield may be overcome by conducting large scale separations using filtration. For example, in some embodiments, a large scale TFF procedure includes use of a 790 cm^2 filter (versus 115 cm^2 in a smaller scale) and/or increased tubing sizes in the filter pump head (*e.g.*, ¼ inch to ½ inch or, *e.g.*, increase by 100% in size).

[0477] In some embodiments, a scaled-up filtration procedure may be performed. In some such embodiments, for example, a certain amount of dried material is filtered. In some such embodiments, protein recovery and safety factors may be within ranges as described in the present

disclosure. In some such embodiments, scaled up procedures involving TFF result in improved recovery and/or safety factors as compared to those procedures performed on a smaller scale.

[0478] In some embodiments, flow rates may need to be adjusted as process scales are adjusted (*e.g.*, increased). For example, in some embodiments, a flow rate of between 5.5 – 7.5 L/min will process a batch through TFF in one hour.

[0479] In some embodiments, tubing is also completely immersed in fluid so that no air may enter the system (relative to filtration procedures where tubing is not immersed and, in such embodiments, foaming of solutions may occur due to air entry). Without being bound by any particular theory, it is contemplated that immersion of the tubing prevents foaming by preventing air entry into the solutions.

[0480] In some embodiments, permeability of a filter (*e.g.*, a TFF filter) decreases substantially after exposure to a stabilizing agent solution. In some such embodiments, however, permeability does not further decrease after filtration is performed (*e.g.*, after TFF process is run). Accordingly, in some embodiments, even if flow rate decreases after exposure to a stabilizing agent, filtered materials are unlikely to be further adhering to the filter and further decreasing the flow.

[0481] In some embodiments, when TFF is used, a membrane is washed with between 1 and 30 volume washes. In some embodiments, a membrane is washed with 1-5, 5-10, 10-15, 15-20, 20-25, 25-30 or more volume washes. In some embodiments, a membrane is washed with 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, or more, volume washes. Without being bound by any particular theory, it is contemplated that increased numbers of volume washes will also increase the safety factor of a suspension subjected to tangential flow filtration. For example, in some embodiments, if a suspension is filtered through a 500 kD membrane with 18 volume washes, a safety factor may reach 90. In some embodiments, at least 15 washes are used to reach a safety factor of 20 using a 500 kD membrane, or 31 washes to reach a safety factor of 20 using a 750 kD membrane. One of skill in the art will understand that wash volumes and times may be altered due to factors such as membrane type and contents of material (*e.g.*, suspension) to be filtered.

[0482] In some embodiments, post-processing may include one or more dilutions. The present disclosure provides an insight that, in some embodiments, dilution can facilitate one or more post-processing (*e.g.*, centrifugation, filtration, *etc.*). For example, in some embodiments,

centrifugation and/or filtration may remove a stabilizing agent, unformed polymer, un-incorporated payloads more effectively from diluted nanoparticle solution/suspension than nanoparticle solution/suspension without dilution.

[0483] In some embodiments, a nanoparticle solution/suspension is diluted with a buffer (*e.g.*, ammonium bicarbonate). In some embodiments, a volume ratio of a nanoparticle solution/suspension to a diluting solution is within a range of about 10:1 and about 1:10. In some embodiments, a volume ratio of a nanoparticle solution/suspension to a diluting solution is within a range of about 5:1 and about 1:5. In some embodiments, a volume ratio of a nanoparticle solution/suspension to a diluting solution is within a range of about 4:1 and about 1:4. In some embodiments, a volume ratio of a nanoparticle solution/suspension to a diluting solution is within a range of about 3:1 and about 1:3. In some embodiments, a volume ratio of a nanoparticle solution/suspension to a diluting solution is within a range of about 2:1 and about 1:2. In some embodiments, a volume ratio of a nanoparticle solution/suspension to a diluting solution is about 1:1.

[0484] In some embodiments, post-processing may comprise one or more centrifugation steps. In some embodiments, two or more centrifugation steps may be performed (*e.g.*, in a serial manner). In some embodiments, two or more centrifugation steps are performed with one or more other steps (*e.g.*, filtration, dilution) between centrifugations. In some embodiments, when two or more centrifugation steps are performed, each centrifugation step may be at the same or different speed, same or different temperature and/or for the same or different amount of time. In some embodiments, one or more optional steps between centrifugations may include filtration (*e.g.*, tangential flow filtration).

[0485] In some embodiments, one or more low speed (*e.g.*, 100-750 xg) centrifugation step(s) is/are performed using a nanoparticle composition. Without wishing to be bound to any particular theory, in some embodiments, it is contemplated that low speed centrifugation may aid in collecting large particles which are considered undesirable for a final nanoparticle composition. Without wishing to be bound to any particular theory, in some embodiments, it is contemplated that low speed centrifugation may aid in removing polymers that are not formed into nanoparticles in nanoparticle suspension and/or unincorporated payloads. In some embodiments, a low speed centrifugation step is within a range of approximately 750 xg, 700 xg, 600 xg, 500 xg, 400 xg, 300 xg, 200 xg, 100 xg, or less. In some embodiments, a low speed

centrifugation step is performed within a temperature range of approximately 4 °C to approximately 37 °C. In some embodiments, a low speed centrifugation step is performed at approximately 4 °C. In some embodiments, a low speed centrifugation step is approximately 15 mins to approximately 20 hours or more in duration. In some embodiments, a low speed centrifugation step is approximately 15 mins – 30 mins, 15 mins – 1 hour, 30 mins – 1 hour, 30 mins – 2 hours, 1 hour – 3 hours, 3 hours -5 hours, 5 hours – 8 hours, 5 hours – 10 hours, 10 hours – 15 hours, or 15 hours – 20 hours, or more, in duration.

[0486] Without wishing to be held to a particular theory, it is contemplated that a centrifugation step (*e.g.*, low speed centrifugation) may remove large nanoparticles (*e.g.*, average size larger than 200 nm, 300 nm, 400 nm or 500 nm) from a nanoparticle suspension. For example, in some embodiments, removal of large nanoparticles may facilitate a following filtration step, as it alleviates clogging of a filter.

[0487] In some embodiments, after an initial low speed centrifugation step, one or more additional centrifugation steps may be performed. In some embodiments, centrifugation is performed using supernatant from an initial low-speed centrifugation step. It is contemplated that subsequent centrifugation steps, following an initial low-speed centrifugation step, will further pellet any residual large particles and facilitate collection and removal. In some embodiments, one or more subsequent low speed centrifugation steps is/are performed at speeds of approximately 100-750 xg, for approximately 15 minutes to 20 hours at approximately 4-37 °C. In some embodiments, it is contemplated that centrifugation is insufficient to fully separate and/or collect desired nanoparticle populations (*e.g.*, nanoparticles in a range of 100-500 nm mean size, *e.g.*, 100-300 nm, *etc.*), thus, additional purification steps (*e.g.*, tangential flow filtration) may be performed to collect smaller (*e.g.*, 100-500 nm mean size, *e.g.*, 100-300 nm, *etc.*) nanoparticles.

[0488] In some embodiments, after an initial low speed centrifugation step, one or more additional processing, purification, and/or separation methods (*e.g.*, lyophilization, filtration, centrifugation, tangential flow filtration, protease digestion, ion exchange and use of other resins) may be performed. In some embodiments, one or more purification and/or separation methods may be performed prior to an initial or subsequent (relative to initial) low speed centrifugation step.

[0489] In some embodiments, an optional intermediate speed centrifugation step is performed alone, or in addition to (*i.e.*, before or after) another centrifugation step in the same or different (*e.g.*, low speed or high speed) range, on a nanoparticle composition to pellet the desired nanoparticles. In some embodiments, an intermediate speed centrifugation step is performed after a low speed centrifugation step. In other embodiments, one or more purification and/or separation methods (*e.g.*, filtration, centrifugation, tangential flow filtration, protease digestion, ion exchange and use of other resins) are performed prior to or following an intermediate speed centrifugation step. In some embodiments, an intermediate speed centrifugation step is performed at speeds of approximately 750 xg – approximately 7500 xg. In some embodiments, an intermediate speed centrifugation step is performed at speeds of approximately 1000 xg, 1500 xg, 2000 xg, 2500 xg, 3000 xg, 3500 xg, 4000 xg, 4500 xg, 5000 xg, 5500 xg, 60000 xg, 6500 xg, 7000 xg, or 7500 xg. In some embodiments, an intermediate speed centrifugation step is performed at temperature ranges of approximately 4-37 ℃. In some other embodiments, after an initial intermediate speed centrifuge step one or more additional centrifuge steps are performed on a given supernatant to further pellet nanoparticles present in the solution. In some embodiments, an intermediate speed centrifuge step is performed for approximately 15 minutes to approximately 20 hours. In some embodiments, an intermediate speed centrifuge step is performed for approximately 15 mins – 30 mins, 15 mins – 1 hour, 30 mins – 2 hours, 1 hour – 3 hours, 3 hours -5 hours, 5 hours – 8 hours, 5 hours – 10 hours, 10 hours – 15 hours, or 15 hours – 20 hours, or more, in duration.

[0490] In some embodiments, a high speed centrifugation step is performed alone, or in addition to (*i.e.*, before or after) another centrifugation step in the same or different (*e.g.*, low speed or intermediate speed) range, on a nanoparticle composition to pellet the desired nanoparticles. In some embodiments, a high speed centrifugation step is performed after a low and/or intermediate speed centrifugation step. In some embodiments, one or more purification and/or separation methods (*e.g.*, filtration, centrifugation, tangential flow filtration, protease digestion, ion exchange and use of other resins) are performed prior to or following a high speed centrifugation step. In some embodiments, a high speed centrifugation step is performed at speeds of approximately 8000 xg – 25,000 xg or greater. In some embodiments, a high speed centrifugation step is performed at speeds of approximately 8000 xg, 9000 xg, 10000 xg, 11000 xg, 12000 xg, 13000 xg, 14000 xg, 15000 xg, 16000 xg, 17000 xg, 18000 xg, 19000 xg, 20000

xg, 21000 xg, 22000 xg, 23000 xg, 24000 xg, or 25,000 xg or greater. In some embodiments, a high speed centrifugation step is performed at temperature ranges of approximately 4-37 °C. In some other embodiments, after an initial high speed centrifuge step one or more additional centrifuge steps are performed on a given supernatant to further pellet residual nanoparticles. In some embodiments, a high speed centrifuge step is performed for 15 minutes to approximately 20 hours. In some embodiments, a high speed centrifuge step is performed for 15 mins – 30 mins, 15 mins – 1 hour, 30 mins – 1 hour, 30 mins – 2 hours, 1 hour – 3 hours, 3 hours -5 hours, 5 hours – 8 hours, 5 hours – 10 hours, 10 hours – 15 hours, or 15 hours – 20 hours, or more, in duration.

[0491] In some embodiments, post-processing may include precipitation by gravity. In some embodiment, a precipitation of unincorporated polymers that are not formed into nanoparticles in nanoparticle suspension and/or unincorporated payloads occurs faster than a precipitation of nanoparticles.

[0492] In some embodiments, methods provided by the present disclosure do not require one or more post-processing processes that are required by conventional methods. For example, methods provided by the present disclosure may not involve centrifugation steps. Without wishing to be held to a particular theory, it is contemplated that centrifugation steps may not be necessary because nanoparticles in accordance with the present disclosure have uniform size distribution.

[0493] In some embodiments, post-processing may comprise an ion exchange step (*e.g.*, through filtration), chromatography (*e.g.*, an ion exchange chromatography), which may be performed on a nanoparticle suspension. In some embodiments, ion exchange and/or chromatography may separate a payload that is weakly associated with a nanoparticle. Typically, chromatography separates ions and polar molecules based on their affinity, for example, to the ion exchanger. For example, water-soluble and charged molecules bind to moieties which are oppositely charged by forming ionic bonds to the insoluble stationary phase (*e.g.*, ion exchange resin).

[0494] In some embodiments, a resin, for example, in a column may be used for post-processing of nanoparticles. In some embodiments, post-processing comprising a column (*e.g.*, an ion exchange column) may occur in more than one step of certain provided methods and/or with more than one column. In some embodiments, an amount of resin in a column may vary relative

to an amount of polymer, payload, or even resin used in a different step of a particular embodiment.

[0495] In some embodiments, post-processing may include incubating nanoparticles with an amount of resin (*e.g.*, ion exchange resin) for a period of time. In some embodiments, a period of time may be, *e.g.*, 15 minutes, 30 minutes, 45 minutes, 60 minutes, 75 minutes, 90 minutes, or more.

[0496] In some embodiments, it may be desirable to modify certain key parameters of an ion exchange chromatography to produce desired nanoparticles. For example, a relative amount of ion exchange resin to nanoparticles may be increased, which may be helpful to separate a payload that is weakly associated with a nanoparticle. In some embodiments, nanoparticles may be incubated with a stationary phase longer to achieve a higher degree of separation of weakly associated payloads. In some embodiments, an ion exchange resin with a higher affinity to nanoparticles (*e.g.*, higher retention time) may be helpful to separate payloads that are weakly associated with nanoparticles.

[0497] The present disclosure provides an insight that one or more post-processing steps may be combined, for example to optimize yield of particular populations of nanoparticles. For example, the present disclosure recognizes that, in some embodiments, additional separation steps may be needed to collect desired populations of nanoparticles. For example, in some embodiments, separation by centrifugation at any speed may not sufficiently collect desired nanoparticles from a suspension; thus a combination of separation and collection methods (*e.g.*, centrifugation followed by filtration, *e.g.*, tangential flow filtration) may be used to optimize yield of all and/or desired populations (*e.g.*, particular size and/or payload content) nanoparticles. In some embodiments, a first separation (*e.g.*, low speed centrifugation) step may be performed in order to remove nanoparticles and/or aggregates within a particular size range (*e.g.*, 300-500 nm; 500-1000 nm, or greater than 1000 nm), followed by a second separation (*e.g.*, filtration, *e.g.*, tangential flow filtration) to collect nanoparticles in a desirable size range such as, *e.g.*, 100-200 nm, 100-300 nm, 100-400 nm, 200-400 nm, or 200-300 nm.

[0498] In some embodiments, before nanoparticles are subject to post-processing (*e.g.*, contact with a resin), the amount of free payload to encapsulated payload may be any of a variety of ratios. In some embodiments, a ratio of free payload to encapsulated payload (before post-processing) may be approximately 10:1, 9:1, 8:1:7:1, 6:1, 5:1, 4:1, 3:1, 2:1, 1:1, 1:2, 1:3, 1:4, 1:5,

1:6, 1:7, 1:8, 1:9, 1:10, or any range of such ratios. In some embodiments, the ratio of free payload to encapsulated payload is greater than 10:1. In some embodiments, the ratio of free payload to encapsulated payload is less than 1:10.

[0499] In some embodiments, a certain percentage of nanoparticles comprising encapsulated payload may be lost (*e.g.*, destroyed, retained in a column) during a process of contacting nanoparticles to a resin for, *e.g.*, removal of weakly associated or unassociated payload. In some embodiments, a percentage of nanoparticles lost is at least about 5% to at most about 25%. In some embodiments, a percentage of nanoparticles lost is at least about 25% to at most about 50%. In some embodiments, a percentage of nanoparticles lost is at least about 50% to at most about 75%.

[0500] In some embodiments, post-processing steps may increase yields of particular populations of nanoparticles and/or improve purity of nanoparticle populations/solutions. For example, in some embodiments, post-processing steps may improve yield of nanoparticles that are approximately 100-500 nm in at least one dimension. In some embodiments, post-processing steps may increase yield of nanoparticles that are approximately 100-200 nm in at least a single dimension. In some embodiments, post-processing steps may increase yield of nanoparticles that are approximately 100-300 nm in at least a single dimension. In some embodiments, post-processing steps may increase yield of nanoparticles that are approximately 100-400 nm in at least a single dimension.

[0501] In some embodiments, post-processing may be or comprise warming provided nanoparticles to a temperature above room temperature (*e.g.*, within a range of about 30 to 50°C). Without wishing to be held by a particular theory, a payload weakly associated with a nanoparticle may be released at a temperature near the glass transition temperature of a provided payload. For example, in some embodiments, a payload weakly associated with a nanoparticle may be released at a temperature within a range of about 30 to 50°C.

[0502] In some embodiments, post-processing may be or comprise one or more of lyophilization, electrodialysis, collection of nanoparticles by separation of one or more components of a provided composition (*e.g.*, filtration, *e.g.*, ultrafiltration, tangential flow filtration; *e.g.*, centrifugation (including, *e.g.*, continuous flow centrifugation which may be or comprise flow in an aqueous buffer and extraction while spinning, potentially with nanoparticles under centrifugal force for extended periods of time, such as several hours; *e.g.*, use of column and/or resin

purification, *e.g.*, ion exchange resin), and/or removal of free or weakly associated payload by protease digestion.

[0503] In some embodiments, post-processing does not comprise one or more of electrodialysis, collection of nanoparticles by filtration, tangential flow filtration, removal of free or weakly associated payload by protease digestion, centrifugation (including, *e.g.*, continuous flow centrifugation which may be or comprise flow in an aqueous buffer and extraction while spinning, with nanoparticles under centrifugal force for many hours), and/or use of an ion exchange resin. For example, in some embodiments, components of nanoparticle compositions may be further separated using filtration. In some such embodiments, nanoparticles may be filtered. In some embodiments, filtration may occur through a column comprising a medium (*e.g.*, a resin). In some embodiments, filtration may occur through a membrane.

[0504] In some embodiments, post-processing may be or comprise treating provided nanoparticles (*e.g.*, comprising a polymer and a payload) with at least one protease (*e.g.*, papain) or a chaotropic agent (*e.g.*, sodium iodide) to remove (*e.g.*, partially or totally digest) some or substantially all of a payload associated with an outer surface of a nanoparticle. In some embodiments, a protease may be in a suspension or solution. In some embodiments, a protease may be associated with a carrier (*e.g.*, a bead). In some embodiments, chromatography is performed to separate the digested payload by the protease. In some embodiments, provided nanoparticles are treated with at least one protease prior to a chromatography step. In some embodiments, provided nanoparticles are treated with at least one protease during or substantially simultaneously with a chromatography step. In some embodiments, provided nanoparticles are treated with at least one protease and not subjected to a chromatography step.

[0505] In some embodiments, a protease may be selected from the group consisting of papain, proteinase K, trypsin, chymotrypsin, any other protease derived from plant, animal or bacterial sources that could be deemed pharmaceutically compatible, and combinations thereof.

[0506] In some embodiments, a protease may be used at a weight ratio of nanoparticles to protease within a range of about 1000:1 to 1:1 (*e.g.*, 100:1, 10:1, 5:1, *etc.*).

[0507] In some embodiments, an amount (*e.g.*, concentration in a suspension or solution, ratio of molecules of protease to nanoparticles) of protease may be chosen to ensure that it is sufficient to digest payload associated with a surface of a nanoparticle within 30 minutes or one hour.

[0508] In some embodiments, nanoparticles are treated with a protease for between 5 and 60 mins (*e.g.*, approximately 10, 20, 30, 40, or 50 minutes). In some embodiments, provided nanoparticles are treated with a protease for a time period sufficient to at least partially degrade any payload that is exposed on the surface of the nanoparticle, while not compromising the integrity of the polymer such that additional payload is released.

[0509] In some embodiments, nanoparticles are treated with a protease at a temperature within a range of between 0 and 37 °C. In some embodiments, post-processing is performed under conditions in which temperature is controlled (*e.g.*, external heat and/or cooling may be applied). In some embodiments, post-processing is performed under ambient conditions.

[0510] In some embodiments, any one or combination of post-processing steps (*e.g.*, those discussed above) may be used to isolate nanoparticle species with one or more desirable characteristics (*e.g.*, maximum desirable level of payload encapsulation). In some embodiments, a nanoparticle species with desirable (*e.g.*, elevated, including maximized) payload encapsulation has approximately 10 – 90 μg payload/mg polymer. In some such embodiments, a nanoparticle with desirable payload encapsulation is in a size range of approximately 100 – 500 nm. In some embodiments, an amount of payload encapsulation is in a range of approximately 40 – 80 μg/mg of polymer. In some embodiments, a desirable size range is approximately 100-300 nm.

In some embodiments, an amount of free (*e.g.*, unencapsulated) payload in a given composition comprising nanoparticles is low enough that there is little to no risk of inducing an allergic reaction when administered to a subject with an allergy to the payload. In some embodiments, amount of payload encapsulation corresponds to a safety factor. In some such embodiments, a safety factor indicates that a quantity of free payload is not great enough to result in risk of anaphylaxis, when administered to a subject with an allergy to the payload. In some embodiments, an increased safety factor corresponds to a higher encapsulation rate and/or higher percentage of removal of any remaining free payload from a provided nanoparticle composition prior to administration. In some embodiments, a desirable payload encapsulation range corresponds to a particular safety factor (*e.g.*, as measured by an equation, *e.g.*, Equation 1 as described in Example 3). In some embodiments, free payload may be reduced by one or more separation steps as provided herein and/or one or more wash steps. It will be understood by those of skill in the art that separation and/or wash steps may be altered to both optimize free payload reduction and nanoparticle retention.

Coating Nanoparticles

[0511] In some embodiments, provided methods may include a step of coating nanoparticles. In some embodiments, a dry coating agent is directly added to a nanoparticle suspension. In some embodiments, a coating agent solution is added to a nanoparticle suspension. As discussed further herein, those skilled in the art are aware of a variety of coating agents that can be utilized in the preparation of nanoparticles, and of solvent systems that can be utilized to prepare appropriate solutions of such coating agents. In some embodiments, a combination of nanoparticles and coating agents is stirred and/or sonicated to form coated nanoparticles. In some embodiments, a combination of nanoparticles and coating agents may be sonicated for time within a range of about 0.1 to 10 seconds per mL of the combination.

[0512] In some embodiments, nanoparticles are coated with one or more immunomodulatory agents (e.g., TLR ligands). In some embodiments, nanoparticles are coated with one or more lipids. In some embodiments, nanoparticles may be coated with a complex coating agent. In some embodiments, nanoparticles are coated with one or more lipids – e.g., which, in many embodiments, may be or comprise lipids naturally found in microbial cells. In some embodiments, nanoparticles are coated with a lipid extract, for example a cellular lipid extract, e.g., from a mammalian cell or a microbial cell. In some embodiments, nanoparticles are coated with a microbial lipid extract, for example from *E. coli*.

[0513] In some embodiments, coating is performed at a temperature within a range of about 0 to 25 °C. In some embodiments, coating is performed without application of heat from an external source. In some embodiments, coating is performed without application of cooling from an external source. In some embodiments, coating is performed under conditions in which temperature is controlled (*e.g.*, external heat and/or cooling may be applied).

[0514] In some embodiments, a solution comprising coated nanoparticles is lyophilized to form a solid dispersion (*e.g.*, a powder). In some embodiments, a coated nanoparticle suspension is subjected to freeze-drying, lyophilization, or other drying strategy so that such solid nanoparticle dispersion is obtained.

[0515] In some embodiments, provided technologies include one or more steps of drying nanoparticles.

[0516] In some embodiments, a solid dispersion of coated nanoparticles may be milled, sifted, or sieved, so that the solid dispersion may have a desired particle size distribution.

Additional Components

[0517] In some embodiments, compositions as described herein may include one or more additional components not specifically named in the description above. Those skilled in the art, reading the present disclosure, will be aware of a variety of additional components that can be included in such compositions. To give but some examples, in some embodiments, additional components may comprise one or more dissolution aids, emulsifiers, preservatives, solubilizers, surfactants, viscosity modifiers, salts, sugars, buffers, crystallization inhibitor *etc.* It will be understood by those of skill in the art that any additional components may desirably be modified to maintain a particular composition or portion thereof. For example, in some embodiments, an additional component may naturally occur in a crystalline form that is not particularly compatible with a nanoparticle formulation. In some such embodiments, one of skill in the art will recognize and know how to modify such a component (*e.g.*, by obtaining a more granulated form, or by using processing methods, such as, *e.g.*, lyophilization of an aqueous solution containing the component), to make the component more amenable to a particular nanoparticle formulation as described herein.

[0518] In some embodiments, post-processing may be or comprise addition of one or more agents or additional components and/or one or more additional steps as described herein. For example, in some embodiments, a sugar (*e.g.*, trehalose, sucrose, glucose, fructose, sorbitol) may be added to a composition comprising nanoparticles. In some embodiments, sugar (*e.g.*, trehalose, sucrose, glucose, fructose, sorbitol) is added in a ratio of approximately 0.5:20 mg /mg PLG to approximately 20: 0.5 mg / mg PLG. In some embodiments, a ratio of a sugar (*e.g.*, trehalose, sucrose, glucose, fructose, sorbitol) to PLG is 1:1 to 2:1 mg/ mg PLG. In some embodiments, a ratio of a sugar (*e.g.*, trehalose, sucrose, glucose, fructose, sorbitol) to PLG is 5:1 – 15:1. In some embodiments, a ratio of a sugar (*e.g.*, trehalose, sucrose, glucose, fructose, sorbitol) to PLG is 7:1 – 11:1. In some such embodiments, following addition of one or more components to a composition comprising nanoparticles (*e.g.*, trehalose, sucrose, glucose, fructose, sorbitol), one or more additional steps (*e.g.*, lyophilization, spray drying) may be performed.

[0519] In some embodiments, a dissolution aid may be added to a nanoparticle suspension (*e.g.*, comprising nanoparticles coated with a stabilizing agent). In some embodiments, a dissolution aid is selected from the group consisting of sugars (*e.g.*, trehalose, mannitol, lactose, glucose), hydrophilic polymers (*e.g.*, polyethylene glycol, polyvinylpyrrolidone, polyvinylpyrrolidone vinyl acetate copolymer) and combinations thereof.

[0520] In some embodiments, a dissolution aid may be pre-processed in order to facilitate incorporation into a suspension and/or production of a product for use in a pharmaceutical composition. The present disclosure provides the insight that, for example, in production and subsequent administration of a pharmaceutical composition, an important feature in any clinical trial is for an active ingredient to have a similar texture and/or appearance as inactive ingredients, such that, *e.g.*, a placebo will not be readily distinguishable from an active compound. For example, in some embodiments, trehalose and/or glucose granules are crystalline and larger than nanoparticles of suspensions disclosed herein. In some such embodiments, it is contemplated that a favorable approach is to have a dissolution agent that is more similarly sized and textured to nanoparticles as produced according to the methods described herein. Therefore, in some embodiments, micronized trehalose and/or glucose is used anywhere that trehalose and/or glucose or an equivalent is used, in accordance with the present disclosure. In some such embodiments, if micronized trehalose and/or glucose is not available, trehalose and/or glucose is mixed with water, lyophilized and ground to produce a micronized equivalent of trehalose and/or glucose.

[0521] In some embodiments, a weight ratio of a dissolution aid to polymer is within a range of about 20:0.5 to 0.5:20, 15:5 to 5:15, 11:1 to 1:11, 7:1 to 1:7, 5:1 to 1:5, 5:1 to 1:1, or 3:1 to 1:1.

Drying

[0522] In some embodiments, a nanoparticle preparation may be dried (*e.g.*, by lyophilization, spray drying, *etc.*). In some embodiments, a preparation is dried without being frozed (*e.g.*, without being subjected to temperatures below freezing).

[0523] In some embodiments, drying of a nanoparticle preparation produces a dry cake; in some embodiments, such dry cake may comprise nanoparticles together with at least one other agent (*e.g.*, stabilizing agents, sugars [which in some cases may be considered stabilizing agents], *etc.*, including combinations thereof).

[0524] In some embodiments, a dry nanoparticle preparation is resuspended in a buffer. In some embodiments, a buffer comprises ammonium bicarbonate. In some embodiments, a 10 mM ammonium bicarbonate buffer is used to resuspend a dried cake comprising nanoparticles, a stabilizing agent, and a sugar. One of skill in the art will recognize that buffers may be altered in composition and concentration in accordance with a given process and in consideration of factors such as components of given compositions.

[0525] In some embodiments, a nanoparticle preparation may not be dried.

[0526] In some embodiments, a nanoparticle preparation may be frozen. In some embodiments, a nanoparticle preparation may be frozen in a liquid form (*e.g.*, solution, suspension, frozen droplet, *etc.*). In some embodiments, a nanoparticle preparation may be frozen to be stored and/or transported. In some embodiments, a frozen nanoparticle solution/suspension may be unfrozen (*e.g.,* for further transport, aliquoting, and/or storage, or shortly before administration). In some embodiments, a dosage form is frozen.

[0527] In some embodiments, a nanoparticle preparation may be dried via spray drying. In some embodiments, a nanoparticle preparation is introduced into a drying chamber using a nozzle, atomizer, *etc*. In such embodiments, fine droplets of a nanoparticle preparation are formed. Fine droplets contacting with the drying fluid may be flowed into and through a drying chamber.

[0528] In some embodiments, a drying chamber for spray drying is filled with a drying gas at elevated temperature. In some embodiments, temperature of a drying chamber is within a range of 100-200° C. In some embodiments, temperature of a drying chamber is within a range of 100-150° C. In some embodiments, temperature of a drying chamber is within a range of 150-200° C. In some embodiments, temperature of a drying chamber is within a range of 50-100° C.

[0529] In some embodiments, spring drying is beneficial for preserving burst release of a payload from nanoparticles. For example, in some embodiments, burst release (*e.g.*, related to porosity) of payload from nanoparticles before spray drying is substantially similar to burst release of payload from nanoparticles after spray drying (*e.g.*, resuspended nanoparticles after drying). Without wishing to be bound by any theory, it is contemplated that spray drying is helpful to maintain structural integrity of nanoparticles in a dry cake.

[0530] In some embodiments, a nanoparticle preparation may be dried via freeze drying (*e.g.*, lyophilization). In some embodiments, lyophilization is performed at temperature within a range of -100° C to -30° C. In some embodiments, lyophilization is performed at temperature within a

range of -100° C to -50° C. In some embodiments, lyophilization is performed at temperature within a range of -80° C to -0° C. In such embodiments, a crystallization inhibitor may be added to a nanoparticle preparation prior to lyophilization.

[0531] In some embodiments, lyophilization may form pores on nanoparticles. Such pores may increase burst release. In such embodiments, a sugar selected from the group consisting of sucrose, glucose, fructose, sorbitol and combinations thereof may be preferred than trehalose. In some embodiments, a combination of trehalose and a sugar selected from the group consisting of sucrose, glucose, fructose, sorbitol may be selected. Without wishing to be bound by any theory, it is contemplated that sucrose, glucose, fructose, or sorbitol preserve porosity of nanoparticles during lyophilization, compared to trehalose. In some embodiments, a sugar may be or comprise trehalose.

Freezing

[0532] In some embodiments, a nanoparticle preparation may be frozen. For example, in some embodiments, a nanoparticle preparation may be flash frozen (*e.g.*, using liquid N_2). In some embodiments, a nanoparticle preparation may be frozen slowly. It is contemplated that, in some embodiments, freezing may form pores and/or crystals in a preparation.

[0533] In some such embodiments, a cryoprotective agent may be added. For example, in some embodiments, a preparation may include one or more cryoprotective agents including, *e.g.*, sucrose, glucose, fructose, sorbitol, trehalose, PEG, PVA, agar, one or more gums (*e.g.*, guar gum, xanthan gum, carob bean gum, locust bean gum, *etc.*). In some such embodiments, one or more combinations of agents may be preferred over another. In some embodiments, a preparation may include sorbitol as a cryoprotective agent. In some embodiments, a preparation may include trehalose as a cryoprotective agent.

[0534] In some embodiments, a sealing agent (e.g., that reduces adhesions of nanoparticles, e.g., chitosan) may be added before or during a freezing process.

[0535] In some embodiments, a nanoparticle preparation may not be dried. Without wishing to be bound by any particular theory, the present disclosure provides an insight that a freezing process (e.g., when compared to lyophilization or other post-processing) may reduce a burst rate (e.g., payload amount released in first 15 minutes, when nanoparticles are exposed to a physiological condition).

[0536] In some embodiments, a freezing step is performed at a temperature range of about -200 °C to -0 °C, -150 °C to -0 °C, -100 °C to -0 °C, -50 °C to -0 °C, -30 °C to -0 °C, -200 °C to -150 °C, -150 °C to -100 °C, or -100 °C to -50 °C. In some embodiments, a freezing step is performed for about 10 mins, 15 mins, 20 mins, 25 mins, 30 mins, 35 mins, 40 mins, 45 mins, 50 mins, 55 mins, or 60 mins.

[0537] In some embodiments, a nanoparticle preparation may be frozen and thawed one, two, three, four, five, six, seven, eight, nine, ten or more times. In some embodiments, a nanoparticle preparation is stable through one or more freezing and thawing processes; in some embodiments, provided nanoparticle preparations and/or compositions that include them are stable through (e.g., have been demonstrated to be stable through) 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more freeze/thaw cycles.

Methods of Treatment

[0538] The present disclosure provides, among other things, technologies that may be used in prophylactic and/or therapeutic approaches, *e.g.*, for one or more disease, disorders and/or conditions. For example, the present disclosure provides technologies that may be used to treat a subject at risk of a disease, disorder or condition (*e.g.*, risk of allergic reaction, *e.g.*, risk of viral infection, *e.g.*, flu, *etc.*) by providing, *e.g.*, a nanoparticle composition that can, *e.g.*, decrease risk of an individual ever developing symptoms of a disease, disorder and/or condition even in the event an individual is exposed to, *e.g.*, an allergen, a virus, *etc*. The present disclosure also provides technologies that may be used to treat a subject with a disease, disorder and/or condition (*e.g.*, cancer, *e.g.*, infection) such that after a subject has been determined to have such a disease, disorder, or condition, a composition in accordance with the present disclosure is administered to treat the subject.

[0539] The present disclosure provides, among other things, methods of administering to a subject in need thereof a nanoparticle composition including a plurality of nanoparticles, each of which is comprised of a biodegradable or biocompatible polymer, and at least one of a preparation of a payload and/or at least one preparation of a coating agent associated with the external surface of the nanoparticle.

[0540] In some embodiments, provided nanoparticle compositions are administered to a subject in need thereof so that, when administered, the payload (*i.e.*, comprising protein to which subject

is allergic) is hidden from immune system components for at least a period of time. In some embodiments, encapsulated contents of provided nanoparticle compositions are released into the system of a subject to whom a composition has been administered over a period of time. In some such embodiments, payload of compositions (comprising protein to which a subject is allergic) are released over a period of time such that the subject does not have an anaphylactic reaction when exposed to encapsulated contents of the nanoparticle. In some such embodiments, it is contemplated that such administration and exposure, repeated and with payload amount increased over a period of time, will result in a desensitization to one or more components of a payload. In some such embodiments, treatment of a subject (*e.g.*, for a period of time) with a sensitization and/or allergy (*e.g.*, history of anaphylactic reaction to) a payload in a provided composition will result in decreased incidence and/or risk of reaction when exposed to one or more components of a payload of a provided nanoparticle composition. Without being bound by any particular theory, it is contemplated that such administration will alter immune responses in subjects such that responses that mediate anaphylactic reactions will occur less frequently or will not occur, and responses that mediate tolerance to one or more proteins will function more frequently or at every exposure to one or more components of a payload in a provided nanoparticle composition.

[0541] In some embodiments, the present disclosure provides methods of treating various diseases, disorders and/or conditions. In some embodiments, provided compositions may be administered to a subject for treatment and/or prevention of allergy, infection, cancer, and combinations thereof. Exemplary suitable compositions include those described herein.

[0542] In some embodiments, a disease is an autoimmune disease. In some embodiments an autoimmune disease is one in which disease pathogenesis is driven by a Th1, Th2, Th17, and/or Treg response. In some embodiments, an autoimmune disease is one in which disease pathogenesis is driven by a B cell response. In some embodiments, an autoimmune disease is one in which disease pathogenesis is driven by an innate immune response.

[0543] In some embodiments, an autoimmune disease is associate with an autoantibody. In some embodiments, an autoimmune disease is one associate with an antibody as described in **Table 6**.

Table 6: Autoimmune diseases associated with known autoantibodies.

Autoantibody	Associated Autoimmune Disease
Anti-centromere	CREST syndrome, primary biliary cirrhosis, proximal scleroderma
Anti-dsDNA	Systemic lupus erythematosus (SLE)
Anti-gp210	Primary biliary cirrhosis
Anti-histone	SLE
Anti-Jo1	Polymyositis, dermatomyositis
Anti-LA/SS-b	Sjogrens syndrome
Anti-p62	Primary biliary cirrhosis
Anti-PM-Scl	Polymyositis/ systemic sclerosis overlap syndrome
Anti-RNP	Mixed connective-tissue disease, SLE
Anti-Ro/SS-A	sLE, Sjogrens syndrome, neonatal heart block
Anti-Smith	SLE
Anti-sp100	Primary biliary cirrhosis
Anti-topoisomerase	scleroderma

[0544] In some embodiments, an autoimmune disease is achalasia, Addison's disease, adult Still's disease, agammaglobulinemia, alopecia areata, amyloidosis, ankylosing spondylitis, anti-GBM/anti-TBM nephritis, antiphospholipid syndrome, autoimmune angioedema, autoimmune dysautonomia, autoimmune encephalitis, autoimmune hepatitis, autoimmune inner ear disease, autoimmune myocarditis, autoimmune oophoritis, autoimmune orchitis, autoimmune pancreatitis, autoimmune retinopathy, autoimmune urticaria, axonal & neuronal neuropathy, Balo's disease, Behcet's disease, benign mucosal pemphigoid (mucous membrane pemphigoid), bullous pemphigoid, Castleman disease, celiac disease, Chagas disease, chronic Lyme disease, chronic inflammatory demyelinating polyneuropathy, chronic recurrent multifocal osteomyelitis, Churg-Strauss syndrome, cicatricial pemphigoid, Cogan's syndrome, cold agglutinin disease, complex regional pain syndrome, congenital heart block, Coxsackie myocarditis, CREST syndrome, Crohn disease, dermatitis herpetiformis, dermatomyositis, Devic's disease (neuromyelitis optica), Discoid lupus, Dressler's syndrome, endometriosis, eosinophilic esophagitis, eosinophilic fasciitis, erythema nodosum, essential mixed cryoglobulinemia, Evans syndrome, fibromyalgia, fibrosing alveolitis, giant cell arteritis (temporal arteritis), giant cell myocarditis, glomerulonephritis, Goodpasture's syndrome, granulomatosis with polyangiitis, Graves' disease, Guillain-Barre syndrome, Hashimoto thyroiditis, hemolytic anemia, Henoch-Schonlein purpura, herpes gestationis (pemphigoid gestationis), hidradenitis suppurative (acne inversa), IgA nephropathy, IgG4-related sclerosing disease, immune thrombocytopenic purpura,

inclusion body myositis, interstitial cystitis, juvenile arthritis, juvenile type I diabetes, juvenile myositis, Kawasaki disease, Lambert-Eaton syndrome, lichen planus, lichen sclerosus, ligneous conjunctivitis, linear IgA disease, Meniere's disease, microscopic polyangiitis, mixed connective tissue disease, Mucha-Habermann disease, multifocal motor neuropathy, multiple sclerosis, myasthenia gravis, myelin oligodendrocyte glycoprotein antibody disorder, myositis, narcolepsy, neonatal lupus, neutropenia, ocular cicatricial pemphigoid, optic neuritis, Palindromic rheumatism, Pediatric autoimmune neuropsychiatric disorders associated with streptococcus infections, paraneoplastic cerebellar degeneration, paroxysmal nocturnal hemoglobinuria, pars planitis, Parry Rombery syndrome, Parsonage-Turner syndrome, pemphigus, peripheral neuropathy, perivenous encephalomyelitis, pernicious anemia, POEMs syndrome, polyarteritis nodosa, polyglandular syndromes (*e.g.*, types I, II, or III), polymyalgia rheumatica, polymyositis, postmycardial infarction syndrome, postpericardiotomy syndrome, primary biliary cirrhosis, primary sclerosing cholangitis, progesterone dermatitis, progressive hemifacial atrophy, psoriasis, psoriatic arthritis, pulmonary alveolar proteinosis, pure red cell aplasia, pyoderma gangrenosum, Raynaud's phenomenon, reactive arthritis, relapsing polychondritis, restless leg syndrome, retroperitoneal fibrosis, rheumatic fever, rheumatoid arthritis, sarcoidosis, Schmidt syndrome, scleritis, scleroderma, Sjogren syndrome, Stiff person syndrome, Susac's syndrome, sympathetic ophthalmia, systemic lupus erythematosus, Takayasu's arteritis, temporal arteritis, thrombocytopenic purpura, thrombotic thrombocytopenic purpura, thyroid eye disease, Tolosa-Hunt syndrome, transverse myelitis, type I diabetes, ulcerative colitis, undifferentiated connective disease, uveitis, vasculitis, vitiligo, Vogt-Koyanagi-Harada disease, and warm autoimmune hemolytic anemia.

[0545] In some embodiments, an autoimmune disease comprises multiple autoimmune diseases (*e.g.*, Schmidt syndrome, autoimmune polyendocrine syndrome type 2). In some embodiments, an autoimmune disease is related to a genetic predisposition (celiac disease, Hashimoto's thyroiditis, Graves disease, Addison's disease). In some embodiments, an autoimmune disease is precipitated by an infection or injury. In some embodiments, an autoimmune disease is idiopathic.

[0546] In some embodiments, the present disclosure provides vaccine compositions comprising a nanoparticle population comprising one or more payloads, or precursor(s) thereof, that activate antigen-specific T cells, for example wherein such one or more payloads is/are displayed (or, if a

nucleic acid, may encode an agent that is displayed) by an MHC class I complex or an MHC class II complex.

[0547] In some embodiments, a provided vaccine composition comprises an immune adjuvant. In some embodiments, an immune adjuvant is provided from one or more bacterial sources. In some embodiments, an immune adjuvant comprises a cellular lysate (e.g., microbial lysate), or a cellular lysate fraction. In some embodiments, an immune adjuvant is a mucosal immune adjuvant.

[0548] In some embodiments, an adjuvant is a Th2 adjuvant. In some embodiments an adjuvant is alum. In some embodiments, an adjuvant is from **Table 7**.

Table 7: Exemplary Th2 adjuvants

Category	Example	Comments
Bioactive lipids	phytoprostanes	Mimic PGE to suppress IL-12, activate DCs to promote TH2 polarization
Toxins	Cholera toxin (CT)	The most widely used model of oral sensitization to food proteins uses the mucosal adjuvant CT
Glycans	Ara h 1; *Schistosoma* glycoproteins	Facilitated uptake and activation of DCs via recognition by C-type lectin receptors
Nonmammalian biopolymer	Chitin	Innate immune activation leading to eosinophilia and TH2 polarization
Proteases	Papain; Der p 1	Activation of innate immune cells via protease-activated receptors and other pathways; perturbation of epithelial barrier function
Enterotoxin	Staphylococcus enterotoxin B (SEB)	SEB could induce the upregulation of TIM-4 on DCs isolated from the small intestine
Superallergens	Plant lectins; gp120; low-molecular-weight peanut antigen	Binding to nonvariant regions of IgE or other mechanisms for direct activation of mast cells and macrophages
Thymic stromal lymphopoietin	Human thymic stromal lymphopoietin	TSLP-exposed DCs could support the differentiation of

Category	Example	Comments
		proinflammatory TH2 cells producing IL-4, IL-5, IL-13, and TNF-α.

[0549] In some embodiments, the present disclosure provides a vaccine composition comprising first and/or second nanoparticle populations, wherein the first nanoparticle population comprises a first payload, or precursor(s) thereof, that activates first antigen-specific T cells; and the second nanoparticle population comprises a second payload, or precursor(s) thereof, that activates second antigen-specific T cells. In some embodiments, the first payload is displayed by (or encodes an agent that is displayed by) an MHC class I complex. Alternatively or additionally, in some embodiments, the second payload is displayed by (or encodes an agent that is displayed by) an MHC class II complex. In some such embodiments, the first and second nanoparticle populations are included in a same composition.

Treating Allergy

[0550] The present disclosure provides, among other things, methods and compositions for the treatment and/or prevention of allergy. In some embodiments, provided nanoparticle compositions are useful as vaccines to prevent and/or delay the onset of an allergic reaction. In some embodiments, provided nanoparticle compositions are useful as vaccines to lessen the severity and/or duration of a future allergic reaction. In some embodiments, provided nanoparticle compositions are useful as therapeutics to alleviate and/or arrest an allergic reaction in progress.

[0551] In some embodiments, methods and compositions of the present disclosure are provided to a subject in incremental doses, which doses may escalate (*e.g.*, in frequency, quantity, *etc.*) over time (*e.g.*, slowly desensitize likelihood of an allergic reaction). For example, in some embodiments, methods and compositions of the present disclosure are used and provided in escalating doses of, *e.g.*, an allergen, over a period of time such that upon continued and repeated exposure to escalating doses of a particular composition over a period of time the likelihood that an individual will react lessens (*e.g.*, as dose escalates, tolerance to an allergen increases and desensitization to an allergen may be achieved). In some embodiments, the subject in need thereof is suffering from an allergic condition as herein described, including, but not limited to

allergic rhinitis, asthma, atopic eczema, anaphylaxis, insect venom, drug allergies, food allergies, and/or combinations thereof.

[0552] In some embodiments, provided nanoparticle compositions may be used for treatment and/or prevention of allergies associated with insect allergens. Examples of common insect allergens include, but are not limited to, proteins from insects (*e.g.*, fleas, ticks, ants, cockroaches, and bees), drugs, and rubber.

[0553] In some embodiments, provided nanoparticle compositions may be used for treatment and/or prevention of allergies associated with local allergic dermatitis. Local allergic dermatitis may develop within a short time after exposure to latex and generally includes symptoms of urticaria or hives. The reaction is thought to be allergic and triggered by direct contact, not inhalation (Sussman *et al.*, 1991, *JAMA*, 265:2844; incorporated herein by reference). Symptoms of immediate systemic hypersensitivity vary from skin and respiratory problems (*e.g.*, urticaria, hives, rhinoconjunctivitis, swelling of lips, eyelids, and throat, wheezing, and coughing) to anaphylaxis which may progress to hypotension and shock. Such a reaction may be triggered by inhalation or skin exposure to the allergen.

[0554] In some embodiments, provided nanoparticle compositions may function to suppress and/or decrease a subject's T_H2-type responses and/or enhance and/or increase a subject's T_H1-type responses. In some embodiments, provided nanoparticle compositions may function to enhance and/or increase a subject's T_H2-type responses and/or suppress and/or decrease a subject's T_H1-type responses. In some embodiments, a subject's T_H2-type responses are enhanced through targeting of a cell surface receptor for CpG oligonucleotides (*e.g.*, DEC205). In some embodiments, T_H2-type responses and/or subject's T_H1-type responses may be monitored once provided nanoparticle compositions are administered.

[0555] In some embodiments, provided nanoparticle compositions effectively treat and/or prevent all of a subject's allergies falling into a particular class of allergy. In some embodiments, exemplary "classes" of allergies include, but are not limited to, anaphylactic allergies and non-anaphylactic allergies. In some embodiments, exemplary "classes" of allergies include, but are not limited to food allergies, insect allergies, pet dander allergies, pollen allergies, grass allergies, rubber allergies, and so forth. Thus, in some embodiments, provided nanoparticle compositions may be useful for treating all of a subject's food allergies. In some embodiments, exemplary "classes" of allergies include, but are not limited to, particular

individual foods which contain multiple allergens. For example, there are at least eleven known peanut allergen proteins. Thus, in some embodiments, a "class" of allergies is "peanut" allergy, and provided nanoparticle compositions may be useful for treating all of a subject's allergies associated with all seven different peanut allergen proteins.

[0556] In some embodiments, provided nanoparticle compositions may be useful for treating and/or preventing a single allergy, even though no allergy-specific antigen is included. In some embodiments, provided nanoparticle compositions may be useful for treating and/or preventing multiple different allergies. In some embodiments, provided nanoparticle compositions may be useful for treating and/or preventing substantially all of a subject's allergies. For example, subjects suffering from and/or susceptible to allergy are frequently allergic to more than one allergen, *e.g.*, at least 1, at least 2, at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, at least 10, at least 15, at least 20, at least 25, or more different allergens. Thus, in some embodiments, a provided nanoparticle composition may be used for treating and/or preventing at least 1, at least 2, at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, at least 10, at least 15, at least 20, at least 25, or more different allergies in a single patient. In some embodiments, a provided nanoparticle composition is administered to a subject suffering from and/or susceptible to multiple different allergies, *e.g.*, at least 1, at least 2, at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, at least 10, at least 15, at least 20, at least 25, or more different allergies, such that the subject's symptoms are reduced and/or improved. In some embodiments, a provided nanoparticle composition is administered to a subject suffering from and/or susceptible to multiple different allergies, *e.g.*, at least 1, at least 2, at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, at least 10, at least 15, at least 20, at least 25, or more different allergies, such that onset of the subject's symptoms is delayed.

[0557] In some embodiments, a provided composition maybe used as an oral vaccine to treat allergy. One of the major benefits of oral vaccines is the ability to generate both mucosal and systemic immunity. While oral vaccines have been developed previously, but they have been almost entirely directed to prevention of infectious disease, and have met with widely varying levels of success. For example, oral vaccines have been developed for anthrax, cholera, gastroenteritis, infant diarrhea, malaria, measles, and tuberculosis, among others (*see* Aziz *et al.*, Oral Vaccines: New Needs, New Possibilities, 2007, BioEssays 29.6: 591-604; *see also* Silin *et al.*, Oral Vaccination: Where are we?, Exp. Opin. Drug Deliv., 2007, 4(4):323-340, both of

which are hereby incorporated by reference in their entirety). Part of the reason for such unpredictable results is the complex nature of the gut mucosa. Briefly, the base of the mucosa in the gut is lined by gut- or mucosa-associated lymphoid tissue, with underlying lamina propria that is rich in intraepithelial lymphocytes (sometimes referred to as diffuse lymphoid tissue). The majority of T-cells in the gut mucosa are either αβ or γδ types. Both CD4 and CD8 cells are found in the gut mucosa, which also carries B cells, monocytes/macrophages, dendrocytes and other immune cells. In fact, the gut is known to house ~90% of the total number of immunocompetent cells in the human body, with circulating lymphocytes only comprising ~2% of the total lymphocytes (*see* Silin *et al.*). Furthermore, the gut is known to accommodate ~80% of all immunoglobin or Ig-producing cells and releases 2 to 3 times more secretory IgA that the total output of circulating IgG (*see* Silin *et al.*). Accordingly, any therapy that is exposed to the gut environment has the potential to engender a wide variety of responses and be affected by any of several immune or other cells.

[0558] In order to have an effective oral vaccine to treat allergy, effective presentation of one or more antigens to an antigen presenting cell (APC) is required. Although M-cells and Peyer's patches are popular targets of oral therapies, additional targets include, but are not limited to, enterocytes, mesenteric lymph nodes, and intestinal epithelial cells. Each APC may be targeted by various embodiments. Oral immunization is known to generate significant quantities of secretory IgA (sIgA), which is known to play a major role in mucosal defense against pathogens. However, the value of sIgA is questionable when one considers non-mucosal pathogens or conditions. Various embodiments recognize this and do not trigger large amounts of sIgA release, instead substantially generating a Th2 response.

[0559] The present disclosure recognizes, among other things, that there are several major known barriers to providing effective oral vaccines including but not limited to proteolytic degradation of antigens in the gut, tuning of proper release profile in the intestine, and problems delivering enough antigen in a reasonable sized dose. Additionally, the development of oral tolerance to an antigen is thought to be a major point of concern in developing oral vaccines in general. Oral tolerance is a phenomenon where oral antigen exposure can lead to immune tolerance and a suppression of the systemic immune response to subsequent challenges. The development of oral tolerance is not an automatic feature of oral antigen exposure, but rather depends on several factors including, but not limited to, age of subject, MHC restriction, delivery

site, nature, size and dose of antigen, degree of antigenic uptake, and processing and frequency of administration of antigen. Oral tolerance is thought to be mediated by several immunological mechanisms including: induction of regulatory T-cells (suppressors) that downregulate specific cytokines including IL-4, IL-10, and TGF-β, functional of clonal deletion of effector cells, and antibody-mediated suppression (*see* Silin *et al.*).

[0560] In some embodiments, provided compositions are able to present antigen to APCs without inducing oral tolerance. For example, in some embodiments, provided compositions may be administered buccally. Without wishing to be held to a particular theory, it is contemplated that high density of mast cells in buccal area facilitate decreasing oral tolerances. Without wishing to be held to a particular theory, it is possible certain embodiments are able to present larger quantities of antigen to the immune system than traditionally known methods of oral immunization. It is suspected that oral tolerance may manifest, at least in part, due to very small amounts of antigen being presented to APCs (*see* Silin *et al.*, Overcoming immune tolerance during oral vaccination against actinobacillus pleuropneumoniae, 2002, J Vet. Med. 49:169-175). In some embodiments, provided compositions present antigens to APCs in such a manner as to promote immune tolerance. Without wishing to be held to a particular theory, it may be advantageous to promote immune tolerance in some clinical circumstances, such as in cases of anaphylaxis, autoimmune disease, or certain infectious diseases including, but not limited to, dengue fever and RSV.

Treating Infectious Disease

[0561] The present disclosure provides, among other things, methods and compositions for the treatment and/or prevention of an infectious disease. In some embodiments, provided nanoparticle compositions are useful as vaccines to prevent and/or delay the onset of an infectious disease. In some embodiments, provided nanoparticle compositions are useful as vaccines to lessen the severity and/or duration of a future infectious disease. In some embodiments, provided nanoparticle compositions are useful as therapeutics to alleviate and/or arrest an infectious disease in progress.

[0562] In some embodiments, provided nanoparticle compositions may be administered once, twice, three times, four times or more. In some embodiments, it may be sufficient to administer provided nanoparticle compositions once (optionally followed by a single booster). In some

embodiments, it is possible, although less desirable, to administer provided nanoparticle compositions to an infected individual to achieve a therapeutic response. Dosing may need to be adjusted accordingly.

[0563] In some embodiments, provided nanoparticle compositions may administrated with other prophylactic or therapeutic compounds. As a non-limiting example, a prophylactic or therapeutic compound may be an adjuvant or a booster. In some embodiments, an adjuvant or a booster may be given after an earlier administration of the prophylactic composition. The time of administration between the initial administration of the prophylactic composition and an adjuvant or a booster may be, but is not limited to, 1 minute, 2 minutes, 3 minutes, 4 minutes, 5 minutes, 6 minutes, 7 minutes, 8 minutes, 9 minutes, 10 minutes, 15 minutes, 20 minutes 35 minutes, 40 minutes, 45 minutes, 50 minutes, 55 minutes, 1 hour, 2 hours, 3 hours, 4 hours, 5 hours, 6 hours, 7 hours, 8 hours, 9 hours, 10 hours, 11 hours, 12 hours, 13 hours, 14 hours, 15 hours, 16 hours, 17 hours, 18 hours, 19 hours, 20 hours, 21 hours, 22 hours, 23 hours, 1 day, 36 hours, 2 days, 3 days, 4 days, 5 days, 6 days, 1 week, 10 days, 2 weeks, 3 weeks, 1 month, 2 months, 3 months, 4 months, 5 months, 6 months, 7 months, 8 months, 9 months, 10 months, 11 months, 1 year, 18 months, 2 years, 3 years, 4 years, 5 years, 6 years, 7 years, 8 years, 9 years, or more than 10 years.

[0564] In some embodiments, provided compositions are able to induce both mucosal and systemic immunity. In some embodiments, a provided composition maybe used as an oral vaccine to treat and/or prevent an infectious disease. In some embodiments, provided compositions are able to present antigen to APCs without inducing oral tolerance. In some embodiments, oral administration of a provided composition may be beneficial for infections transmitted mainly through the mucosal tissue of the respiratory tract. Such infections are known that the mucosal immune system, rather than the systemic immune system, plays fundamental roles in the host's defense against the infections. In some embodiments, oral administration of a provided composition may induce respiratory mucosal immunity, enhancing protection from infections.

[0565] In some embodiments, the subject in need thereof is suffering from an infection caused by, but not limited to viruses, prions, bacteria, viroids, macroparasites, fungi, and/or combinations thereof. In some embodiments, the subject is suffering from a primary infection. In some embodiments, the subject is suffering from a secondary infection. In some

embodiments, the subject is suffering from an active symptomatic infection. In some embodiments, the subject is suffering from an active asymptomatic infection (*i.e.,* infection is active, but does not produce noticeable symptoms; *e.g.,* silent or subclinical infection). In some embodiments, the subject is suffering from a latent infection (*i.e.*, inactive or dormant infection).

[0566] Exemplary infections that may be treated by some embodiments include, but are not limited to actinomycosis, African sleeping sickness, AIDS, anthrax, hemorrhagic fevers, bacterial pneumonia, candidiasis, cellulitis, Chagas disease, chickenpox, cholera, C. difficile infection, coronavirus disease 2019 (COVID-2019) Creutzfeldt-Jakob disease, dengue fever, diphtheria, ebola, enterococcus infection, food poisoning, gangrene, gonorrhea, streptococcal infections, hepatitis A-E, herpes, hookworm, mononucleosis, leishmaniosis, leprosy, Lyme disease, malaria, measles, meningitis, mumps, conjunctivitis, pertussis, rabies, respiratory syncytial virus, rhinovirus, rubella, SARS, scabies, sepsis, shingles, syphilis, tetanus, trichinellosis, tuberculosis, tularemia, viral pneumonia, West Nile fever, and yellow fever.

Treating Cancer

[0567] The present disclosure provides, among other things, methods and compositions for the treatment and/or prevention of cancer. In some embodiments, provided nanoparticle compositions are useful as vaccines to prevent and/or delay the onset of a cancer. In some embodiments, provided nanoparticle compositions are useful as therapeutics to alleviate and/or arrest a cancer in progress.

Dosing

[0568] In some embodiments, provided nanoparticle and/or pharmaceutical compositions are administered according to a dosing regimen sufficient to achieve a desired immunological reaction. For example, in some embodiments, a dosing regimen is sufficient to achieve a desired immunological reaction if its administration to a relevant patient population shows a statistically significant correlation with achievement of the desired immunological reaction.

[0569] In some embodiments, the desired immunological reaction is a reduction in the degree and/or prevalence of symptoms of a disease, disorder or condition (*e.g.*, allergy, infection and/or cancer) of at least about 20%, about 25%; about 30%; about 31%, about 32%, about 33%, about 34%, about 35%, about 36%, about 37%, about 38%, about 39%, about 40%, about 41%, about

42%, about 43%, about 44%, about 45%, about 46%, about 47%, about 48%, about 49%, about 50%, about 51%, about 52%, about 53%, about 54%, about 55%, about 56%, about 57%, about 58%, about 59%, about 60%, about 61%, about 62%, about 63%, about 64%, about 65%, about 66%, about 67%, about 68%, about 69%, about 70%, about 71%, about 72%, about 73%, about 74%, about 75%, about 76%, about 77%, about 78%, about 79%, about 80%, about 81%, about 82%, about 83%, about 84%, about 85%, about 86%, about 87%, about 88%, about 89%, about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, about 99%, or more.

[0570] In some embodiments, a provided nanoparticle and/or pharmaceutical composition is administered according to a dosing regimen sufficient to achieve a reduction in the degree and/or prevalence of symptoms of a disease, disorder or condition (*e.g.*, allergy, infectious disease, cancer) of a specified percentage of a population of patients to which the composition is administered. In some embodiments, the specified percentage of population of patients to which the composition was administered is at least about 5%, about 10%, about 15%, about 20%, about 25%, about 30%, about 31%, about 32%, about 33%, about 34%, about 35%, about 36%, about 37%, about 38%, about 39%, about 40%, about 41%, about 42%, about 43%, about 44%, about 45%, about 46%, about 47%, about 48%, about 49%, about 50%, about 51%, about 52%, about 53%, about 54%, about 55%, about 56%, about 57%, about 58%, about 59%, about 60%, about 61%, about 62%, about 63%, about 64%, about 65%, about 66%, about 67%, about 68%, about 69%, about 70%, about 71%, about 72%, about 73%, about 74%, about 75%, about 76%, about 77%, about 78%, about 79%, about 80%, about 81%, about 82%, about 83%, about 84%, about 85%, about 86%, about 87%, about 88%, about 89%, about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, about 99%, or more.

[0571] To give but a few illustrative examples, in some embodiments, administration of at least one provided nanoparticle and/or pharmaceutical composition according to a dosing regimen is sufficient to achieve a reduction in the degree and/or prevalence of a disease, disorder or conditions (*e.g.*, allergy, infectious disease, cancer) of at least about 20% in at least about 50% of the population of patients to which the composition was administered. In some embodiments, administration of at least one provided nanoparticle and/or pharmaceutical composition according to a dosing regimen is sufficient to achieve a reduction in the degree and/or prevalence

of a disease, disorder or conditions (*e.g.*, allergy, infectious disease, cancer) of at least about 30% in at least about 50% of the population of patients to which the composition was administered.

[0572] In some embodiments, at least one provided nanoparticle and/or pharmaceutical composition is administered according to a dosing regimen sufficient to achieve a delay in the onset of symptoms of a disease, disorder or conditions (*e.g.*, allergy, infectious disease, cancer). In some embodiments, at least one provided nanoparticle and/or pharmaceutical composition is administered according to a dosing regimen sufficient to prevent the onset of one or more symptoms of a disease, disorder or conditions (*e.g.*, allergy, infectious disease, cancer).

[0573] In some embodiments, a provided dosing regimen comprises or consists of a single dose. In some embodiments, a provided dosing regimen comprises or consists of multiple doses, separated from one another by intervals of time that may or may not vary. In some embodiments, a provided dosing regimen comprises or consists of dosing once every 20 years, once every 10 years, once every 5 years, once every 4 years, once every 3 years, once every 2 years, once per year, twice per year, 3 times per year, 4 times per year, 5 times per year, 6 times per year, 7 times per year, 8 times per year, 9 times per year, 10 times per year, 11 times per year, once per month, twice per month, three times per month, once per week, twice per week, three times per week, 4 times per week, 5 times per week, 6 times per week, daily, twice daily, 3 times daily, 4 times daily, 5 times daily, 6 times daily, 7 times daily, 8 times daily, 9 times daily, 10 times daily, 11 times daily, 12 times daily, or hourly.

[0574] In some embodiments, a provided dosing regimen comprises or consists of an initial dose with one or more booster doses. In some embodiments, one or more booster doses are administered 1 day, 2 days, 3 days, 4 days, 5 days, 6 days, 1 week, 2 weeks, 1 month, 2 months, 6 months, 1 year, 2 years, 5 years, 10 years, or longer than 10 years after the initial dose. In some embodiments, an initial dose comprises a series of doses administered over a period of time. For example, in some embodiments, an initial dose comprises a series of 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, or more doses administered at regular intervals, *e.g.*, intervals that are close in time to one another, such as 5 minute intervals, 10 minute intervals, 15 minute intervals, 20 minute intervals, 25 minute intervals, 30 minute intervals, 45 minute intervals, hourly intervals, every 2 hours, *etc.*

[0575] In some embodiments, an initial dose and booster doses contain the same amount of provided nanoparticles and/or nanoparticle composition. In some embodiments, an initial dose

and booster doses contain different amounts of provided nanoparticles and/or nanoparticle composition. In some embodiments, dosing escalates from a starter dose to a target dose. In some embodiments, the escalation is over the span of 1 day, 2 days, 3 days, 4 days, 5 days, 6 days, 1 week, 2 weeks, 1 month, 2 months, 6 months, 1 year, 2 years, 5 years, 10 years, or longer than 10 years after the initial dose. Course of a day, week, month, or year.

[0576] In certain embodiments, provided nanoparticles and/or nanoparticle compositions are administered at dosage levels sufficient to deliver from about 0.001 mg/kg to about 100 mg/kg (*e.g.*, of payload, nanoparticles, or nanoparticle composition), from about 0.01 mg/kg to about 50 mg/kg, preferably from about 0.1 mg/kg to about 40 mg/kg, from about 0.5 mg/kg to about 30 mg/kg, from about 0.01 mg/kg to about 10 mg/kg, from about 0.1 mg/kg to about 10 mg/kg, or from about 1 mg/kg to about 25 mg/kg, of subject body weight per day, one or more times a day. In some embodiments, provided nanoparticles and/or nanoparticle compositions are formulated into a unit dose. In some embodiments, a unit dosage is about 1 μg, 10 μg, about 25 μg, about 50 μg, about 100 μg, about 250 μg, about 500 μg, about 1mg, 10 mg, about 25 mg, about 50 mg, about 100 mg, about 250 mg, about 500 mg, about 1 g, about 5 g, about 10 g, about 25 g, about 50 g, about 100 g, or more than about 100 g. In some embodiments, the amount of provided nanoparticles and/or nanoparticle composition present in a particular unit dose depends on the subject to which the composition is to be administered. To give but a few examples, in some embodiments, a unit dose appropriate for a mouse is smaller than a unit dose that is appropriate for a rat, which is smaller than a unit dose that is appropriate for a dog, is smaller than a unit dose that is appropriate for a human.

[0577] In some embodiments, a provided dosing regimen comprises or consists of administration of multiple doses over the course of the subject's entire lifespan. In some embodiments, a provided dosing regimen comprises administration of multiple doses over the course of several years (*e.g.*, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, 50, 60, 70, 80, 90, or 100 years). In some embodiments, a provided dosing regimen comprises or consists of multiple doses over the course of 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, or 12 months.

[0578] In some embodiments, when provided nanoparticles and/or compositions are used in the treatment of allergy, prior to the first dose, a subject's baseline allergic response is determined by one or more of a variety of methods, including, but not limited to, (1) performing a prick skin test (PST) of one or more of the subject's 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17,

18, 19, 20, or more than 20 allergens, and measuring the wheal and flare response to the PST; (2) measuring blood serum IgE levels; (3) noting the subject's own description of her typical symptoms (*e.g.*, nature, severity, and/or duration of symptoms) upon exposure to one or more of her 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, or more than 20 allergens; (4) exposing the subject to a certain dose of one or more of her 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, or more than 20 allergens (*e.g.*, if only a small or nonexistent risk of anaphylaxis); (5) measuring expression (*e.g.*, levels, spatial distribution, temporal distribution, *etc.*), of one or more molecular markers, including, but not limited to, T-cell markers CD4+ and/or CD8+; (6) performing a basophil histamine release assay; and/or combinations thereof. In some embodiments, a subject's allergic response is monitored using any combination of methods, *e.g.*, methods (1) – (6) described above, throughout the course of the treatment regimen and/or after the treatment regimen is completed, *e.g.*, at regular intervals. In some embodiments, allergic response is monitored daily, weekly, bi-weekly, monthly, 6 times per year, 4 times per year, 3 times per year, 2 times per year, once per year, every 2 years, every 5 years, and/or every 10 years, *etc.*

[0579] In some embodiments, a subject is challenged with a single allergen and/or multiple allergens, *e.g.*, a subset of the subject's allergens (*e.g.*, allergens to which the subject is known to be allergic) and/or all of the subject's allergens (*e.g.*, allergens to which the subject is known to be allergic). In some embodiments, allergy challenge is performed after 1 week, 2 weeks, 1 month, 2 months, 6 months, and 1 year after initiation of treatment.

[0580] In some embodiments, provided nanoparticles and/or compositions may be administered via any medically acceptable route. For example, in some embodiments, a provided composition may be administered via intravenous administration; intradermal administration; transdermal administration; oral administration; subcutaneous administration; transmucosal administration; and/or combinations thereof. In some embodiments, exemplary routes of transmucosal administration include, but are not limited to buccal administration; nasal administration; bronchial administration; vaginal administration; rectal administration; sublingual administration; and/or combinations thereof.

Routes of Administration

[0581] In some embodiments, provided nanoparticle compositions may be formulated for any appropriate route of delivery. In some embodiments, provided nanoparticles and/or nanoparticle compositions may be formulated for any route of delivery, including, but not limited to, bronchial instillation, and/or inhalation; buccal, enteral, interdermal, intra-arterial (IA), intradermal, intragastric (IG), intramedullary, intramuscular (IM), intranasal, intraperitoneal (IP), intrathecal, intratracheal instillation (by), intravenous (IV), intraventricular, mucosal, nasal spray, and/or aerosol, oral (PO), as an oral spray, rectal (PR), subcutaneous (SQ), sublingual; topical and/or transdermal (*e.g.*, by lotions, creams, liniments, ointments, powders, gels, drops, *etc.*), transdermal, vaginal, vitreal, and/or through a portal vein catheter; and/or combinations thereof. In some embodiments, the present disclosure provides methods of administration of provided nanoparticle compositions via mucosal administration. In some embodiments, the present disclosure provides methods of administration of provided nanoparticle compositions via oral administration. In some embodiments, the present disclosure provides methods of administration of provided nanoparticle compositions via sublingual administration.

[0582] Without being bound by any particular theory, the present disclosure contemplates that a feature of the provided technologies is its amenability to oral and/or mucosal administration. That is, the present disclosure provides technologies that, among other things, can be formulated for delivery into an oral cavity of a subject. In some embodiments, provided nanoparticles and/or nanoparticle preparations may be formulated for oral administration. In some embodiments, oral administration may be or comprise enteral administration. In some embodiments, oral administration may not comprise enteral administration. For example, the present disclosure contemplates that, in some embodiments, delivery of provided nanoparticles and/or nanoparticle preparations to a restricted area of the oral cavity (*e.g.*, sublingual and/or buccal) may provide advantages for absorption by and contact with particular cell types that are in higher density in sublingual and/or buccal areas versus other cell types which may exist in higher densities in other areas of the oral cavity and/or gastrointestinal tract. In some embodiments, oral administration is buccal, sublabial, and/or sublingual administration. In some embodiments, dosage forms for oral administration include a tablet (*e.g.*, to swallow, chew or dissolve or melt in water or sublingually, *e.g.*, time-release or sustained-release), capsule (*e.g.*, chewable capsule *e.g.*, with a coating that dissolves in the stomach or bowel to release the medication there, *e.g.*, time-release or sustained-release), paste, gum, frozen droplet, tablet,

capsule, powder, granule, tea, drop, liquid medication (e.g., sublingual immunotherapy), and syrup. In some embodiments, any solid or semi solid format (*e.g.*, powder, granule, *etc.*) may be mixed with an amount of liquid and administered/placed into, *e.g.*, a sublingual, *e.g.*, buccal area.

[0583] In some embodiments, provided nanoparticles and/or nanoparticle preparations for oral administration may be formulated in a form of powder. In such embodiments, nanoparticle powder may be stored and/or transferred in a vial. For example, in some embodiments, a patient may add water to a vial containing nanoparticle power. A patient may extract a nanoparticle solution from a vial and administer buccally, sublabially, and/or sublingually. In some embodiments, provided nanoparticles and/or nanoparticle preparations for oral administration may be formulated in a frozen tablet that liquifies at room temperature. A frozen tablet may be stored and/or transferred under about 0, -1, -2, -3, -4, -5, -6, -7, -8, -9 or -10 °C.

[0584] In some embodiments nanoparticles are formulated in a phosphate buffer for oral administration. In such embodiments, nanoparticles and phosphate buffer are packaged in single-use ampoules. In such embodiments, nanoparticles are stored at -20°C.

[0585] In some embodiments, the present disclosure provides a method comprising steps of administering to a subject in need thereof a nanoparticle composition comprising a nanoparticle population having one or more payloads, or precursor(s) thereof, that activate antigen-specific T cells, wherein the nanoparticle composition is administered orally, sublingually or buccally.

[0586] In another aspect, the present disclosure provides a method comprising steps of (i) administering to a subject in need thereof a first nanoparticle composition comprising a first nanoparticle population having a first payload, or precursor(s) thereof, that activate a first antigen-specific T cell; and administering to the subject a second nanoparticle composition comprising a second nanoparticle population having a second payload, or precursor(s) thereof, that activate a second antigen-specific T cell, wherein the first and/or second nanoparticle compositions are administered orally, sublingually or buccally.

Combination Therapy

[0587] In some embodiments, provided therapy (*e.g.*, provided nanoparticles and compositions) can be administered in combination with at least one other therapy, so that the subject receives at least some benefit from both. In some embodiments, a subject may have previously received or be currently receiving at least one other therapy. In some embodiments, the at least one other

therapy is administered to a subject who has previously received or is currently receiving nanoparticle therapy as described herein. For example, useful in the treatment of one or more diseases, disorders, or conditions treated by the relevant provided pharmaceutical composition, so the subject is simultaneously exposed to both. In some embodiments, a provided nanoparticle composition is utilized in a pharmaceutical formulation that is separate from and distinct from the pharmaceutical formulation containing another therapeutic agent. In some embodiments, a provided nanoparticle composition is admixed with the composition comprising another therapeutic agent. In other words, in some embodiments, a provided nanoparticle composition is produced individually, and the provided nanoparticle composition is simply mixed with another composition comprising another therapeutic agent.

[0588] The particular combination of therapies (substances and/or procedures) to employ in a combination regimen will take into account compatibility of the desired substances and/or procedures and the desired therapeutic effect to be achieved. In some embodiments, provided nanoparticle compositions can be administered concurrently with, prior to, or subsequent to, one or more other therapeutic agents (*e.g.*, desired known allergy therapeutics).

[0589] It will be appreciated that the therapies employed may achieve a desired effect for the same disorder (for example, a provided nanoparticle composition useful for treating allergy may be administered concurrently with a known allergy therapeutic that is also useful for treating allergy), or they may achieve different effects (for example, a provided nanoparticle composition that is useful for treating allergy may be administered concurrently with a therapeutic agent that is useful for alleviating adverse side effects, for instance, inflammation, nausea, *etc.*). In some embodiments, provided nanoparticle compositions in accordance with the present disclosure are administered with a second therapeutic agent that is approved by the U.S. Food and Drug Administration (FDA).

[0590] It will be appreciated that the therapies employed may achieve a desired effect for the same disorder (for example, a provided nanoparticle composition useful for treating an autoimmune disease may be administered concurrently with an autoimmune disease therapeutic that is also useful for treating an autoimmune disease), or they may achieve different effects (for example, a provided nanoparticle composition that is useful for an autoimmune disease may be administered concurrently with a therapeutic agent that is useful for alleviating adverse side effects, for instance, inflammation, nausea, *etc.*).

[0591] As used herein, the terms "in combination with" and "in conjunction with" mean that the provided nanoparticle compositions can be administered concurrently with, prior to, or subsequent to, one or more other desired therapeutics. In general, each substance will be administered at a dose and/or on a time schedule determined for that agent.

Allergy Therapies

[0592] For example, in some embodiments, provided nanoparticles and/or compositions for the treatment of allergy may, in some embodiments, be administered in combination with, for example, one or more antihistamines (*i.e.*, histamine antagonist), corticosteroids including glucocorticoids; epinephrine (adrenaline); theophylline (dimethylxanthine); cromolyn sodium; anti-leukotrienes; anti-cholinergics; decongestants; mast cell stabilizers; immunotherapy (progressively larger doses of a specific allergen); monoclonal anti-IgE antibodies (*e.g.*, omalizumab); and/or combinations thereof.

[0593] Exemplary antihistamines include, but are not limited to Azelastine; Brompheniramine; Buclizine; Bromodiphenhydramine; Carbinoxamine; Cetirizine; Cyclizine; Chlorpheniramine; Chlorodiphenhydramine; Clemastine; Cyproheptadine; Desloratadine; Dexbrompheniramine; Deschlorpheniramine; Dexchlorpheniramine; Dimetindene; Diphenhydramine (Benadryl); Doxylamine; Ebastine; Embramine; Fexofenadine; Levocetirizine; Loratadine; Olopatadine (Patanol); Phenindamine (Nolahist and Thephorin); Pheniramine (Avil); Phenyltoloxamine; Promethazine; Pyrilamine; Rupatadine; Tripelennamine; Triprolidine; and/or combinations thereof.

[0594] Exemplary corticosteroids and glucocorticoids include, but are not limited to Beclometasone dipropionate and Beclomethasone (Clenil, Qvar, Beconase AQ, Alanase, Vancenase); Budesonide (Rhinocort, Rhinosol, Pulmicort, Budicort, Symbicort, Noex); Ciclesonide (Alvesco, Omnaris, Omniair); Flunisolide (Aerobid); Fluticasone (Veramyst); Fluticasone (Flonase); Mometasone and Mometasone furoate (Nasonex); Triamcinolone (Nasacort AQ); Prednisone; Methylprednisolone (Depo-Medrol); Triamcinolone (Kenalog); and/or combinations thereof.

[0595] Exemplary forms of cromolyn sodium include, but are not limited to, Rynacrom; Nasalcrom; Prevalin; Intal; Optocrom; Optrex; Gastrocrom; Intercron; and/or combinations thereof.

[0596] Exemplary anti-leukotrienes and leukotriene inhibitors (or modifiers) include, but are not limited to Montelukast (Singulair, Montelo-10, and Monteflo); Zafirlukast (Accolate, Accolcit, Vanticon); Pranlukast; Zileuton (Zyflo, Zyflo CR); and/or combinations thereof.

[0597] Exemplary anti-cholinergics include, but are not limited to, Benztropine (Cogentin); Bupropion (Zyban, Wellbutrin); Chlorphenamine (Chlor-Trimeton); Combivent (Ipratropium bromide and Albuterol); Dextromethorphan; Dimenhydrinate (Dramamine); Diphenhydramine (Benadryl, Sominex, Advil PM, *etc.*); Doxacurium; Glycopyrrolate (Robinul); Hexamethonium; Ipratropium bromide (Atrovent, Apovent, Ipraxa , Aervoent); Mecamylamine; Oxitropium (Oxivent); Oxybutinin (Ditropan, Driptane, Lyrinel XL); Tiotropium (Spiriva); Tolterodine (Detrol, Detrusitol); Tubocurarine; and/or combinations thereof.

[0598] Exemplary decongestants include, but are not limited to, Ephedrine; Levo-methamphetamine; Naphazoline; Oxymetazoline; Phenylephrine; Phenylpropanolamine; Propylhexedrine; Synephrine; Tetrahydrozoline; and/or combinations thereof.

[0599] Exemplary mast cell stabilizers include, but are not limited to, Cromoglicic acid; Ketotifen and Ketotifen fumarate (Zaditor, Zaditen, Alaway, Zyrtec Itchy-Eye Drops, Claritin Eye); Methyl xanthines; and/or combinations thereof.

[0600] In some embodiments, exemplary known allergy therapeutics that can be administered in combination with provided nanoparticle compositions in accordance with the present disclosure include, but are not limited to, any of the therapeutics described in US Patent Numbers 5,558,869, 5,973,121, 6,835,824, 6,486,311, and/or 7,485,708, and/or in US Patent Publication Numbers 2003/0035810, 2003/0202980, 2004/0208894, 2004/0234548, 2007/0213507, 2010/0166802, and/or 2011/0027298, all of which are incorporated herein by reference.

Infectious Disease Therapies

[0601] For example, in some embodiments, provided nanoparticles and/or compositions for the treatment of infectious disease may, in some embodiments, be administered in combination with, for example, one or more sulfaniliamides; folic acid analogs; beta-lactams such as penicillins, cephalosporins, and carbapenems; aminoglycosides such as streptomycin, kanamycin, neomycin, and gentamycin; tetracyclines such as chlortetracycline, oxytetracycline, and doxycycline; macrolides; lincosamides; streptogramins; fluoroquinolones, rifampin, mupirocin, cycloserine,

aminocyclitols, glycopeptides, oxazolidinones, and derivatives/analogs and/or combinations thereof.

[0602] Exemplary antiviral agents include, but are not limited to Abacavir, Aciclovir, Acyclovir, Adefovir, Amantadine, Amprenavir, Ampligen, Arbidol, Atazanavir, Atripla, Boceprevirertet, Cidofovir, Combivir, Darunavir, Delavirdine, Didanosine, Docosanol, Edoxudine, Efavirenz, Emtricitabine, Enfuvirtide, Entecavir, Entry inhibitors, Famciclovir, Fomivirsen, Fosamprenavir, Foscarnet, Fosfonet, Imunovir, Idoxuridine, Imiquimod, Indinavir, Inosine, Interferon type III, Interferon type II, Interferon type I, Interferon, Lamivudine, Lopinavir, Loviride, Maraviroc, Moroxydine, Methisazone, Nelfinavir, Nevirapine, Nexavir, Nucleoside analogues, Oseltamivir, Peginterferon alfa-2a, Penciclovir, Peramivir, Pleconaril, Podophyllotoxin, Raltegravir, Reverse transcriptase inhibitors, Ribavirin, Rimantadine, Ritonavir, Pyramidine, Saquinavir, Stavudine, Tea tree oil, Telaprevir, Tenofovir, Tenofovir disoproxil, Tipranavir, Trifluridine, Trizivir, Tromantadine, Truvada, Valaciclovir, Valganciclovir, Vicriviroc, Vidarabine, Viramidine, Zalcitabine, Zanamivir, Zidovudine, and derivatives/analogs and/or combinations thereof.

[0603] Exemplary antifungal agents include, but are not limited to polyene agents such as amphotericin, candicidin, filipin, hamycin, natamycin, nystatin, and rimocidin; imidazole, triazole and thiazole agents such as bifonazole, butoconazole, clotrimazole, econazole, fenticonazole, isoconazole, ketoconazole, miconazole, omoconazole, oxiconazole, sertaconazole, sulconazole, tioconazole, alboconazole, fluconazole, isavuconazole, posaconazole, ravuconazole, terconazole, voriconazole, and abafungin; allylamines such as amorolfin, butenafine, naftafine, and terbinafine; and echinocandins such as anidulafungin, caspofungin, and micafungin and derivatives/analogs and/or combinations thereof.

[0604] As an additional example, in some embodiments, provided nanoparticles and/or compositions for the treatment of infectious disease may be administered in combination with, for example, an antibiotic such as an antibacterial agent, an antiviral agent, and/or an antifungal agent. In some embodiments, provided pharmaceutical compositions may be administered in combination with a vaccine.

Cancer Therapies

[0605] As an additional example, in some embodiments, provided nanoparticles and/or compositions for the treatment of cancer may be administered in combination with, for example,

surgery, radiation therapy, chemotherapy, targeted therapy, immunotherapy, hormonal therapy, angiogenesis inhibitor, and/or other anticancer therapies and/or medications.

[0606] In some embodiments, provided nanoparticles and/or compositions for the treatment of cancer may be administered in combination with therapeutic agents (*e.g.*, alkylating agents, antimetabolite agents, chemotherapeutic, cytotoxic agents, radioactive ion, and/or other therapeutic agent).

[0607] Exemplary alkylating agents include, but are not limited to polyfunctional alkylating agents such as cyclophosphamide (Cytoxan), mechlorethamine, melphan (Alkeran), chlorambucil (Leukeran), thiopeta (Thioplex), and busulfan (Myleran); procarbazine, dacarbazine, altretamine, cisplatin, and derivatives/analogs and/or combinations thereof.

[0608] Exemplary antimetabolite agents include, but are not limited to methotrexate; purine antagonists such as mercaptopurine (6-MP), thioguanine (6-TG), fludarabine phosphate, cladribine, and pentostatin; pyrimidine antagonists such as fluorouracil, cytarabine, and azacitidine; plant alkaloids such as vinblastine (Velban), vincristine (Oncovin), etoposide (VP-16), teniposide (Vimon), topotecan (Hycamtin), irinotecan (Camptosar), paclitaxel (Taxol), and docetaxel (Taxotere) and derivatives/analogs and/or combinations thereof.

[0609] Exemplary other anticancer agents include, but are not limited to amsacrine; hydroxyurea (Hydrea); asparaginase (El-spar); mitoxantrone (Novantrone); mitotane; retinoic acid, bone marrow growth factors, amifostine, and derivatives/analogs and/or combinations thereof.

[0610] Exemplary cytotoxic agents include, but are not limited to 1-dehydrotestosterone, actinomycin D, CC-1065 colchicin, cytochalasin B, daunorubicin, dihydroxy anthracin dione, doxorubicin, emetine, ethidium bromide, etoposide, glucocorticoids, gramicidin D, lidocaine, maytansinoids, *e.g.*, maytansinol, mithramycin, mitomycin, mitoxantrone, procaine, propranolol, puromycin, taxol, tenoposide, tetracaine, vinblastine, vincristine and derivatives/analogs and/or combinations thereof.

[0611] Exemplary radioactive ions include, but are not limited to iodine (*e.g.*, iodine 125 or iodine 131), strontium 89, phosphorous, palladium, cesium, iridium, phosphate, cobalt, yttrium 90, Samarium 153 and praseodymium. Other therapeutic agents include, but are not limited to, antimetabolites (*e.g.*, methotrexate, 6-mercaptopurine, 6-thioguanine, cytarabine, 5-fluorouracil decarbazine), alkylating agents (*e.g.*, mechlorethamine, thioepa chlorambucil, CC-1065, melphalan, carmustine (BSNU) and lomustine (CCNU), cyclothosphamide, busulfan,

dibromomannitol, streptozotocin, mitomycin C, and cis-dichlorodiamine platinum (II) (DDP) cisplatin), anthracyclines (*e.g.*, daunorubicin (formerly daunomycin) and doxorubicin), antibiotics (*e.g.*, dactinomycin (formerly actinomycin), bleomycin, mithramycin, and anthramycin (AMC)), anti-mitotic agents (*e.g.*, vincristine, vinblastine, taxol and maytansinoids), and derivatives/analogs and/or combinations thereof.

[0612] Exemplary cytotoxic nucleosides and/or cytotoxic nucleoside analogues include, but are not limited to adenosine arabinoside, cytarabine, cytosine arabinoside, 5-fluorouracil, fludarabine, floxuridine, ftorafur, 6-mercaptopurine, cytarabine, gemcitabine, troxacitabine, decitabine, tezacitabine, DMDC, cladribine, clofarabine, 5-azacytidine, 4′-thio-aracytidine, cyclopentenylcytosine and 1-(2-C-cyano-2-deoxy-beta-D-arabino-pentofuranosyl)-cytosine, fludarabine phosphate, N4-behenoyl-1-beta-D-arabinofuranosylcytosine, N4-octadecyl-1-β-D-arabinofuranosylcytosine, N4-palmitoyl-1-(2-C-cyano-2-deoxy-beta-D-arabino-pentofuranosyl)cytosin-e, P-4055 (cytarabine 5′-elaidic acid ester), Capecitabine, N4 alkyl and aralkyl carbamates of 5′-deoxy-5-fluorocytidine and the implication is that these compounds will be activated by hydrolysis under normal physiological conditions to provide 5′-deoxy-5-fluorocytidine. Pyrazolo[3,4-D]-pyrimidines, allopurinol, azathioprine, capecitabine, cytosine arabinoside, fluorouracil, mercaptopurine, 6-thioguanine, acyclovir, ara-adenosine, ribavirin, 7-deaza-adenosine, 7-deaza-guanosine, 6-aza-uracil, G-aza-cytidine, thymidine ribonucleotide, 5-bromodeoxyuridine, 2-chloro-purine, and inosine, and derivatives/analogs and/or combinations thereof.

[0613] In some embodiments, provided nanoparticles and/or compositions for the treatment of cancer may be administered, for example, in combination with immune checkpoint inhibitors. Exemplary checkpoint inhibitors include, but are not limited to PD-1, TIM-3, VISTA, A2AR, B7-H3, B7-H4, BTLA, CTLA-4, IDO, KIR and LAG3.

Kits

[0614] The present disclosure provides kits comprising provided nanoparticles, nanoparticle compositions, and/or pharmaceutical compositions. In some embodiments, a kit may comprise (i) at least one provided nanoparticle composition; and (ii) at least one pharmaceutically acceptable excipient; and, optionally, (iii) instructions for use.

[0615] In some embodiments, kits include multiple (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, or more) doses of provided nanoparticles and/or nanoparticle compositions. In some embodiments, kits include multiple (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, or more) populations of provided nanoparticles having different functional elements (*e.g.*, microbial mimic entities). In some embodiments, multiple populations of provided nanoparticles are packaged separately from one another in provided kits. In some embodiments, provided kits may include provided compositions and one or more other therapeutic agents intended for administration with the provided compositions.

[0616] In some embodiments, the present disclosure provides pharmaceutical packs or kits including provided nanoparticles and/or nanoparticle compositions to be used in treatment methods according to the present disclosure. In some embodiments, pharmaceutical packs or kits include preparations or pharmaceutical compositions containing provided nanoparticles and/or nanoparticle compositions in one or more containers filled with optionally one or more additional ingredients of pharmaceutical compositions in accordance with the present disclosure. In some embodiments, the pharmaceutical pack or kit includes an additional approved therapeutic agent for use in combination therapies, as described herein. Optionally associated with such container(s) can be a notice in the form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceutical products, which notice reflects approval by the agency of manufacture, use, or sale for human administration.

[0617] In some embodiments, kits are provided that include provided nanoparticle compositions and instructions for use. Pharmaceutical doses or instructions therefor may be provided in a kit for administration to an individual suffering from and/or susceptible to a disease, disorder or condition (*e.g.*, allergy, infectious disease, cancer).

EXEMPLIFICATION

[0618] These Examples describe exemplary methods for preparation of certain polymer nanoparticles (*e.g.*, polymer nanoparticles comprising a payload and/or a coating) in accordance with the present disclosure. Representative nanoparticle manufacturing processes are described below. One of skill in the art will appreciate that certain conditions and specific values as described herein may be changed as desired.

Example 1: Preparation of polymer nanoparticles comprising protein

[0619] This Example describes an exemplary method for preparation of certain polymer nanoparticles (*e.g.,* polymer nanoparticles comprising a payload and/or a coating) in accordance with the present disclosure. One of skill in the art will appreciate that certain conditions and specific values as described herein may be changed as desired.

Steps:

1) An aqueous payload solution was prepared at room temperature by dissolving a payload comprising protein (*e.g.*, peanut protein as, *e.g.*, crude peanut extract) in water (*e.g.*, here, crude peanut extract) and DNA (*e.g.*, sheared *E. coli* DNA). Sonication and/or homogenization were used as required to obtain a substantially homogenous solution of protein and water.

2) An organic polymer solution was prepared by dissolving polymer (here, PLG) into organic solvent (here, DMSO), using magnetic stirring to generate an organic PLG solution. The temperature was maintained between approximately 25°C – 30°C, which prevents DMSO from freezing, lowers viscosity of the PLG solution, and increases speed at which PLG dissolves.

3) The aqueous payload solution of step 1 was then added to the organic polymer solution of step 2 and combined to achieve a mixture of substantial homogeneity.

4) The mixture of step 3 (organic solution of step 1 and aqueous solution of step 2) was then subjected to rotary evaporation until approximately 93% of the original mass was removed.

5) 1-propanol was added to the concentrated mixture of step 4 in the presence of mechanical stirring. The temperature was maintained at about 16 °C. This step generates a precipitate of the PLG, peanut protein and DNA.

6) The nanoparticle suspension of step 5 was mixed with aqueous PVA solution. The temperature of the aqueous PVA was about 4 °C. Without being held to a particular theory, it is contemplated that the addition of PVA is helpful in minimizing aggregation of the nanoparticles in suspension and/or otherwise stabilizing formed nanoparticles.

7) The stabilized nanoparticle suspension of step 6 was filtered through a TFF membrane. Without wishing to be held to a particular theory, it is contemplated that this step may remove one or more of unformed polymer molecules, unassociated stabilizing agents, solvent system of nanoparticle suspension.

8) The filtrate of step 7 was centrifuged at a low speed in order to remove large nanoparticles (*e.g.*, average size larger than 200 nm, 300 nm, 400 nm or 500 nm). The temperature was maintained at about 4 °C. Without wishing to be held to a particular theory, it is contemplated that the separated large nanoparticles have larger average diameter and lower density that the other nanoparticles.

9) The supernatant of step 8 was filtered through a TFF membrane. The second filtration step removed free protein.

10) The resulting solution comprising loaded nanoparticles, trace PVA, trehalose, and water was then dried or frozen.

Example 2: Preparation of polymer nanoparticles comprising protein

[0620] This Example describes another exemplary method for preparation of certain polymer nanoparticles (*e.g.*, polymer nanoparticles comprising a payload and/or a coating) in accordance with the present disclosure. One of skill in the art will appreciate that certain conditions and specific values as described herein may be changed as desired.

Steps:

1) An aqueous payload solution was prepared at room temperature by dissolving a payload comprising protein (*e.g.*, peanut protein as, *e.g.*, crude peanut extract) in water (*e.g.*, here, 6 mg/mL crude peanut extract) and DNA (*e.g.*, sheared *E. coli* DNA). Sonication and/or homogenization were used as required to obtain a substantially homogenous solution of protein and water.

2) An organic polymer solution was prepared by dissolving polymer (here, PLG) into organic solvent (here, DMSO), at 1.9 mg/mL using magnetic stirring to generate an organic PLG solution. The temperature was maintained between approximately 25°C – 30°C, which prevents DMSO from freezing, lowers viscosity of the PLG solution, and increases speed at which PLG dissolves.

3) The aqueous payload solution of step 1 was then added to the organic polymer solution of step 2 and combined to achieve a mixture of substantial homogeneity. The volumetric ratio of DMSO (organic/"payload") solution: aqueous (water/"payload" solution) was 94:6. This ratio may be varied to suit other applications or desired embodiments, including as described in the present disclosure.

4) The mixture of step 3 (organic solution of step 1 and aqueous solution of step 2) was then subjected to rotary evaporation at between 50-85 rpms, 160-250 mbar, and approximately 65-75 ℃ until approximately 93% of the original mass was removed (approximately 2 hours).

5) 1-propanol was added to the concentrated mixture of step 4 in the presence of mechanical stirring. The volumetric ratio of the concentrated mixture of step 4 to 1-propanol was 1:73. This step generates a precipitate of the PLG, peanut protein and DNA. Without being held to a particular theory, it is contemplated that the precipitation temperature and stirring conditions may be important to control the size of the nanoparticles.

6) The nanoparticle suspension of step 5 was mixed with 1% aqueous PVA solution in the presence of mechanical stirring. The volume ratio of the nanoparticle suspension of step 5 to the aqueous PVA solution was 1:4.5. Without being held to a particular theory, it is contemplated that the addition of PVA is helpful in minimizing aggregation of the nanoparticles in suspension and/or otherwise stabilizing formed nanoparticles.

7) The stabilized nanoparticle suspension of step 6 was diluted with cold ammonium bicarbonate buffer. The volume ratio of the stabilized nanoparticle suspension of step 6 to the buffer was 1:1.

8) The suspension of step 7 was filtered through a TFF membrane with a MWCO of 500 kilodaltons (*e.g.*, MiniKros/KrosFlo/*etc.*) at a pressure of about 10 psi, at a shear rate of 10,000 s^{-1}, at a flux rate of between 7 and 8 mL/min for 1 hour.

9) The suspension of step 8 was concentrated 6.5 times, and then washed about 10 times with ammonium bicarbonate to remove all PVA and free non-encapsulated protein.

10) An aqueous solution of Organic *E. coli.* Extract ("OEE") was prepared by adding water to OEE powder (in this Example, to a concentration of 2 g/L OEE).

11) The mixture was sonicated to make a solution of concentrated OEE micelles in water ("OEE Solution"), which was then mixed with the loaded nanoparticle solution of step 12, sonicated, and then combined with trehalose at a concentration of such that the final amount of dehydrated trehalose was 2 times the mass of the purified nanoparticles (*e.g.*, for 0.5 g/L OEE, the concentration of trehalose is 1 g/L). The addition of trehalose in this

step constitutes the vast majority of trehalose in the final formulation because the prior TFF step reduces trehalose concentration to negligible levels.

12) The resulting solution comprising loaded nanoparticles, trace PVA, trehalose, OEE micelles, and water was then lyophilized.

Example 3: Nanoparticle Dissolution Test

[0621] Safety factor of nanoparticles is a term used to quantify free protein as compared to encapsulated protein to determine how much protein is encapsulated versus not, with a greater amount of encapsulated protein being considered safer. Determination of safety factor requires two preparations: a nanoparticle total protein preparation and a nanoparticle free protein preparation as described herein. Solutions and safety factor determination was conducted as follows:

Materials

[0622] The following **Tables 8** and **9** contain the equipment and reagents used to analyze solutions for peanut protein content using the bicinchoninic acid (BCA) assay kit.

Table 8. Reagents utilized for the quantitation of peanut protein using BCA assay.

Reagents	**Catalog Number**
Pierce BCA Protein Assay Kit	ThermoFisher 23225
10 N NaOH	Fisher SS2551
Lyophilized Peanut Extract	Greer Labs (Lot:302581)
MilliQ H2O	Millipore Advantage A10 System
1.5 mL PS disposable cuvettes (quartz cuvettes are also appropriate)	Brand 759015
Anti-static weigh funnels (XS)	Fisher NC9078021
3 mL Falcon graduated transfer pipets	Fisher 13-680-50

Table 9. Equipment utilized for the quantitation of peanut protein using BCA assay.

Equipment Used	**Model Number**
UV-VIS Spectrophotometer	(Now Agilent) Varian Cary 50
Thermo Scientific Super-Nuova+ hot plate	Thermo SP88857190
Rainin 200 µL variable volume Pipetman	Gibson N/A
Rainin 2 mL LTS variable volume Pipet-Plus	Gibson N/A
Sartorius micro balance	Sartorius ME5
Vortex Genie 2	Scientific Industries Model G-560

Assay Procedure

[0623] The procedure used to analyze solutions as disclosed herein, for peanut is described in the present example. This procedure also uses a Pierce BCA assay in accordance with manufacturer's instructions and can be accessed at hypertext transfer protocol secure assets.thermofisher.com/TFS-Assets/LSG/manuals/MAN0011430_Pierce_BCA_Protein_Asy_UG.pdf), which also describes two manufacturer recommended protocols, "Standard" (sample incubation at 37°C) and "Enhanced" (sample incubation 60°C). This Example uses the "Enhanced" protocol to analyze lyophilized peanut extract (LPE).

[0624] Quantification of free and total protein entails three parts, as follows:

1. Prepare materials for the standard curve;
2. Prepare nanoparticles for analysis (*i.e.*, sample preparation, which differs for measurement of total protein versus free (*e.g.*, unencapsulated) protein); and
3. Run the BCA assay on materials from 1 and 2

Preparation of Standard Curve

[0625] 1. Prepare 100% stock standard at 200 µg protein/mL using a 10 mL type A volumetric pipette (Dissolve 2.8 mg of LPE in 10 mL 0.1N NaOH); note that lyophilized peanut extract has a 71.5% assay value so 2.8 mg LPE = 2.0 mg protein.

[0626] 2. Prepare standard solutions by diluting 100% standard as shown in **Table 10**.

Table 10. Description of standard solution preparation using type A volumetric pipettes.

Standard Dilution	100% Standard Volume (mL)	0.1N NaOH volume (mL)
50%	2	2
25%	1	3
10%	1	9
5%	2 mL of 10% std*	2

* 5% Standard Dilution is a serial dilution using excess of the 10% standard. This can also be prepared by diluting 1 mL of 100% standard into 19 mL of diluent.

[0627] An exemplary standard curve was prepared using LPE standards dissolved in 0.1N NaOH, demonstrating desirable linearity is observed between 2 to 200 µg/mL. Absorbance values can be found in **Table 11**. Samples for a standard curve are typically prepared using one

replicate for each concentration, therefore any uncertainty in a given standard curve can be assessed using the standard error calculated for the slope and intercept of that curve.

Table 11. Absorbance values for standard curve at 562 nm (n = 1 replicates) with slope of 0.00322 ± 0.00003 (absorbance/µg/mL) and y-intercept of 0.0142 ± 0.003 (absorbance).

[protein] (µg/mL)	Blank corrected absorbance at 562	% Recovery
2.0	0.015	20%
19.9	0.079	102%
49.7	0.181	105%
99.4	0.331	99%
198.7	0.653	100%

* Error is calculated using standard linear regression statistics.

Sample Preparation

[0628] Total protein content was determined as follows:

1. Weigh out approximately 4 mg of nanoparticles into an appropriately sized scintillation vial (*e.g.*, 4 mL for 4 mg nanoparticles) in duplicate. Typically, nanoparticles are stabilized in PVA and trehalose, so the actual mass that is weighed out is corrected for the expected mass of the excipients.

a. A typical sample includes of ~33% PLG nanoparticles with a peanut loading of about 50 µg Peanut/mg PLG. In the present Example, 12 mg of material is equivalent to 4 mg of nanoparticles.

2. Add 2 mL of 0.1N NaOH using a type A volumetric pipette and vortex for about 30 seconds to suspend the powder. The volume of 0.1N NaOH can be changed if expected potency of nanoparticles is not 50 µg Peanut/mg PLG.

3. Allow samples to hydrolyze overnight (~16 hours) on rotating mixer or rocker table. Ensure all samples are visually fully hydrolyzed (samples should be clear with no evidence of undissolved solids).

4. Analyze solutions for peanut content using BCA assay. (Procedure can be found below)

[0629] Free protein content was determined as follows:

1. Weigh 12 mg of nanoparticles into a 4 mL scintillation vial in duplicate or triplicate.

a. An exemplary sample includes ~33% PLG nanoparticles with a peanut loading of about 50 µg Peanut/mg PLG. In this Example, 36 mg of material would be equivalent to 12 mg of nanoparticles.

b. Manufacturing aims to achieve low levels of un-encapsulated protein. As such, it is important to achieve a high enough nanoparticle concentration in ammonium bicarbonate such that low levels of un-encapsulated protein are detectable, and ideally quantifiable. It is contemplated that with more material available, it may desirable to increase nanoparticle concentration to better quantify low levels of free protein. An ideal final concentration of nanoparticle suspension would be 20 mg nanoparticles/mL.

2. Add 2 mL of 10 mM Ammonium bicarbonate and vortex until powder is evenly suspended. The volume of ammonium bicarbonate (and mass of nanoparticles) can be varied depending on the expected free protein (*see* 1b, above).

3. Take a 1 mL aliquot and centrifuge at 380,000 RCF for 8 minutes at 25°C. This force is sufficiently high that the majority of nanoparticles spin down and only the solubilized peanut protein remains in the supernatant.

4. Take a portion of the supernatant using a 1.5 mL transfer pipette and filter through 0.1 µm PVDF centrifuge filter (Millipore, catalog number UFC40VV00) by spinning down sample at 10,000 RCF for 4 minutes. (Note: remove supernatant away from the pellet since the pellet is easily disturbed).

5. Sample the filtrate and analyze using BCA assay. (Procedure can be found below)

[0630] BCA assay was performed as follows:

1. Prepare assay working reagent (WR) by mixing 50 parts of regent A and 1 part of regent B (both regents provided in the BCA assay kit) into an Erlenmeyer flask. The amount of WR depends on the number of samples being analyzed. For example, 24 samples requires 50 mL of WR. This can be prepared by adding 50 mL of regent A and 1 mL of regent B into a 50 mL Erlenmeyer flask and allowing to stir for at least 1 minute on a stir plate (typically stirring at 300 RPM).

2. Add 100 µL of sample and 2 mL of reagent to 4 mL clear scintillation vials using a 100 µL Pipetman and a 2 mL variable volume Pipet-Plus. Samples are typically analyzed in the order in which WR is added. For instance, if the 100% standard sample is the

first to be mixed with WR, the 100% standard sample would be the first sample analyzed using a spectrophotometer which minimizes error associated with continuation of the reaction between the BCA reagent and peanut protein at room temperature.

3. Mix by vortexing samples for ~10s using a Vortex Genie 2 at a setting of 8, and incubate for 30 minutes at 60°C in water bath. A water bath can be set up by adding water to a 100 mm x 190 mm crystallizing dish and heating to 60°C using a temperature controlled hot plate. The water level of the bath should be high enough such that all of the liquid in the 4 mL vials is submerged below the water line. Sample incubation time is critical and affects sensitivity range for the assay.

4. After 30 minutes, remove vials from the water bath and allow samples to equilibrate to RT. Samples are typically allowed to equilibrate for about 10 minutes.

5. Transfer the colored solutions into disposable 1.5 mL semi micro cuvettes (disposable or quartz) using disposable transfer pipettes and measure the absorbance spectra from 500 nm to 600 nm using UV-VIS spectrophotometer. A spectrum is collected for potential troubleshooting. For example, baseline shifts can be indicative of scattering from undissolved solids.

6. Subtract the absorbance measurement of a blank solution (typically 0.1 N NaOH) from the 562 nm absorbance of all samples and standards. Use the slope and intercept of the standard curve to determine the protein concentration of each unknown sample.

Nanoparticle Total Protein Preparation

[0631] To determine safety factor, approximately 4 mg of loaded nanoparticles prepared in accordance with the protocol of Example 1 were weighed into a scintillation vial in replicates. To vials containing nanoparticles, 2 mL of 0.1 N NaOH were added using a type A volumetric pipette and vortexed for ~ 30 seconds to fully suspend nanoparticle powder. Volume of 0.1 N NaOH may be altered accordingly, including, *e.g.*, in consideration of expected potency of nanoparticles being measured. Suspended nanoparticles in 0.1 N NaOH were allowed to hydrolyze overnight (approximately 16 hours) on a rotating mixer or rocker table prior to processing for protein quantification.

Nanoparticle Free Protein Preparation

[0632] Approximately 12 mg of nanoparticles were weighed into a scintillation vial in replicate. To vials containing nanoparticles, 2 mL of 10 mM ammonium bicarbonate were added and vortexed until nanoparticles were uniformly suspended. Volume of 10 mM ammonium bicarbonate may be altered accordingly, including, *e.g.*, in consideration of expected amount of free protein. After resuspension, 1 mL of solution was centrifuged at 380,000 RCF for 8 minutes at 25 ℃ to pellet nanoparticles. An aliquot of supernatant was then removed with a 1.5 mL transfer pipette and filtered through a 0.1 um PVDF centrifuge filter by spinning the sample at 10,000 RCF for 4 minutes. The resulting filtrate was then used for protein quantification.

Protein Quantification

[0633] In the present Example, protein quantification was performed using a BCA assay kit. It is understood by a person of ordinary skill in the art that alternative methods may be used to quantify protein (*e.g.*, Bradford assay). To 2 mL of BCA working reagent in a 4 mL clear scintillation vial, 100 µL of a sample (*e.g.*, total protein from nanoparticles, free protein from nanoparticles) was added, vortexed for approximately 10 seconds and incubated for 30 minutes at 60 ° C in a water bath, with a water level high enough such that all liquid inside the vial(s) was submerged below the water line. After 30 minutes, vials containing BCA reagent and sample were removed from the water bath and allowed to equilibrate to room temperature for approximately 10 minutes. The solutions were then transferred into disposable 1.5 mL semi microcuvettes (*e.g.*, disposable or quartz) using disposable transfer pipettes and absorbance spectra were measured from 500 nm to 600 nm using a UV-VIS spectrophotometer. Normalization of sample readings was performed by subtracting the absorbance measurement of a blank solution (*e.g.*, 0.1 N NaOH) from the 562 nm absorbance of all samples and standards. The slope and intercept of the standard curve was used to determine protein concentration in each measured sample.

Calculations and Interpretation

[0634] Prior to data interpretation, protein concentrations measured in part B must be converted to mass of protein per mass of starting material, taking into account any actual concentrations and dilutions. This correction enables conversion of the total protein concentration measured in

the BCA assay to a potency value that reports mass of protein per mass of the solid intermediate.

[0635] Calculation of Safety factor (SF) (which may alternatively be referred to as Encapsulation Factor) also involves normalizing both protein concentrations to a unit mass of solid intermediate. Safety factor can then be calculated using Equation 1.

$$SF = \frac{total\ protein - free\ protein}{free\ protein} \quad (1)$$

[0636] The following notes may be considered for this and future assays:

[0637] Potency (protein mass per mass of solid intermediate) is not the same as the mass of protein per mass of nanoparticles. To perform this measurement, a sample workup, analogous to those described in the present Example, must be performed to remove excipients from the nanoparticles including centrifuging the nanoparticles and washing the pellet to remove unbound excipients. The lyophilized pellet can then be weighed and hydrolyzed directly to release free protein from a known mass of nanoparticles.

[0638] Higher sensitivity and accuracy for quantitation of low peanut protein concentrations can be achieved by forcing the standard curve through zero in addition to adding replicate preparation of standards at the lower range of protein concentrations.

[0639] The recommended masses in the sample preparation sections can be scaled up to higher concentrations or higher amounts for greater accuracy.

[0640] The enhanced protocol is linear from 5 µg LPE/mL to 250 µg LPE/mL. This range can be changed by varying the incubation time at 60°C. Longer incubation times will result in higher sensitivity at the lower concentrations but decreased sensitivity at the higher concentrations.

[0641] The assay is a kinetic assay that continually develops overtime even at room temperature. Although the rates significantly decrease at room temperature, there is a practical limitation to the number of samples that can be prepared and analyzed at a time. The time elapsed between your first and last sample is generally one hour or less, however, in some situations, more or less elapsed time may be desired.

[0642] The stability of the peanut protein in 0.1N is not well understood and is an area that should be further developed. As such, solutions should be analyzed as soon as the PLG appears to be fully hydrolyzed.

Example 4: Exemplary Vaccines

[0643] This example describes PLG nanoparticles encapsulating various types of antigens (*e.g.*, allergens, infectious agents) in various payload formats (*e.g.*, protein, nucleic acid – and particularly mRNA encoding relevant proteins and/or epitopes thereof), and assessment of the extent and/or type of immune responses (*e.g.*, T cell and/or antibody responses they can elicit).

1. OVA vaccines

[0644] PLG nanoparticles encapsulating OVAmRNA and/or OVA protein in accordance with the present disclosure can serve as a vaccine to elicit OVA-specific T cell and B cell responses.

[0645] PLG nanoparticles encapsulating OVAmRNA alone (OVAmRNA-Enanos), are expected to elicit CD8 T cell/class I restricted responses, elicit OVA-specific CD4 T cells and CD4 driven antibody responses demonstrating cross presentation.

[0646] PLG nanoparticles encapsulating OVA alone (OVA-Enanos), are expected to elicit CD4 T cell/class II restricted responses, elicit OVA-specific CD8 T cells.

[0647] Additionally, the delivery of PLG nanoparticles encapsulating OVAmRNA in the presence of full-length OVA or OVA peptides support the activation of CD4, CD8 T cells and B cell antibody responses. The activation may allow the presentation of OVA peptides that can bind both MHC class I and class II and thus activate both CD8 and CD4 T cells.

[0648] Two separate PLG nanoparticle populations (OVAmRNA-Enanos and OVA-Enanos), when delivered together, activate CD4 T cells, CD8 T cells and antibody responses as effectively as when delivering both in the same single Enano.

[0649] In some embodiments, a cell penetrating peptide, *e.g.*, MPG, may be attached to nanoparticle surfaces, *e.g.*, to facilitate cross presentation and CD8 T cell responses.

2. COVID vaccines

[0650] PLG nanoparticles encapsulating SPIKEmRNA (SPIKEmRNA-Enanos) serve as a vaccine to elicit SPIKE-specific antibody responses.

[0651] Mice are immunized with either SPIKEmRNA-Enanos or OVAmRNA-Enanos as a control. After weeks, serum from individual mice are tested for either anti-spike or anti-OVA antibodies.

3. Murine OVA model

[0652] A murine OVA model consisting of OT-1 CD8 Ts and OT-2 CD4 Ts from T cell receptor transgenic mice is utilized to determine immune responses.

[0653] In vivo assays provide information on the numbers of responding OVA-specific T (CD4 and CD8 cells) and antibody production. In vitro assays will test the ability of dendritic cells to present and activate CD4 and CD8 T cells (no antibody response).

a. In vitro

[0654] OVA vaccines: Bone marrow derived dendritic cells (BMDCs) are cultured with both CFSE labelled OT-2 CD4 T cells and/or OT-1 CD8 T cells from T cell receptor transgenic mice respectively in the presence of OVA, OVA-Enanos, or OVAmRNA-Enanos. After days, analysis of OT-1 and OT-2 T cell proliferation and cytokine production are performed.

[0655] COVID vaccines: BMDCs are exposed to GFP labeled SPIKE-Enano alone and localization of SPIKE-Enano is determined.

b. In vivo analysis:

[0656] OVA vaccines: OT-2 T cells and OT-1 T cells are transferred into mice along with OVA, OVA-Enanos, or OVAmRNA-Enanos. Analysis of CD4 and CD8 T cell proliferation by CFSE and analysis of antibody production are performed.

[0657] COVID vaccines: Normal BALB/c mice are exposed to SPIKEmRNA-Enanos or OVA-Enanos. Blood samples are collected at time points and analyzed.

4. Reagents:

[0658] The purity/integrity of the OVAmRNA/SPIKEmRNA/Enanos are determined with assays.

[0659] Spike mRNA is provided, encapsulated and tested.

[0660] Localization of the SPIKEmRNA-Enanos is determined, for example, by exposing BMDC for various times with SPIKEmRNA-Enano (*e.g.*, GFP). Amounts of mRNA to be used for in vitro and in vivo studies are determined.

[0661] Cell penetrating peptide, MPG, may be purchased and attached to nanoparticles if necessary.

Example 5: Preparation of nanoparticles comprising protein

[0662] This example describes an exemplary method for preparation of certain nanoparticles (e.g., polymer nanoparticles comprising a payload and/or a coating) in accordance with the present disclosure. One of skill in the art will appreciate that certain conditions and specific values as described herein may be changed as desired.

[0663] Briefly, an aqueous payload solution (1) was mixed with an organic polymer solution (2) to achieve a mixture of substantial homogeneity and then subjected to rotary evaporation until approximately 93% of the original mass was removed. The mixture was nanoprecipitated by slow addition of the DMSO solution into IPA/DMSO followed by the LPS/Water solution addition under homogenization. The prepared nanoparticles were then treated with TFF to remove organic solvents and concentrated. Centrifugation was used to eliminate residual free peanut after the TFF concentration step.

1) An aqueous payload solution was prepared at room temperature by dissolving a payload comprising protein (e.g., peanut protein as, e.g., crude peanut extract) in water (e.g., here, crude peanut extract) and DNA (e.g., sheared *E. coli* DNA). Sonication and/or homogenization were used as required to obtain a substantially homogenous solution of protein and water.

2) An organic polymer solution was prepared by dissolving polymer (here, PLG) into organic solvent (here, DMSO), using magnetic stirring to generate an organic PLG solution. The temperature was maintained between approximately 25°C – 30°C, which prevents DMSO from freezing, lowers viscosity of the PLG solution, and increases speed at which PLG dissolves.

3) The aqueous payload solution of step 1 was then added to the organic polymer solution of step 2 and combined to achieve a mixture of substantial homogeneity.

4) The mixture of step 3 (organic solution of step 1 and aqueous solution of step 2) was then subjected to rotary evaporation until approximately 93% of the original mass was removed.

5) The mixture of step 4 comprising the payload/polymer preparation (e.g., peanut mixture/DMSO/PLG) was dispensed underneath a non-solvent system layer (e.g., IPA + DMSO), where the non-solvent system layer and the payload/polymer preparation layer (e.g., peanut mixture/DMSO/PLG) do not mix together.

6) An overhead stirrer was set up so that the top edge of the fin was at the interface between the aqueous layer and non-solvent layer. Stirring was performed until nanoprecipitation was complete and the solution was homogenous.

7) Immediately after solution was homogeneous, a sample of the material was measured on DLS to ensure that the particles were formed with a diameter (~150 nm

8) The nanoprecipitation (i.e., the population of nanoparticles) was homogenized by adding a lipid to the nanoprecipitation using a peristaltic pump at about 1.2 L/min while recirculating the material with the homogenizer set to about 10,000 rpm.

9) The population of nanoparticles was diluted using PBS and ammonium bicarbonate.

10) The stabilized population of nanoparticles from step 9 was filtered through a TFF membrane. Without wishing to be held to a particular theory, it is contemplated that this step may remove one or more of unformed polymer molecules, unassociated stabilizing agents, solvent system of nanoparticle suspension. The filters were washed with 10x 10 mM PBS.

11) Optionally, the filtrate of step 10 was centrifuged at a low speed in order to remove large nanoparticles (e.g., average size larger than 200 nm, 300 nm, 400 nm or 500 nm). The temperature was maintained at about 4 °C. Without wishing to be held to a particular theory, it is contemplated that the separated large nanoparticles have larger average diameter and lower density that the other nanoparticles.

12) The supernatant from step 10 or step 11 was filtered through a TFF membrane. The filtration step removed free protein.

13) Optionally, the resulting solution comprising a population of loaded nanoparticles, trace LPS, trehalose and water was then dried or frozen.

Example 6: Preparation of nanoparticles comprising protein

[0664] This example describes an exemplary method for preparation of certain nanoparticles (e.g., polymer nanoparticles comprising a payload and/or a coating) in accordance with the present disclosure. One of skill in the art will appreciate that certain conditions and specific values as described herein may be changed as desired.

1) An aqueous payload solution was prepared at room temperature by dissolving a payload comprising protein (e.g., peanut protein as, e.g., crude peanut extract) in water (e.g.,

here, 6 mg/mL crude peanut extract) and DNA (e.g., sheared *E. coli* DNA). Sonication and/or homogenization were used as required to obtain a substantially homogenous solution of protein and water.

2) An organic polymer solution was prepared by dissolving polymer (here, PLG) into organic solvent (here, DMSO), at 1.9 mg/mL using magnetic stirring to generate an organic PLG solution. The temperature was maintained between approximately 25°C – 30°C, which prevents DMSO from freezing, lowers viscosity of the PLG solution, and increases speed at which PLG dissolves.

3) The aqueous payload solution of step 1 was then added to the organic polymer solution of step 2 and combined to achieve a mixture of substantial homogeneity. The volumetric ratio of DMSO (organic/"payload") solution: aqueous (water/"payload" solution) was 94:6. This ratio may be varied to suit other applications or desired embodiments, including as described in the present disclosure.

4) The mixture of step 3 (organic solution of step 1 and aqueous solution of step 2) was then subjected to rotary evaporation at between 50-85 rpms, 160-250 mbar, and approximately 65-75 ℃ until approximately 93% of the original mass was removed (approximately 2 hours).

5) A non-solvent system was prepared by adding 100% isopropyl alcohol (IPA) to dimethyl sulfoxide (DMSO) in a beaker at a ratio of about 10:1 and stirring until mixed. The payload/polymer preparation (e.g., peanut mixture/DMSO/PLG) was dispensed underneath a non-solvent system layer (i.e., IPA + DMSO) with a stripette by inserting the stripette all the way at the bottom of the beaker and slowly expelling the aqueous payload (e.g., peanut mixture/DMSO/PLG) to form a single layer on the bottom of the beaker, where the non-solvent system layer and the payload/polymer preparation layer (e.g., peanut mixture/DMSO/PLG) do not mix together. The volume ratio of payload/polymer preparation to non-solvent system was about 1:4.

6) An overhead stirrer was set up so that the top edge of the fin was at the interface between the aqueous layer and non-solvent layer. Stirring was started at 70 rpm and left there until the nanoprecipitation was complete. Stirring was increased to 150 rpm until the solution was homogenous.

7) Immediately after solution was homogeneous, a sample of the material was measured on DLS to ensure that the particles were formed with a diameter (~150 nm, 200nm for PLG nanoparticles). The DLS for the nanoprecipitation (i.e., the population of nanoparticles) revealed a Z-average = 205.8 nM and PDI = 0.112.

8) An aqueous solution of LPS was prepared by adding water to LPS powder to a concentration of 0.2 mg/mL. The nanoprecipitation (i.e., the population of nanoparticles) was homogenized by adding the 0.2 mg/mL LPS solution to the nanoprecipitation using a peristaltic pump at 1.2 L/min while recirculating the material with the homogenizer set to 10,000 rpm. Analysis following homogenization using (DLS revealed a Z-average = 237.6 and PDI = 0.095.

9) The population of nanoparticles was diluted using 10 mM PBS and 10 mM ammounium bicarbonate (Sigma Aldrich PN: 09830-500G Lot: BCBZ3540). The volume ratio of the stabilized population of nanoparticles from step 8 to the buffer was about 1:1.

10) The stabilized population of nanoparticles from step 9 was filtered through a 8500cm2 TFF membrane with a MWCO of 750 kilodaltons (e.g., MiniKros/KrosFlo/etc.) using the following parameters. A reservoir was filed with 10 mM ammonium bicarbonate and the inlet line was placed into the diluted nanoparticles. Target shear was set to 25k with no added pressure on retentate. Pump was set to 10 L/minute. Concentration lasted about 13 minutes. Diafiltration was performed by placing the inlet into a 10x PBS wash beaker. Target shear was set at 25k with no added pressure on retentate. Pump was set to 10 L/minute, Diafiltration lasted about 14 minutes with about 1620 mL of material collected. DLS/zeta characterization for the material collected had 254.9 Z average and a PDI = 0.08. Concentration of the population of lipid nanoparticles at the end of the first filtration step was = 6.75 mg/mL).

11) The TFF filer from step 10 was washed with 10x 10mM PBS. 12) The supernatant was filtered through a 790 cm2 TFF membrane with a MWCO of 750 kilodaltons (e.g., MiniKros/KrosFlo/etc.) using the following parameters: The second TFF reservoir was filled with 10 mM PBS and the inlet line was placed into the washed material from the previous TFF. Target shear was set to 25k with no added pressure on retentate. Pump was set to 6260 rpm. Concentration lasted about 12 minutes. Permeate flow rate was measured at 300 mL/min. Diafiltration was performed by placing the inlet into a 5x PBS wash beaker. Target shear was set at 25k with no added pressure on retentate. Pump was set to 6260 rpm. Diafiltration lasted

about 16 minutes. Permeate flow rate was measured at 120 mL/min. Concentration of the population of lipid nanoparticle at the end of the second filtration step was 59.14 mg/mL.

12) Material from the second filtration was recirculated with the permeate capped for 3 minutes before collection and was gravity collected. Volume of material collected 184.92 mL (volume before samples were taken out). DLS/zeta characterization for the material collected had a Z average of 236.8 and a PDI = 0.14.

13) Optionally, the material from the second filtration containing the population of nanoparticles were then combined with various excipients to assess stability following one or more freeze-thaw cycles.

Example 7: Ova protein and Ova mRNA encapsulation

[0665] This example describes PLG nanoparticles encapsulating Ovalbumin (OVA) proteins or mRNA encoding relevant an OVA mRNA.

[0666] Payload/polymer preparations were prepared as described in Examples 1, 2, 6, and/or 7. Briefly, for OVA protein, an aqueous payload solution was prepared at room temperature by dissolving a payload comprising protein (*e.g.*, OVA protein) in water (*e.g.*, here, 5 mg/mL OVA protein) and DNA (*e.g.*, sheared *E. coli* DNA). Briefly, for mRNA encoding an OVA protein, an aqueous payload solution was prepared at room temperature by dissolving a payload comprising protein (*e.g.*, mRNA encoding an OVA protein) in polyethyleneimine (PEI) and acetate buffer (*e.g.*, here, 5 mg/mL mRNA encoding an OVA protein) and DNA (*e.g.*, sheared *E. coli* DNA). Without wishing to be bound by any particular theory, positively charged PEI binds to the negatively charged mRNA.

[0667] Sonication and/or homogenization were used as required to obtain a substantially homogenous solution of protein and water.

[0668] An organic polymer solution was prepared by dissolving polymer (here, PLG) into organic solvent (here, DMSO), at 1.9 mg/mL using magnetic stirring to generate an organic PLG solution. The temperature was maintained between approximately 25°C – 30°C, which prevents DMSO from freezing, lowers viscosity of the PLG solution, and increases speed at which PLG dissolves.

[0669] The aqueous payload solution of step 1 was then added to the organic polymer solution of step 2 and combined to achieve a mixture of substantial homogeneity. The volumetric ratio of

DMSO (organic/"payload") solution: aqueous (water/"payload" solution) was 94:6. This ratio may be varied to suit other applications or desired embodiments, including as described in the present disclosure.

[0670] The mixture of step 3 (organic solution of step 1 and aqueous solution of step 2) was then subjected to rotary evaporation at between 50-85 rpms, 160-250 mbar, and approximately 65-75 ℃ until approximately 93% of the original mass was removed (approximately 2 hours).

[0671] 1-propanol was added to the concentrated mixture of step 4 in the presence of mechanical stirring. The temperature was maintained at about 16 °C. This step generates a precipitate of the PLG, OVA protein or mRNA encoding an OPVA protein, and DNA.

[0672] The nanoparticle suspension of step 5 was mixed with aqueous PVA solution. The temperature of the aqueous PVA was about 4 °C. Without being held to a particular theory, it is contemplated that the addition of PVA is helpful in minimizing aggregation of the nanoparticles in suspension and/or otherwise stabilizing formed nanoparticles.

[0673] DLS assessment the nanoprecipitation including the PLG, OVA protein, and DNA revealed PDI between 0.06 and 0.14 and Z averages between 131 and 197, as shown in **Table 12** below.

Table 12. DLS assessment of nanoparticles comprising PLG, OVA protein, and DNA

Condition	Z-ave (nm)	Num. (nm)	PDI	Zeta (mV)
PLG only – PVA	194	191	0.06	-10
PLG only – LPS	147	132	0.06	-43
PLG+OVA – PVA	179	169	0.08	-9
PLG+OVA – LPS	131	98	0.14	-15

[0674] BCA of the nanoprecipitation including the PLG, OVA protein, and DNA revealed encapsulation of the OVA protein, as described by the values in **Table 13**.

Table 13. BCA of nanoparticles comprising PLG, OVA protein and DNA.

	OVA (µg/mL)	µg OVA/mgPLG	% Loading

PLG/PVA	7.7	1.5	-
PLG(OVA)/PVA	151	31	34 %
PLG/LPS	2.25	0.45	-
PLG(OVA)/LPS	475	97	107 %

[0675] DLS assessment of the nanoprecipitation comprising PLG, mRNA encoding an OVA protein, and DNA revealed PDI between 0.06 and 0.14 and Z averages between 131 and 197, as shown in **Table 14** below.

Table 14. DLS assessment of nanoparticles comprising PLG, mRNA encoding an OVA protein, and DNA

Condition	Z-ave (nm)	Num. (nm)	PDI	Zeta (mV)
PLG + PEI – PVA	310	282	0.16	7
PLG + PEI – LPS	283	236	0.26	42
PLG+RNA – PVA	239	222	0.09	-15
PLG+RNA – LPS	172	146	0.14	-40
PLG only – PVA	187	184	0.03	-9
PLG+RNA+PEI spike – PVA	192	186	0.06	-23
PLG only – LPS	144	127	0.06	-44
PLG+RNA+PEI spike – LPS	145	128	0.13	-40

Example 8: Preparation of nanoparticles comprising protein

[0676] The present example describes an exemplary method for preparation of certain nanoparticles (*e.g.*, polymer nanoparticles comprising a payload and/or a coating) in accordance with the present disclosure. One of skill in the art will appreciate that certain conditions and specific values as described herein may be changed as desired.

[0677] Polymer nanoparticles comprising a coating and containing payload comprising allergen and adjuvant were prepared via a nanoprecipitation method and were purified away from residual solvent and free cargo by transverse flow filtration. In the exemplary method described in the present Example, the polymer nanoparticle comprises PLG, the coating comprises LPS, the allergen is peanut, and the adjuvant is sheared *E. coli* genomic DNA.

[0678] Briefly, 78.1515 g of PLG was dissolved in 8.976 kg of DMSO on a magnetic stir plate until homogenous.

Example 9: Assaying reactivity of nanoparticles with whole blood basophil activation test (BAT)

[0679] The present example describes an exemplary method for assaying reactivity of certain polymer nanoparticles (*e.g.*, polymer nanoparticles comprising a payload and/or a coating) with whole blood BAT. One of skill in the art will appreciate that certain conditions and specific values as described herein may be changed as desired. One of skill in the art will also appreciate that assaying nanoparticle reactivity (e.g., as described in the present Example) provides insight to nanoparticle safety and/or toxicity.

[0680] Steps:

1) Basophil stimulation buffer (BSB) was prepared by adding IL-3 at 2 ng/mL in PBS. An antibody cocktail comprising antibodies against CD63, CD123, CD203c, and HLA-DR was also added to prepare BSB.

2) Whole-blood aliquots (50 uL) were mixed with equal volumes of one of the following:

i. BSB alone;

ii. BSB with the addition of peanut protein at serial 10-fold dilutions (from 10 to 1 x 10^{-3} µg/mL total protein);

iii. BSB with the addition of polyclonal anti-IgE antibody (1 µg/mL, positive control);

iv. BSB with the addition of N-formyl-methionyl-leucyl-phenylalanine (1 μmol/L, IgE-independent positive control);

v. BSB with the addition of PN-nanos (concentration from neat to 10^{-3} μg/mL of total protein); or

vi. BSB with the addition of empty Nanos at amount equal of that at the PN-nanos at the highest total peanut protein concentration condition.

3) Stimulation was performed at 37 °C for 20 minutes in a water bath.

4) Immediately after step 3 (stimulation), 0.6 mL of Optilyse C Lysing fixing solution (Beckman) was added to each sample and samples were incubated for 10 minutes at room temperature in the dark.

5) Next, 600 μL of 1xPBS was added to each sample and samples were incubated for 10 minutes at room temperature in the dark.

6) Samples were stored overnight at 4 °C. Samples stored for greater than 24 hours were centrifuged at 300 xg for 5 minutes. 1000 μL of supernatant was aspirated, and 100 μL of staining buffer was added to each sample and samples were vortexed.

7) Flow cytometry was used to assess basophil activation.

Example 10: Scaleable production of highly loaded protein nanoparticles for immune modulation

[0681] The present example demonstrates an efficient, high-yield and large-scale production of payload-containing polymer nanoparticles that has overcome issues that have thus far limited use of such nanoparticles for delivery of therapeutics. The present example specifically exemplifies PLG nanoparticles with a complex payload – including both protein (in particular, a relatively crude peanut extract including peanut allergen proteins) and nucleic acid (specifically, *E. coli* DNA) components – and furthermore including a surface coating (of *E. coli* lipid extract, including LPS). The present example documents effective delivery of and, moreover, effective immune modulation (reducing an allergic reaction) with respect to, the protein payload component.

[0682] Technologies described herein, and exemplified in the present example, have a versatile and modifiable payload capacity; manufacturing conditions are amenable to incorporation even of payloads traditionally considered to be "fragile" – such as protein, carbohydrate (*e.g.*, polysaccharide), and/or nucleic acid payloads. Furthermore, as demonstrated here, provided technologies achieve incorporation (and effective delivery) of complex payloads.

[0683] As documented herein, provided technologies are particularly useful and effective in achieving immune modulation. For example, components (such as *E. coli* DNA and/or lipids) that favor the generation of desired immune responses can be incorporated into nanoparticles and can influence immune responses directed toward other payload components (e.g., nanoparticles as shown in **FIG. 1A**).

[0684] Technologies exemplified herein manufacture nanoparticles by an inhomogeneous precipitation process that runs counter to the accepted wisdom that homogeneous precipitation conditions are required for scalable nanoparticle formation (e.g., as described in **FIG. 1B**).

[0685] The present example demonstrates that, when delivered orally, these nanoparticles effectively activate the immune system. Furthermore, the present example demonstrates that encapsulated peanut allergen protein elicits ~10-fold lower levels of activation of basophils isolated from peanut allergic patients than does unencapsulated peanut protein, thus providing ex vivo evidence of their safety for use in humans in the context of anaphylactic allergy. Thus, a engineered PLG nanoparticle delivery system as described herein constitutes a drug delivery platform that can be manufactured at pharmaceutical scales and whose properties make it particularly useful for clinical applications.

[0686] Immune modulation and desensitization is an area of significant current research that applies to clinical indications from oncology to food allergy. The present disclosure provides a new nanoparticle (NP) process and NP constructs that encapsulate payloads (e.g., protein and/or nucleic acid payloads) and address this broad range of therapeutic challenges. As an example, provided technologies can generate polylactide-co-glycolide (PLG) NP constructs useful as orally deliverable therapeutics for individuals with peanut allergies.

[0687] Currently, allergen immunotherapy (AIT) is the only approved clinical treatment for allergies in humans that can modulate the course of allergic disease (specifically, that can decrease allergen-specific IgE, Th2 cytokines, and anaphylaxis). Oral administration of AIT has

been shown to successfully modify allergic responses to food allergens; however, this success required multiple administrations of increasing doses of "unmasked" sensitizing allergens, which gives rise to undesirable adverse reactions that limit its therapeutic value, diminish its clinical use, and compromise its safety (Hughes et al., 2022). In initial clinical trials, a significant number of patients taking Palforzia, an FDA-approved oral AIT for peanut allergy, had an anaphylactic reaction prior to reaching the maintenance dose (FDA et al., 2020). Furthermore, the majority of participants regained reactivity upon discontinuation of the drug.

[0688] The present disclosure appreciates that NP encapsulation can effectively "hide" administered allergens from a subject's immune system, so that anaphylactic risk can be dramatically reduced (see, for example, WO2014/165679). However, commercial translation of the concept of allergen desensitization with NPs has been impeded by the lack of a process to produce highly loaded PLG at scale. The present disclosure provides a new process that overcomes this translation limitation and enables the production of 5 wt% loaded PLG NPs of 300 nm size. In addition, the process provided herein achieves incorporation of a DNA adjuvant together with protein allergen and includes an activated glycol-phospholipid adjuvant coating to stimulate antigen presenting cell uptake in, for example, a sublingual application.

[0689] Without wishing to be bound by any particular theory, we note that technologies provided herein provide, among other things, at least four important recognitions and advancements. One is realizing that the protein loss during classical PLG encapsulation may be partly or wholly because the precipitation of the PLG matrix has been predominantly done into an aqueous phase. Since the protein being encapsulated is soluble in the aqueous phase, there is a driving force for its escape from the precipitating PLG matrix. The smaller the NP that is being produced, the faster is that escape since the diffusion escape times scale with the square of the particle size. Therefore, the present disclosure provides an insight that use of an organic antisolvent (e.g., n-propanol as exemplified herein), which is an antisolvent for both the PLG and protein dissolved in DMSO. Consequently, there is no driving force for the loss of protein; this enables the loadings of peanut protein of 5 %.

[0690] Additionally, a further innovation provided by the present disclosure is incorporation of the glycol-phospholipid adjuvant coating on the NP. This coating is not applied from solution, but is rather "spread" or "smeared" on the NP surface; the present disclosure provides an insight that such incorporation may be achieved, for example, analogously spreading of red blood cell

membranes on the surfaces of NPs as reported by Zhang and Muzykantov (Fang et al., 2012; Villa et al., 2016).

[0691] Still further, the present disclosure provides an innovation is that NPs are formed in the propanol solution without the use of a stabilizer. Universally, in previous PLG studies, a polymeric stabilizer has had to be added to the antisolvent stream to prevent NP aggregation during precipitation. Most often for microparticles, these are polyvinyl alcohol polymers. In methods and compositions described herein, the NPs are self-stabilizing and produce NPs with extraordinarily narrow polydispersities such as 0.9-1.0 as shown in **Table 15**.

[0692] Yet another innovation provided by the present disclosure is that uniform NPs can be produced by such a globally heterogeneous precipitation process. The universal conventional understanding of how to control particle size and uniformity during precipitations has been to conduct the precipitation under uniform supersaturation to drive uniform precipitation kinetics (Horn et al., 2001). This is accomplished either by using intense mixing to homogenize the solvent/antisolvent concentrations , or to use microfluidics to reduce dimensions so that the diffusion time scales are short and solvent uniformity is rapidly achieved (Mahajan et al., 1993; Johnson et al., 2003; Martins et al., 2018). The present disclosure demonstrates that an intentionally heterogeneous, layered two-fluid process with mild mixing produces the desired NPs. The process is termed a "Tequila Sunrise" precipitation, since the dye layered visualization experiments (**FIG. 1C**) are reminiscent a cocktail of the same name. Quite surprisingly, this layered system allows scale up from laboratory scale to production scale (e.g., in this example, a scale involving 190 L batches that enable production of NPs containing 4 g of protein that would provide 2500 doses). This intentionally heterogeneous precipitation means that particle formation occurs continuously under a time-dependent concentration driving force, while still producing uniform NPs.

[0693] Among other things, the present example describes the development of the Tequila Sunrise precipitation process, and then post-processing steps that generate a final purified NP dispersion by tangential flow ultrafiltration (TFF). The dispersion can be stabilized with cryoprotectants, if desired; such a cryoprotectant might be particularly useful when it is desired that frozen samples be stored prior to being thawed and administered. The present example describes the application of these techniques to generate nanoparticles encapsulating either proteins from a crude peanut extract or chicken ovalbumin.

[0694] The present example further describes results of biological assays that demonstrate TLR activation by NPs carrying selected adjuvants, substantial reduction in the propensity of nanoencapsulated peanut antigen to cause peanut allergen-induced basophil activation using basophils from peanut allergic patients, and antigen-specific T cell responses induced in vivo through oral administration of OVA-encapsulated nanoparticles to OVA-responsive mice.

RESULTS

I. Design of PLG nanoparticles for peanut allergy desensitization via sublingual/buccal administration.

[0695] The design of PLG constructs described in the present example takes into consideration promising options to develop NPs for successful treatment by AIT, based on previous findings. These include: encapsulation of the allergen within the construct; addition of multiple modulating components (sheared *E. coli* DNA and lipids such as *E. coli*-based lipids or lipid extract) as adjuvants to drive IFN-γ producing CD4 and CD8 T cells that successfully suppress responses associated with allergy; and arrangement of the modulating components to achieve a powerful synergistic effect.

[0696] FIGS. 1A-B provide a schematic representation of the particle's construction and a block diagram of the steps in the process. The PLG matrix encapsulates both (i) peanut protein allergens to deliver for the AIT as well as (ii) sheared *E. coli* DNA as an adjuvant to activate TLR 9. Coating the nanoparticle is a mixture of *E. coli* phospholipids and sodium deoxycholate both to provide colloidal stability and to activate TLRs (TLR4) on dendritic cells of the immune system.

[0697] The particular lipid extract of *E. coli* utilized in the present example, obtained from Avanti Polar Lipids (Alabaster AL), consists mainly of phosphatidylethanolamine, phosphatidylglycerol and cardiolipin as well as an additional 18% by mass of the polar components of *E. coli* cell membrane.

A. Protein carrier phase preparation:

[0698] This detailed description is for the large scale peanut protein encapsulation process utilized in the present example, which produces 1.7 L of concentrated peanut protein NPs at final scale. There are several aspects of the physical chemistry of the system that are significant.

[0699] The matrix PLG (50:50 poly(DL-lactide-co-glycolide) with a 0.26-0.54 dL/g inherent viscosity from Lactel (B6010-1, Birmingham, AL) was dissolved in DMSO at a concentration of 100 mg of peanut protein per gram of PLG. The protein is not soluble in pure DMSO, but is soluble at pH 9.00 in an ammonium bicarbonate (AmBic) buffer at pH 9.0, as is the DNA, which is added at 4 mg/100 mg peanut protein. Peanut and DNA solution (493 ml) at 1.7 wt% peanut is added to 8.976 kg of DMSO containing PLG. A clear solution is obtained. However, the resulting solution is too dilute in PLG to precipitate and form NPs in isopropanol (IPA). Therefore, the PLG and peanut concentrations were raised to 27.4 mg/ml PLG and 2.7 mg/ml protein by rotary evaporation at 2 mTorr and 75 C. In this concentration step, 67 % of the solution mass was removed. The resulting solution appeared clear, but quite likely contains nucleating or phase separating protein and PLG by virtue of depletion flocculation, which is known to occur in protein and polymer solutions. This possibility is supported by the fact that direct dissolution of the PLG, protein and DNA at the final concentrations was not achieved, even with moderate heating.

[0700] 3645 ml of antisolvent IPA was introduced into a 10L vessel followed by 405 mL of DMSO. 1.7 L of concentrated protein PLG solution, which has a density of ~ 1.1 g cm-3 was carefully layered under the IPA/DMSO solution. The resulting volume ratio of antisolvent to DMSO phase was 0.8. A four-bladed stirring paddle with a diameter of 114 mm was placed at the interface in the 217 mm diameter vessel. The impeller blade was placed at the interface with 67 % of the blade in the DMSO layer. The overhead stirrer was set to 80 rpms to provide gentle stirring for 2 min, at which time the entire vessel was turbid indicating the layering had been eliminated. At that point the stirring speed was increased to 150 rpm for 1 min.

[0701] The goal of the process had been 200-350 nm NPs, which is expected to optimize NP uptake by the cells involved in antigen processing and presentation. With pure IPA in the antisolvent phase the NP size was smaller than this goal. By adding 10% DMSO to the IPA phase, the antisolvent quality was decreased, which slowed the precipitation and resulted in the desired NP size. The sizes for each step of the process is given in **Table 15**.

Table 15: Nanoparticle sizes at various steps in the process.

Step	Size (nm)	Polydispersity (PDI)	Reproducibility of size (nm) (standard deviation for 3 separate runs)	Zeta Potential (mV)
Initial precipitation	180	0.1	2	N/A
After TFF 1	330	0.2	9	-33*
After TFF2	330	0.2	11	-34*
After *E. coli* extract coating	305	0.2	10	-40
After freeze thaw	320	0.2	10	N/A

* Two entries with asterisks are for NPs made in earlier runs

[0702] The observed particle size distributions were exceedingly narrow and reproducible, as shown by using a Zetasizer Nano-ZS (Malvern Instruments, Southboro, MA) at 25 °C with a detection angle of 173°. A representative size distribution is shown in **FIG. 1D**. DLS data were processed with Malvern's software using a cumulant model for distribution analysis. The cumulant analysis is defined in International Organization for Standardization (ISO) standard document 13321. The calculations of PDI are defined in the ISO standard document 13321:1996 E. PDI's of 0.1 or less are considered monodisperse. For three separate experimental runs using the Tequila Sunrise precipitation process, the z-average size varied by only 2 nm (standard deviation, **Table 15**) and the PDIs were 0.09, 0.10 and 0.9. A representative scanning electron microscopy image of exemplary nanoparticles described herein is shown in **FIG. 1E**.

B. Coating with deoxycholate:

[0703] Although these particles were stable as formed, during the subsequent concentration by tangential flow ultrafiltration (TFF) they aggregated. To enhance NP repulsions, electrostatic stabilization was incorporated by coating them with the negatively charged amphiphilic bile salt—deoxycholate. The efficacy of this approach had been demonstrated previously with emulsion-based processing of NPs (Shalgunov et al., 2017).

[0704] Sodium deoxycholate was added at 0.11 mg.mL-1 in a 10 mM pH 8.2 AmBic buffer at a volume ratio of approximately 3:1 aqueous buffer to IPA dispersion. The addition was performed using a high shear mixer (Silverson, L5M-A), mounted with an in-line mixing assembly operated at 10,000 rpm for 6 minutes. The deoxycholate concentration was calculated to create a 2 nm thick uniform coating on the surfaces of the NPs. The final volume of the dispersion is ~190 L. In addition to providing steric stabilization of the NP during processing, Hughes et al. have shown that the highly negatively charged NP enhances immune cell uptake (Hughes et al., 2012).

C. Tangential Flow Ultrafiltration for solvent removal and concentration:

[0705] The present example utilized a scalable tangential flow ultrafiltration (TFF) process to remove solvents and concentrate the NP dispersion. This concentration was accomplished in two TFF steps. The first step used a modified poly(ethersulfone) membrane (Repligen, hollow fiber, mPES) with a 750 kDa pore size. The unit had surface area of 41,000 cm2 and was operated at a shear rate of 19,.000 s-1 with a transmembrane pressure of 30 psi. These conditions were chosen to minimize NP deposition on the membrane surface during TFF. In the first step, 190 L was concentrated by 14X and diafiltered with 20X volume of 10 mM phosphate buffer, 20 mM NaCl and 0.08 mg/ml deoxycholate. TFF took 2 hours. The added deoxycholate maintains charge and stability of the NPs since labile deoxycholate passes through the membrane along with the unencapsulated peanut protein and DNA. The holdup volume in the large TFF unit was too large to enable concentration to the desired final concentration of peanut protein of 2mg.mL-1 . Therefore, a second, smaller scale TFF unit with the same mPES membrane (8,500 cm2 area, and 16,400 s-1 shear rate) was used for the final 3.6 X wash and concentration. The final solution comprised 10mM phosphate buffer at pH 9 and 0.08 mg.mL-1 deoxycholate. The volume was reduced from 13.5 L to 1.7 L.

D. Coating with *E. coli* lipid extract:

[0706] The lipid extract is insoluble in aqueous buffer. Therefore, its deposition on the NP surface involves mechanical shear of the lipid oil onto the interface. This is accomplished by high shear homogenization with a Silverson L5M-A multifunctional Lab Mixer mounted with a standard head and high shear screen, operated at 10, 000 rpm for 4 minutes. The mechanical deposition results in some aggregation, which is minimized by continued shear. Shearing decreased the NP size from 330 nm to 305 nm. Spinning down the NPs and measuring the lipid concentration in the supernatant showed that 56% of the lipid was associated with the NP surface.

E. Freeze Thaw Stability:

[0707] One application of these nanoparticles is to serve as an orally deliverable agent with which to suppress allergic reactivity in patients with peanut allergy. For this purpose, in some embodiments, the final dosage form is in a container (e.g., in a packet or an ampoule) containing 1 mL of liquid dispersion. This can be shipped and stored frozen, and then thawed and

administered. After an evaluation of a number of cryoprotectants, it was found that 100 mg/ml trehalose provided stability during freeze thaw (**Table 16**). NPs 305 nm before freeze thaw were 320 nm after storage for 7 days at -80 °C.

Table 16: Sizes of three successive lots of PLG particles prepared by nanoprecipitation from the multiphase system at a 2.9 g scale with respect to encapsulated protein.

Sample	Z-Ave (nm)	Number Mean (nm)	Polydispersity Index (PDI)
Nanoprecipitation A	183	165	0.09
Nanoprecipitation B	187	173	0.10
Nanoprecipitation C	194	175	0.09

II. Design of PLG nanoparticles encapsulating ovalbumin.

[0708] NPs encapsulating OVA were prepared to demonstrate that the Tequila Sunrise process described herein is a platform that can be applied to proteins in general. These experiments were run at 1.18 g OVA scale. The Tequila Sunrise precipitation was identical to that performed for the peanut protein NPs described herein. However, there were two differences in the process. The first was that the deoxycholate was not required to stabilize the NPs. This is likely attributable to the greater hydrophilicity of the OVA protein relative to peanut proteins. This is consistent with the fact that albumin, another hydrophilic protein, is by itself sufficient to adequately stabilize nanoparticles in the commercial paclitaxel-albumin drug Abraxane, without the need for additional components to add electrostatic repulsions (Desai, 2016; Miele et al., 2009). The second difference is that the E Coli lipid extract was added immediately after nanoprecipitation rather than after an intermediate deoxycholate addition.

A. Synthesis of ovalbumin containing nanoparticles

[0709] Ovalbumin (1.18g) (Sigma) was dissolved in 27.41 g of DI water with magnetic stirring. To this was added 4.63 mL of a 10 mg/mL solution of DNA (SPL) in water. The pH was adjusted to 8.9 with 1 N NaOH (110 µL) and it was further diluted with an additional 27.41 g of

water giving a 19.8 mg/mL and 0.777 mg/mL solution of ovalbumin and DNA respectively in pH 8.9 water.

[0710] A solution of 11.58 g of PLG in 1.33kg of DMSO was prepared with stirring for 20 minutes until the PLG was dissolved. To this DMSO solution, the DNA and protein solution was slowly added. The solution was stirred for an additional 20 minutes and then transferred to a 3 L round bottomed flask. Rotary evaporation at 2 Torr in a 75° C water bath concentrated to solution to a final mass of 465 g.

[0711] Nanoprecipitation of the OVA particles was performed again by controlled mixing of the DMSO solution of PLG, protein and DNA with a solution of DMSO and isopropanol. A 4 L beaker was charged with 1.273 kg of IPA and 198 g of DMSO. Using a glass tube connected to a peristaltic pump, 440 g of the DMSO solution of protein, DNA and PLG was pumped gently under the isopropanol/DMSO layer such that two discrete layers are formed. An overhead stirrer with a stainless-steel stirring shaft and cross-shaped impellor inserted such that ¾ of the impellor was in the bottom DMSO solution was set to stir at 50 rpm for 2.5 minutes. The stirring rate was increased to 150 rpm and continued until a white homogeneous solution was obtained. By DLS, the particles were found to be 286 nm with a PDI of 0.15. This material was then homogenized with a solution of *E. coli* lipid extract (Avanti Polar Lipids, Alabaster AL) comprising 1.44 g in 7.2 kg of water, prepared with overhead stirring using a homogenizer (Silverson L5M-A,East Longmeadow, MA). Mixing involved pumping the *E. coli* extract solution into the nanoprecipitation mixture at 1.2 L.min-1 while recirculating through the homogenizer at 10,000 RPM. Once all the *E. coli* extract was added the solution was homogenized for an additional 2 minutes. At the end of the homogenization, the DLS size was 225 nm with a PDI of 0.11, as shown in **Table 17**.

[0712] Prior to TFF the material was diluted with 18.8 L of 10 mM AmBic. Two TFF loops were run. The first TFF loop (Repligen, Waltham MA), hollow fiber, mPES, 750 kDa, 8500 cm2) was preconditioned with 10 mM AmBic. At 1.4 k s-1 shear the solution was concentrated to 2000 mL and then diafiltered against 40 L of a buffer consisting of 10 mM potassium phosphate, 60 mM NaCl and 0.1 mg/mL *E. coli* lipid extract. After the 20-fold wash, 1200 mL of the resulting solution (69 % of the total) was transferred to a second TFF loop (repligen, hollow fiber, mPES, 750 kDa, 115 cm2) which had been preconditioned with a buffer solution of 10 mM phosphate and 0.1 mg/mL *E. coli* lipid extract. The particle solution was first concentrated

to 280 mL and then washed 3.5-fold with the same buffer solution at 15 k s-1 shear and then concentrated to a final volume of 153 mL. This solution was then divided in two. One half had *E. coli* lipid extract added (10 mg), while both halves had trehalose (750 mg) added.

Table 17: Sizes of ovalbumin NPs during processing steps.

Sample	Z-average diameter (nm)	Number-average diameter (nm)	Polydispersity (PDI)
Nanoprecipitation	286	251	0.15
Homogenization	225	207	0.11
End TFF 1	231	214	0.10
End TFF 2	233	192	0.14

III. Physicochemical Characterization of the particles.

[0713] Nanoparticles are characterized by size, zeta potential, and protein loading.

A. Protein concentration in NPs

[0714] A BCA assay was used to measure protein loading in the NPs and the ratio of encapsulated to unencapsulated protein. In brief, the NP dispersion was centrifuged (21.1 krcf for 20 min) to pellet the NPs. The supernatant was removed by filtering through a 100 nm filter and protein in solution assayed by BCA (SI BCA assay).

[0715] To measure level of protein encapsulated in the particles, a separate sample of the dispersion was digested in 0.1 M NaOH overnight to digest the PLG and release the protein. The solution becomes clear as the scattering from the PLG particles is lost as since the ester bonds are hydrolyzed. The final solution was again measured by BCA against a standard of protein that had also been treated with the same concentration of base. The encapsulated protein is then determined by subtracting the free protein from the total protein. For the peanut NPs, 69 % of the initial protein was found to be encapsulated at the end of TFF 2, for a total of 5.1 g of the initial 8.57 g of peanut protein. This 5.1 g of protein corresponds to approximately 2500 doses at the expected loading. The total free protein in the dispersion was 0.2 g. A calculation of the ratio of free to encapsulated protein is employed to generate a metric described as a 'safety factor'. The definition is based on administering a peanut protein dose using our NP dispersion that would be equivalent to the amount of peanut protein that would be given for current protein desensitization

therapy (OIT). In this administered dose, the ratio of the total administered dose over the unencapsulated (free) protein is the safety factor. These peanut particles have a safety factor of 25 which is to say that the patient exposure to unencapsulated peanut protein is reduced 25-fold as compared to the peanut protein exposure associated with standard oral immunotherapy. For the ovalbumin particles, which is our second example protein, the final encapsulation efficiency is 38%, which is higher than obtained by traditional PLG NP processes.

[0716] In addition to the presence of the protein, the present example demonstrates that the protein is not degraded by processing. SDS page gels were run to identify the components in the peanut particles after release from the NP by the base hydrolysis. The same bands can be seen in the sample of the protein.

B. PCR quantification of bacterial DNA:

[0717] The presence of the DNA in the particles was quantified using PCR. Samples of the PLG NPs were aliquoted at 50uL each as "Total" and "Pellet". The tube labeled as "Pellet" was centrifuged at 10,000 rpm for 5 minutes, supernatant was taken out to a separate tube labeled as "Sup", the leftover pellet (nanoparticles) was then resuspended in 250uL of DMSO. The "Total" and "Sup" samples were dissolved in 9 volumes of DMSO. The assay was carried out in a 20 µL reaction containing 0.8nM of *E. coli* primers specific to the ybbW gene, 0.2 nM of the *E. coli* probe (FAM), SprinTaq Master mix (containing 0.5mM of dNTPs, hot-start Taq DNA polymerase and other buffer components), and template DNA.

C. Quantification of the phospholipids:

[0718] The presence of the phospholipids was determined using a phospholipid assay from Abcam. The sample was diluted 1:10 with 5 % solution of Triton X by mixing 100 µL of sample with 900 µL of 5 % Triton X solution in a 1.5 mL Eppendorf tube. The sample was mixed using an end-over-end rotator for 30 min after which the nanoparticles were separated by centrifuging at 21.1 krcf for 20 min. The supernatant was then run neat on the phosphatidylethanolamine (PE, Abcam, AB241005) assay. The assay being able to detect only PE, and the total phospholipid extract being a mixture containing 57 % PE by weight, it was necessary to divide the obtained value by 0.57 to determine total phospholipid amounts.

IV. Immunological Characterization of PLG nanoparticles:

[0719] The NP process and NP constructs described herein overcome significant challenges that have limited PLG NPs for therapeutics. Technologies provided here achieve assemblage of different combinations of adjuvants with encapsulated antigen to create a specifically desired immune response, and furthermore allow scale-up for manufacturing. To assess produced NP constructs, peanut PLG and OVA PLG nanoparticles were prepared to analyze their safety and their efficacy of immune modulation. Since oral delivery has been a challenge due both to poor bioavailability of drug delivery and to low immunogenic potential, one goal of the present disclosure was to demonstrate that NP constructs described herein can provide the necessary signals to induce the desired immune responses when used orally.

A. Analysis of TLR activity of nanoparticles

[0720] To determine whether the *E. coli* lipid extract that coats the nanoparticles and the sheared *E. coli* DNA that is embedded within them are capable of activating their target Toll-like receptors, a commercial service (InvivoGen, San Diego, CA) that performs cell-based bioassays that measure TLR activation was utilized. Activation of TLR-mediated signaling leads to the stimulation of the NF-κB/AP-1 transcription pathway, which can be measured using a reporter gene construct whose expression is responsive to NF-κB/AP-1. In the assays employed in the present study, HEK293 cells are transfected to express a TLR of interest along with a cDNA encoding an NF-κB/AP-1-inducible reporter gene that encodes secreted embryonic alkaline phosphatase reporter gene. Test materials are added to the medium bathing the cells, and colorometric measurement of alkaline phosphatase enzymatic activity in the medium bathing the cells 16-24 hours after addition of the test material provides a measure of the quantity of bioavailable TLR-activating substances associated with the test material. For these studies the test material was comprised of peanut-encapsulating PLG NPs. Prior to being tested in the TLR-activation assay, the peanut PLG NPs were subjected to centrifugation on a sucrose step gradient to separate the NPs from any unincorporated *E. coli* phospholipids and DNA to ensure that that measured TLR activation is produced exclusively by NP-associated TLR ligands. Assays were performed in triplicate. Positive controls were performed by incubating cells expressing a TLR of interest with a standardized preparation that contains an activating ligand of that TLR. For TLR2 the positive control was heat killed Listeria monocytogenes (108 cells/mL), for TLR4 the positive control was *E. coli* K12 LPS (100 ng/mL), and for TLR9 the positive control was CpG ODN 2006 (10 μg/mL). TLR-expressing cells that were not incubated with peanut PLG NPs

were used to establish the baseline signal in the assay. Cells that did not express exogenous TLR were also incubated with peanut PLG NPs, which demonstrated that the peanut PLG NPs did not produce non-specific TLR-independent activation of the NF-κB/AP-1 reporter. The data presented in **FIG. 1F** depict the fold change in reporter expression induced by exposure of cells to the peanut PLG NPs as compared to that detected with control cells that were not exposed to the peanut PLG NPs. For each of the TLRs examined, the data depicted correspond to the fold induction of reporter expression observed at the dilution of peanut PLG NPs that produced maximal induction (1:100 for TLR2 and TLR4, 1:10 for TLR9). These results demonstrate that the bacterial DNA and bacterial lipid incorporated within the peanut PLG NPs and adhered to their surfaces, respectively, are bioavailable and able to activate their target TLR in the context of a cell-based bioassay. Thus, the PLG NPs described herein deliver their peanut protein payload in association with bioactive bacterial adjuvants that allow these NPs to be tuned so as to produce desired immunological responses.

B. Demonstration that basophils from peanut allergic patients are substantially less responsive to encapsulated peanut within NPs when compared to unencapsulated (unmasked) peanut proteins.

[0721] To determine if PLG nanoparticles carrying encapsulated peanut extract and administered orally would increase the safety of the delivery system, human basophils prepared from peanut allergic individuals were exposed to the peanut PLG nanoparticles, unencapsulated peanut protein (unmasked peanut extract) or an empty vehicle control. Basophil responses (%CD63+ basophils) were determined by the basophil activation test (BAT) that measures degranulation following stimulation with peanut, correlating directly with histamine release (REF). As seen in **FIG. 2A**, human basophils from a peanut allergic patient showed greater %CD63+ expression at a lower dose of unencapsulated (unmasked) peanut extract than did human basophils exposed to peanut PLG nanoparticles. In addition, the analysis of 7 individuals, plotted in **Fig. 2C** as the concentration of peanut extract needed to elicit a half maximal basophil response, shows that the dose of peanut PLG nanoparticle required to induce basophil activation is considerably greater (~10 fold) than the dose required by unencapsulated peanut extract.

[0722] These data importantly illustrate that human basophils were approximately 10 times less responsive to the peanut PLG nanoparticles than to the unencapsulated peanut extract or the empty vehicle control. These data provide ex vivo evidence that encapsulating the peanut extract

PLG nanoparticles substantially reduces the level of IgE mediated basophil degranulation, which suggests that encapsulation will potentially greatly increase the safety of oral immunotherapy and of AIT in general.

C. Demonstration that the new PLG nanoparticle process produces nanoparticles that will activate antigen-specific T cell responses in vitro.

[0723] To assess ability of T cells to respond to encapsulated antigen, nanoparticles encapsulating ovalbumin (OVA) were prepared as described, with the PLG matrix encapsulating both OVA proteins as well as sheared *E. coli* DNA as an adjuvant to activate TLR9. On the outside of the particles was a mixture of *E. coli* phospholipids to activate TLR4 on dendritic cells.

[0724] Bone marrow derived dendritic cells (BMDC) were pulsed with OVA PLG nanoparticles or unencapsulated OVA for 2 hours and then cultured with ovalbumin-specific CD8+ and CD4+ T cells derived from T cell receptor transgenic mice (OT-I and OT-II). As can be seen in Fig. 3A-G, both CD4 and CD8 T cells responded by proliferation to the OVA PLG nanoparticles at all doses tested. In addition, the OVA PLG nanoparticles induced a more robust T cell response at lower doses of OVA than did unencapsulated OVA, which showed little response at those two doses. The presence of the OVA protein itself was required for the nanoparticle-induced proliferative response since the empty nanoparticles prepared with only the adjuvants (encapsulated DNA and *E. coli* lipid extract only) did not drive T cell proliferation (Fig.3A-B).

[0725] These findings also show (Fig. 3H-N that a substantial number of OVA specific CD4 T cells express IFN-γ and to a much lesser extent, IL4, IL17 and IL10. Unencapsulated OVA, by comparison, produces little if any cytokine response. Upon stimulation, CD8 T cells show IFN-γ/granzyme expression (Fig. 3M-N (Cytos C) that is considerably higher than unencapsulated OVA.

[0726] These data show that OVA PLG nanoparticle-pulsed dendritic cells induce skewed Th1, inducing primarily IFN-γ producing CD4 T cells. IFN-γ was produced by both CD4 and CD8 T cells, which serves as a significant deterrent of allergy-associated Th2 immunity. This clearly demonstrates that the OVA PLC nanoparticles achieved a desired result of generating the specific type of T cell response necessary for the most successful AIT.

D. Demonstration that the new PLG nanoparticle process leads to nanoparticles that will activate antigen specific T cell proliferative responses in vivo.

[0727] To analyze the ability of OVA PLG nanoparticles and unencapsulated OVA to activate T cell proliferative responses in vivo, isolated OVA-specific CD4 (OT2) and CD8 (OT1) T cells (labeled with CFSE) were adoptively transferred to WT mice. The next day the mice were treated orally with OVA nanoparticles, empty nanoparticles or unencapsulated OVA, and 4 days later tested to assess the levels of T cell proliferative responses. As seen in **FIGS. 4A-B**, the newly created OVA encapsulated nanoparticles were capable of inducing substantially greater numbers of CD4 and CD8 T cells to proliferate compared to nanoparticles without encapsulated OVA.

[0728] These data indicate that provided antigen-encapsulating PLG NPs (produced by provided technologies) activate CD4 and CD8 T cells in vivo. Furthermore, oral administration of OVA PLG NPs generate both Th1-like, and CD8 T cells that are IFN-γ producing, and thus capable of inhibiting Th2 cell responses. Taken together, the manufacturing process and NP construct described herein yield an approach to AIT that is practical from the manufacturing perspective, promising with regard to its potential efficacy, and preferred for offering increased compliance and cost effectiveness. The present Example demonstrated that exemplary peanut PLG nanoparticles disclosed herein, and methods of manufacturing of the same, provide particularly useful technologies for potential allergy treatments.

REFERENCES

Desai, N., Nanoparticle albumin-bound paclitaxel (Abraxane®). Albumin in medicine: Pathological and clinical applications 2016, 101-119.

FDA, P., Palforzia AT. U.S. Food and Drug Administration. (2020). Available online at: https://www.fda.gov/media/134838/download (accessed April 27, 2021).

Horn, D.; Rieger, J., Organic nanoparticles in the aqueous phase—theory, experiment, and use. *Angewandte Chemie International Edition* **2001,** *40* (23), 4330-4361.

Hughes, K. R.; Saunders, M. N.; Landers, J. J.; Janczak, K. W.; Turkistani, H.; Rad, L. M.; Miller, S. D.; Podojil, J. R.; Shea, L. D.; O'Konek, J. J., Masked delivery of allergen in nanoparticles safely attenuates anaphylactic response in murine models of peanut allergy. Frontiers in Allergy 2022, 3, 829605.

Johnson, B. K.; Prud'homme, R. K., Chemical processing and micromixing in confined impinging jets. Aiche Journal 2003, 49 (9), 2264-2282.
Mahajan, A. J.; Kirwan, D., Rapid precipitation of biochemicals. Journal of Physics D: Applied Physics 1993, 26 (8B), B176.
Martins, J. P.; Torrieri, G.; Santos, H. A., The importance of microfluidics for the preparation of nanoparticles as advanced drug delivery systems. Expert opinion on drug delivery 2018, 15 (5), 469-479.
Miele, E.; Spinelli, G. P.; Miele, E.; Tomao, F.; Tomao, S., Albumin-bound formulation of paclitaxel (Abraxane® ABI-007) in the treatment of breast cancer. International journal of nanomedicine 2009, 99-105.
Shalgunov, V.; Zaytseva-Zotova, D.; Zintchenko, A.; Levada, T.; Shilov, Y.; Andreyev, D.; Dzhumashev, D.; Metelkin, E.; Urusova, A.; Demin, O., Comprehensive study of the drug delivery properties of poly (l-lactide)-poly (ethylene glycol) nanoparticles in rats and tumor-bearing mice. Journal of Controlled Release 2017, 261, 31-42.
Villa, C. H.; Anselmo, A. C.; Mitragotri, S.; Muzykantov, V., Red blood cells: Supercarriers for drugs, biologicals, and nanoparticles and inspiration for advanced delivery systems. Advanced drug delivery reviews 2016, 106, 88-103.

Example 11: mRNA Payloads

[0729] The present example describes application of provided technologies to the production of PLG nanoparticles encapsulating mRNA.

[0730] Those skilled in the art will appreciate the significance of mRNA therapeutics, including as vaccines (e.g., to protect against infectious agents and/or tumors), as providing replacement polypeptides (e.g., wild type polypeptides such as enzymes, etc), etc., and furthermore will appreciate the need for technologies that can encapsulate and/or deliver such mRNAs. Those skilled in the art will appreciate that presently commercialized mRNA therapeutics, specifically including SARS-Co-V2 vaccines, are delivered using lipid nanoparticle systems that utilize four different types of lipids, specifically, sterol, phospholipid, cationic and PEGylated lipids. The present disclosure provides alternative delivery technologies, specifically in particular technologies that do not utilize all four such lipid types and/or that utilize polymer nanoparticles, *e.g.,* PLG nanoparticles.

[0731] Among other things, the present disclosure provides an insight that the relatively gentle processes (*e.g.*, Tequila Sunrise) for nanoparticle fabrication in accordance with the present disclosure may well be readily amenable to mRNA payloads. Indeed, successful incorporation of DNA preparations (*e.g.,* sheared *E. coli* DNA and/or isolated CpG DNA) as described herein (see, for example, Example 10) provides evidence of applicability of provided technologies to encapsulation of nucleic acids; the present example specifically describes such application to mRNA agents.

[0732] Thus, in some embodiments, technologies described herein (e.g., in one or more of Examples 1, 2, 5, 6, 7, 8, or 10, and particularly in Example 10 – e.g., including a Tequila Sunrise strategy) are utilized to encapsulate an mRNA payload, such as an mRNA that encodes a polypeptide, which polypeptide may be or comprise, for example, an allergic antigen, an infectious agent antigen, a cancer antigen, a replacement polypeptide, to name a few. In some embodiments, a plurality of different mRNAs (e.g., encoding different polypeptides, which in some embodiments may be or include different forms or variants of the same polypeptide), are incorporated into the same nanoparticles. In other embodiments, only a single type of mRNA is incorporated into any individual nanoparticle, but mixed nanoparticle preparations (*e.g.,* including different RNAs, separately encapsulated) can be prepared, for example, by combination of nanoparticle populations with different individually-encapsulated RNAs.

[0733] Those skilled in the art will further appreciate that, in some embodiments, when an mRNA is used as a payload, it may not be necessary (and, in some embodiments, may not be desirable) to co-encapsulate another nucleic acid (e.g., DNA, such as *E. coli* DNA as described herein) and/or to coat the nanoparticles with *E. coli* lipid extract, or other source of, for example LPS.

[0734] To give but one example, coating with LPS (and/or other lipid) may be particularly useful when, for example, a payload mRNA encodes an allergen to which the recipient individual is or may be allergic, so that immunomodulation mediated by such LPS (toward a Th1-type response, as described herein, may be particularly useful); coating with LPS (and/or other lipid) may not be required, or even particularly beneficial, in certain other contexts (*e.g.,* when an mRNA payload encodes an enzyme replacement).

[0735] In some embodiments, a desired amount of mRNA for encapsulation may be assessed, for example, by a standard method such as, for example, by qRT-PCR and/or by UV

spectroscopy OD260. In some embodiments, degree of encapsulation may be assessed by determining total amount mRNA utilized relative to amount of "free" mRNA after fabrication of nanoparticles as described herein (e.g., as in one or more of Examples 1, 2, 5, 6, 7, 8, or 10, and particularly in Example 10 – e.g., including a Tequila Sunrise strategy).

[0736] If desired, degree of mRNA encapsulation, and/or effectiveness of mRNA delivery to cells with which a nanoparticle preparation that includes an mRNA payload (e.g., via fabrication as described herein) has been contacted, is assessed by detection of an encoded polypeptide. In some embodiments, such an encoded polypeptide may be considered a "reporter" polypeptide that need not have biological function as a payload; in some such embodiments, a reporter polypeptide may be, for example, a detectable polypeptide such as a fluorescent polypeptide (e.g., green fluorescent protein (GFP)), a chemiluminescent polypeptide (e.g., a luciferase polypeptide), or an otherwise detectable polypeptide (e.g., that catalyzes a readily detectable reaction – such as a beta-galactosidase (β-gal) polypeptide.

[0737] In some embodiments, expression of a polypeptide (*e.g.,* a therapeutic polypeptide, as contrasted for example with a "reporter" polypeptide) encoded by a payload mRNA may be assessed, if desired. For example, those skilled in the art will be familiar with technologies such as gel electrophoresis (including Western Blot analysis), immunocytochemistry, immunohistochemistry, etc) that can be utilized to detect polypeptides in or from cells or tissues.

[0738] In some particular embodiments, an encapsulated mRNA (or set of mRNAs) encodes one or more food allergen proteins such as, for example, allergen proteins found in foods such as crustacean shellfish (e.g., shrimp) eggs, fish, milk, peanuts, sesame, soy, tree nuts, wheat, and combinations thereof. In some embodiments, individual mRNAs encoding individual allergen polypeptides may be separately encapsulated; in some embodiments, multiple mRNAs may be co-encapsulated. In some particular embodiments, multiple mRNAs encoding different protein allergens from the same food may be co-encapsulated.

[0739] Alternatively or additionally, in some embodiments, an encapsulated mRNA (or set of mRNAs) encodes one or more infectious agent antigens such as, for example, one or more bacterial, fungal, or viral antigens. Those skilled in the art will be aware of the success of mRNA vaccines encoding coronavirus spike proteins (including specifically the SARS-CoV-2 spike protein, or an antigenic fragment and/or variant thereof), and of reports of mRNA encoding one or more influenza protein antigens.

[0740] Still further alternatively or additionally, in some embodiments, an encapsulated mRNA (or set of mRNAs) encodes one or more cancer vaccine antigens (e.g., which may include one or more neoepitopes that have arisen in the particular subject to whom the vaccine (e.g., a provided nanoparticle composition including an mRNA payload that encodes one or more such neoepitopes).

[0741] Those skilled in the art will appreciate that provided technologies may be applicable to any of a variety of therapeutic polypeptides, potentially including any polypeptide of interest – *e.g.*, that such polypeptides may be delivered via encapsulation of mRNA encoding them in nanoparticles as described herein, followed by administration (e.g., oral administration) of such nanoparticles.

Example 12: GLP-1 Receptor Modulator Payloads

[0742] The present example describes application of provided technologies to the production of PLG nanoparticles encapsulating GLP-1 receptor modulators.

[0743] Those skilled in the art will appreciate the significance of GLP-1 receptor modulators for their burgeoning role in the treatment of type-II diabetes, obesity, and related conditions (*i.e.*, lowering the risk of heart disease, such as heart failure, stroke and kidney disease.). Semaglutide, tirzepatide, exenatide, lixisenatide, dulaglutide, and liraglutide have all recently gained attention for their clinical efficacy, and many other products are under development.

[0744] Most currently commercialized GLP-1 receptor modulator drugs are administered parenterally, typically at least once weekly, and in some cases once daily. *See,* for example, Stretton *et al., Int. Med. J.* doi.org/10.1111/imj.16126, 15 May 2023. The present disclosure appreciates that alternative formulations and/or dosing regimens for GLP-1 receptor modulators may be particularly desirable and/or beneficial to at least some subjects. Moreover, the present disclosure provides an insight that provided technologies may be particularly useful for incorporation, administration, and/or delivery of GLP-1 receptor modulators. Nanoparticle formulations as described herein can be administered via various routes (e.g., orally, parenterally, etc, as noted further below and elsewhere herein), and may be amenable to dosing regimens more palatable to at least some subjects.

[0745] Many known GLP-1 receptor modulators, including marketed therapeutics, are peptide agents. Those skilled in the art, reading the present disclosure, will appreciate that provided

technologies are amenable to incorporation, administration, and/or delivery of such agents in peptide form or as nucleic acids (e.g., DNA or RNA) that encode them.

[0746] One potential advantage of provided PLG nanoparticle technologies for delivery of GLP-1 receptor modulators as described herein is that these technologies can allow administration to the oral cavity (*e.g.*, oral administration, which may achieve or comprise, for example, buccal, and/or sublingual delivery).

[0747] Furthermore, the present disclosure provides an insight that administration via the oral cavity (*i.e.*, oral administration, which may be or comprise sublingual and/or buccal delivery) may be particularly useful in the context of GLP-1 receptor modulators. GLP-1 receptors are located primarily in the gastrointestinal tract, pancreas, central and peripheral nervous systems, cardiovascular systems, kidneys, and lungs. Without wishing to be bound by any particular theory, the present disclosure proposes that oral delivery of GLP-1 receptor modulators, included specifically via provided nanoparticle technologies, may increase drug availability to gastrointestinal GLP-1 receptors and could improve drug availability and/or efficacy.

[0748] As noted herein, the present disclosure provides an insight that the relatively gentle processes (e.g., Tequila Sunrise) for nanoparticle fabrication in accordance with the present disclosure may well be readily amenable to fragile payloads. The present Example teaches that provided nanoparticle technologies may be particularly useful to achieve delivery of GLP-1 receptor modulator agents, and specifically contemplates embodiments in which such agents are incorporated as polypeptides or as nucleic acids that encode them. Indeed, successful incorporation of polypeptide preparations (*e.g.,* crude peanut extract and/or OVA-albumin) as described herein provides evidence of applicability of provided technologies to encapsulation of polypeptides; successful incorporation of sheared *E. coli* DNA and/or CpG oligonucleotides provides evidence of applicability of provided technologies to encapsulation of nucleic acids; Those skilled in the art will appreciate, however, that certain GLP-1 receptor modulator agents, specifically including certain marketed therapeutic agents, include one or more non-natural amino acid analogs, such that administration of a nucleic acid would not achieve delivery of the relevant agent. In some embodiments, such agents are utilized (*e.g.,* incorporated into nanoparticles as described herein) as polypeptides (*e.g.,* including their non-natural amino acids).

[0749] Thus, in some embodiments, technologies described herein (e.g., in one or more of Examples 1, 2, 5, 6, 7, 8, or 10, and particularly in Example 10 – e.g., including a Tequila

Sunrise strategy) are utilized to encapsulate a GLP-1 receptor modulator payload (*e.g.,* polypeptide or nucleic acid encoding it); in many such embodiments, such payload is a polypeptide payload.

[0750] The present Example teaches that, in some embodiments, when a GLP-1 receptor modulator polypeptide (or nucleic acid encoding it) is used as a payload when manufacturing nanoparticles as described herein, it may not be necessary (and, in some embodiments, may not be desirable) to co-encapsulate another nucleic acid (e.g., DNA, such as *E. coli* DNA as described herein) and/or to coat the nanoparticles with *E. coli* lipid extract, or other source of, for example LPS.

[0751] In some embodiments, a desired amount of GLP-1 receptor modulator polypeptide for encapsulation may be assessed (*e.g.,* prior to and/or after encapsulation) using technologies as described herein. For example, polypeptides may be assessed, for example, by one or more of, gel electrophoresis, Western Blot, ELISA, and/or by BCA assay, *etc.* ; nucleic acids may be assessed, for example, by one or more of qRT-PCR, UV spectroscopy OD260, etc . Those skilled in the art will be familiar with technologies such as gel electrophoresis (including western blot analysis), immunocytochemistry, immunohistochemistry, etc.) that can be utilized to detect polypeptides in or from cells or tissues. In some embodiments, degree of encapsulation may be assessed by determining total amount of GLP-1 receptor modulator utilized relative to amount of "free" GLP-1 receptor modulator after fabrication of nanoparticles as described herein (e.g., as in one or more of Examples 1, 2, 5, 6, 7, 8, or 10, and particularly in Example 10 – e.g., including a Tequila Sunrise strategy).

[0752] If desired, degree of GLP-1 receptor modulator encapsulation, and/or effectiveness of GLP-1 receptor modulator delivery to cells with which a nanoparticle preparation that includes an GLP-1 receptor modulator payload (e.g., via fabrication as described herein) has been contacted, is assessed by detection of an encoded polypeptide. In some embodiments, such an encoded polypeptide may be considered a "reporter" polypeptide that need not have biological function as a payload; in some such embodiments, a reporter polypeptide may be, for example, a detectable polypeptide such as a fluorescent polypeptide (e.g., green fluorescent protein (GFP)), a chemiluminescent polypeptide (e.g., a luciferase polypeptide), or an otherwise detectable polypeptide (e.g., that catalyzes a readily detectable reaction – such as a beta-galactosidase (β-gal) polypeptide.

[0753] In some embodiments, a GLP-1 receptor modulator polypeptide may be or comprise one listed below in **Table 18**. In some embodiments, a GLP-1 receptor modulator polypeptide may be or comprise a variant of one listed below in **Table 18**. For example, in some embodiments, a variant may have an amino acid sequence that is at least 80%, 85%, 90%; 95%, 99%, or 100% identical to that of a GLP-1 modulator polypeptide included in **Table 18**.

[0754] In some embodiments, a GLP-1 modulator polypeptide is a natural polypeptide. In some emodiments, a GLP-1 modulator polypeptide is a non-natural polypeptide (those skilled in the art will appreciate that many non-natural GLP-1 modulator polypeptides are referred to as "mimetics" – *e.g.*, "incretin mimetics"; in some embodiments, a non-natural GLP-1 modulator polypeptide includes one or more non-natural residues.

Table 18. Exemplary GLP-1 receptor modulators.

Reference GLP-1 receptor modulator	SEQ ID NO	Sequence	Exemplary Analogues
GLP1 (7-37)	1	HAEGTFTSDVSSYLEGQAAKEF IAWLVKGRG	
GLP1 (7-37) (Arg34 mutant)	2	HAEGTFTSDVSSYLEGQAAKEF IAWLVRGRG	
GLP1 (7-36)	3	HAEGTFTSDVSSYLEGQAAKEF IAWLVKGR	
GIP (1-42)	4	YAEGTFISDYSIAMDKIHQQDF VNWLLAQKGKKNDWKHNITQ	
GIP (1-30)	5	YAEGTFISDYSIAMDKIHQQDF VNWLLAQK	
Dulaglutide (GLP1 (7-37)-Fc fusion protein)	6	HGEGTFTSDVSSYLEEQAAKEF IAWLVKGGGGGGSGGGGSGG GGSAESKYGPPCPPCPAPEAAG GPSVFLFPPKPKDTLMISRTPEV TCVVVDVSQEDPEVQFNWYVD GVEVHNAKTKPREEQFNSTYR VVSVLTVLHQDWLNGKEYKC KVSNKGLPSSIEKTISKAKGQPR EPQVYTLPPSQEEMTKNQVSLT CLVKGFYPSDIAVEWESNGQPE NNYKTTPPVLDSDGSFFLYSRL TVDKSRWQEGNVFSCSVMHEA LHNHYTQKSLSLSLG	
Liraglutide		HAEGTFTSDVSSYLEGQAAKEF IAWLVRGRGE	

Reference GLP-1 receptor modulator	SEQ ID NO	Sequence	Exemplary Analogues
Exenatide (GLP1R agonist)	7	HGEGTFTSDLSKQMEEEAVRLF IEWLKNGGPSSAPPPS	HGEGXFXSDLSKQMEE EAVRLFXEWLKNGGPS SGAPPPS (where X is any amino acid)
Tirzepatide (GLP1R/GIPR dual agonist)	8	YAEGTFTSDYSIYLDKIAQKAF VQWLIAGGPSSGAPPPS	YXEGTFTSDYSIXLDKIA QKAFVQWLIAGGPSSG APPPS (wherein X2 and X13 can be α-amino isobutyric acid (Aib), S39 is L-serinamide, and K20 is N6-{N-(hydrogen icosanedioyl)-γ-Glu-bis[iminobis(ethylenoxy)ac etyl]}-Lysine
NNC0090-2746 (GLP1R/GIPR dual agonist)	9	YAEGTFTSDYSIYLDKQAAKEF VNWLLAGGPSSGAPPPS	
MAR701 (GLP1R/GIPR dual agonist)	10	YAEGTFTSDYSIYLDKQAAKEF VCWLLAGGPSSGAPPPS	
Retratutide (GLP1R/GIPR/GCG R triple agonist)	11	YXQGTFTSDYSIYLDKKAQXAF IEYLLEGGPSSGAPPPS where X at position 2 is A or S where X at position 20 is K or Q	

[0755] Nanoparticle preparations that include and/or deliver a GLP-1 receptor modulator may be characterized, for example, for one or more of: (a) nanoparticle size and/or polydispersity; (b) Zeta potential; (c) extent of encapsulation; (d) timing and/or conditions of release of encapsulated agent; (e) effect of nanoparticle composition and/or agent released therefrom on cell(s) (*in vitro* and/or *in vivo*). In some embodiments, oral administration (*e.g.*, to an appropriate animal model) is assessed. In some embodiments, performance of a nanoparticle composition is compared, for example, with that of the same GLP-1 receptor modulator as a positive comparator, unencapsulated, and/or with empty nanoparticles or other vehicle as a negative comparator.

Example 13: α-Gal Payloads

[0756] The present example describes application of provided technologies to the production of PLG nanoparticles encapsulating a carbohydrate, specifically a disaccharide, specifically galactose-α-1, 3-galactose (α-gal), or an agent that comprises or otherwise delivers such carbohydrate.

[0757] Those skilled in the art will appreciate that α-gal plays a significant role in α-gal syndrome, a type of food allergy where people are allergic to red meat and other products made from mammals. In the United States, α-gal syndrome is often initiated by a bite from a Lone Star tick, which injects α-gal into the bite recipient, triggering an allergic reaction to the α-gal. Once sensitized by such a bite, the subject will often experience mild to severe allergic reactions to red meat, such as beef, pork or lamb. It also can cause reactions to other foods that come from mammals, such as dairy products or gelatins. *See,* for example, Mayo Foundation for Medical Education and Research. (2022, November 15). Alpha-gal syndrome. Mayo Clinic. https://www.mayoclinic.org/diseases-conditions/alpha-gal-syndrome/symptoms-causes/syc-20428608.

[0758] Currently treatment of α-gal syndrome consists of management through antigen avoidance and symptoms management, however no therapeutics are presently available to reduce sensitivity or disease severity. The present disclosure teaches that provided technologies may be particularly useful for the treatment of α-gal syndrome. Specifically, the present disclosure teaches that incorporation of α-gal into nanoparticles as described herein may achieve immune modulation that can alleviate or obviate allergic reactions to α-gal.

[0759] Those skilled in the art will appreciate the significance of carbohydrate therapeutics, including as vaccines (e.g., to protect against infectious agents and/or tumors), as providing replacement carbohydrates (e.g., wild type carbohydrates such as enzymes, etc), etc., and furthermore will appreciate the need for technologies that can encapsulate and/or delivery such carbohydrates (i.e., beyond lipid nanoparticle technologies that include sterol, phospholipid, cationic and PEGylated lipids.

[0760] Among other things, the present disclosure provides an insight that the incorporation of α-gal into nanoparticles for immune modulation through techniques such as SLIT, represents an important step in the treatment of α-gal syndrome.

[0761] the relatively gentle processes (e.g., Tequila Sunrise) for nanoparticle fabrication in accordance with the present disclosure may well be readily amenable to carbohydrate payloads.

[0762] Thus, in some embodiments, technologies described herein (e.g., in one or more of Examples 1, 2, 5, 6, 7, 8, or 10, and particularly in Example 10 – e.g., including a Tequila Sunrise strategy) are utilized to encapsulate a carbohydrate payload which may be or comprise, for example, an allergic antigen, an infectious agent antigen, a cancer antigen, a replacement carbohydrate, to name a few. In some embodiments, a plurality of different carbohydrates may be encapsulated in combination.

[0763] The present Example teaches that, in some embodiments, when α-gal is used as a payload when manufacturing nanoparticles as described herein, it may not be necessary (and, in some embodiments, may not be desirable) to co-encapsulate another nucleic acid (e.g., DNA, such as *E. coli* DNA as described herein) and/or to coat the nanoparticles with *E. coli* lipid extract, or other source of, for example LPS.

[0764] To give but one example, coating with LPS may be particularly useful when, for example, a payload carbohydrate (such as α-gal) is an allergen to which the recipient individual is or may be allergic, so that immunomodulation mediated by such LPS (toward a Th1-type response, as described herein, may be particularly useful).

[0765] In some embodiments, a desired amount of carbohydrate for encapsulation may be assessed. One of skill in the art would understand how to quantify carbohydrates through standard methods such as by Molisch's test, iodine test, etc.. In some embodiments, degree of encapsulation may be assessed by determining total amount of carbohydrate utilized relative to amount of "free" carbohydrate after fabrication of nanoparticles as described herein (e.g., as in one or more of Examples 1, 2, 5, 6, 7, 8, or 10, and particularly in Example 10 – e.g., including a Tequila Sunrise strategy).

[0766] In some particular embodiments, an encapsulated carbohydrate (or set of carbohydrates) encodes one or more food allergen proteins such as, for example, allergenic carbohydrates found in foods such as dairy products, beans, and red meat, and combinations thereof. In some embodiments, individual carbohydrates may be separately encapsulated; in some embodiments, multiple carbohydrates may be co-encapsulated. In some particular embodiments, multiple carbohydrates from the same food may be co-encapsulated.

[0767] Alternatively or additionally, in some embodiments, an encapsulated carbohydrate (or set of carbohydrates) are derived from one or more infectious agent antigens such as, for example, one or more bacterial, fungal, or viral antigens.

[0768] Still further alternatively or additionally, in some embodiments, an encapsulated carbohydrate (or set of carbohydrates) may comprise one or more cancer vaccine antigens (e.g., which may include one or more neoepitopes that have arisen in the particular subject to whom the vaccine (e.g., a provided nanoparticle composition including a carbohydrate that represents one or more such neoepitopes).

[0769] Those skilled in the art will appreciate that provided technologies may be applicable to any or all therapeutic polypeptides – e.g., that such carbohydrates may be delivered via encapsulation of carbohydrates in nanoparticles as described herein, followed by administration (e.g., oral administration) of such nanoparticles.

[0770] Nanoparticle preparations that include and/or deliver α-gal may be characterized, for example, for one or more of: (a) nanoparticle size and/or polydispersity; (b) Zeta potential; (c) extent of encapsulation; (d) timing and/or conditions of release of encapsulated agent; (e) effect of nanoparticle composition and/or agent released therefrom on cell(s) (*in vitro* and/or *in vivo*). In some embodiments, oral administration (*e.g.*, to an appropriate animal model) is assessed. In some embodiments, performance of a nanoparticle composition is compared, for example, with that of unencapsulated α-gal as a positive comparator and/or with empty nanoparticles or other vehicle as a negative comparator.

Example 14: Gene Therapy Vector Payloads

[0771] The present example describes PLG nanoparticles encapsulating nucleic acids for gene therapy.

[0772] Those skilled in the art will appreciate the significance of nucleic acids for gene therapy, including as providing replacement and or supplemental polypeptides (e.g., wild type polypeptides such as enzymes, etc.), etc., and furthermore will appreciate the need for technologies that can encapsulate and/or delivery such nucleic acids for gene therapies (i.e., beyond lipid nanoparticle technologies that include sterol, phospholipid, cationic and PEGylated lipids.

[0773] Those skilled in the art will appreciate that gene therapies can involve several mechanisms including, but not limited to: replacing a disease-causing gene with a healthy copy of the gene; inactivating a disease-causing gene that is not functioning properly; and/or introducing a new or modified gene in to the body to help treat a disease, etc.

[0774] Current delivery methods of gene therapies include plasmid DNA, viral vectors, bacterial vectors, human gene editing technology, and patient-derived cellular gene therapy products.

[0775] Among other things, the present disclosure provides an insight that the relatively gentle processes (e.g., Tequila Sunrise) for nanoparticle fabrication in accordance with the present disclosure may well be readily amenable to nucleic acids for gene therapy payloads. Indeed, successful incorporation of DNA preparations (*e.g.,* sheared *E. coli* DNA and/or isolated CpG DNA) as described herein provides evidence of applicability of provided technologies to encapsulation of nucleic acids; the present disclosure specifically teaches such application to nucleic acid agents.

[0776] Thus, in some embodiments, technologies described herein (e.g., in one or more of Examples 1, 2, 5, 6, 7, 8, or 10, and particularly in Example 10 – e.g., including a Tequila Sunrise strategy) are utilized to encapsulate a nucleic acids for gene therapy payload, such as an mRNA or DNA that encodes a polypeptide, which polypeptide may be or comprise, for example, an allergic antigen, an infectious agent antigen, a cancer antigen, a replacement polypeptide, to name a few. In some embodiments, a plurality of different nucleic acids(e.g., encoding different polypeptides, which in some embodiments may be or include different forms or variants of the same polypeptide).

[0777] Those skilled in the art will further appreciate that, in some embodiments, when a nucleic acid is used as a payload when manufacturing nanoparticles as described herein, it may not be necessary (and, in some embodiments, may not be desirable) to co-encapsulate another nucleic acid (e.g., DNA, such as *E. coli* DNA as described herein) and/or to coat the nanoparticles with *E. coli* lipid extract, or other source of, for example LPS.

[0778] In some embodiments, a desired amount of nucleic acid for encapsulation may be assessed, for example, by a standard method such as, for example, by qRT-PCR and/or by UV spectroscopy OD260. In some embodiments, degree of encapsulation may be assessed by determining total amount nucleic acid utilized relative to amount of "free" nucleic acid after fabrication of nanoparticles as described herein (e.g., as in one or more of Examples 1, 2, 5, 6, 7, 8, or 10, and particularly in Example 10 – e.g., including a Tequila Sunrise strategy).

[0779] If desired, degree of nucleic acid encapsulation, and/or effectiveness of nucleic acid delivery to cells with which a nanoparticle preparation that includes a nucleic acid payload (e.g., via fabrication as described herein) has been contacted, is assessed by detection of an encoded

polypeptide. In some embodiments, such an encoded polypeptide may be considered a "reporter" polypeptide that need not have biological function as a payload; in some such embodiments, a reporter polypeptide may be, for example, a detectable polypeptide such as a fluorescent polypeptide (e.g., green fluorescent protein (GFP)), a chemiluminescent polypeptide (e.g., a luciferase polypeptide), or an otherwise detectable polypeptide (e.g., that catalyzes a readily detectable reaction – such as a beta-galactosidase (β-gal) polypeptide.

[0780] In some embodiments, individual nucleic acids encoding individual polypeptides may be separately encapsulated; in some embodiments, multiple nucleic acids may be co-encapsulated. In some particular embodiments, multiple nucleic acids encoding different proteins may be co-encapsulated.

[0781] Still further alternatively or additionally, in some embodiments, an encapsulated nucleic acid (or set of nucleic acids) encodes one or more cancer vaccine antigens (e.g., which may include one or more neoepitopes that have arisen in the particular subject to whom the vaccine (e.g., a provided nanoparticle composition including an mRNA payload that encodes one or more such neoepitopes).

[0782] Those skilled in the art will appreciate that provided technologies may be applicable to any or all therapeutic polypeptides – e.g., that such polypeptides may be delivered via encapsulation of nucleic acids encoding them in nanoparticles as described herein, followed by administration (e.g., oral administration) of such nanoparticles.

Example 15: TLR Activation

[0783] The present example describes activation of TLR signaling by some embodiments of the described invention.

[0784] Specifically, a nanoparticle preparation referred to herein as "NP-PN1", which is a therapeutic agent that includes an *Arachis hypogaea* peanut extract and sheared *E. coli* DNA encapsulated in poly(lactic-co-glycolic acid) (PLG) nanoparticles coated with *E. coli* lipid extract containing lipopolysaccharide ("LPS"), was assessed. The particles of NP-PN1 are formulated in a vehicle containing phosphate buffer and trehalose for oral administration. The concept behind NP-PN1 is that encapsulating the peanut extract within PLG nanoparticles "hides" the peanut allergens present in the extract from the recipient's immune system, therefore reducing risk of anaphylaxis relative to, for example, systemic administration of peanut allergens as is done in

standard immunotherapy. *See,* for example, Gernez Y, Nowak-Wegrzyn A. Immunotherapy for food allergy: are we there yet? J Allergy Clin Immunol. (2017) 5:250–72. Doi: 10.1016/j.jaip.2016.12.004. Furthermore, inclusion of sheared *E. coli* DNA (a TLR9 agonist) within the nanoparticles, and *E. coli* lipid extract that includes LPS (a TLR 4 agonist) on the nanoparticle surfaces is expected to encourage the recipient's immune system to respond to the nanoparticles, and their contents, as it would to a bacterial agent – i.e., with a Th1-type response. *See,* for example, Srivastava, K. D.; Siefert, A.; Fahmy, T. M.; Caplan, M. J.; Li, X.-M.; Sampson, H. A., Investigation of peanut oral immunotherapy with CpG/peanut nanoparticles in a murine model of peanut allergy. J Allergy Clin Immunol.2016, 138 (2), 536-543. E4. Such a strategy can shift a recipient's immune response to peanut allergens away from a Th2-type response, with its attendant anaphylaxis risk, toward a Th1-type response, which is expected to suppress future Th2-type reactions. The present study analyzes the ability of NP-PN1 to induce activation of TLR4 and TLR9.

[0785] The active agent in NP-PN1 is a PLG nanoparticle that encapsulates an allergenic peanut extract, together with sheared bacterial DNA. The nanoparticle is coated with a bacterial lipid extract containing lipopolysaccharide (LPS). Each of the peanut extract, sheared DNA, and lipid extract and PLG components is sourced from third-party vendors.

[0786] The ability of NP-PN1 to activate specific TLRs (i.e., TLR 2, 4, and 9) was tested. Cell lines with expression of the secreted embryonic alkaline phosphatase reporter gene driven by activation of TLRs were incubated with NP-PN1. Expression of alkaline phosphatase was evaluated by colorimetric measurement.

[0787] To determine whether the E. coli lipid extract coating NP-PN1 and the sheared E. coli DNA embedded within NP-PN1 are capable of activating their target Toll-like receptors (TLR 4 and TLR 9, respectively), a commercial service (InvivoGen, San Diego, CA) performed cell-based bioassays to measure TLR activation. Activation of TLR-mediated signaling leads to stimulation of the NF-κB/AP-1 transcription pathway; activation of this pathway can be measured using a reporter gene construct wherein expression is responsive to NF-κB/AP-1. For the assays employed herein, HEK293 cells are transfected to express a TLR of interest along with a cDNA encoding an NF-κB/AP-1-inducible reporter gene that encodes the secreted embryonic alkaline phosphatase reporter gene. Test materials are added to the medium bathing the cells, and colorimetric measurement of alkaline phosphatase enzymatic activity in the

medium bathing the cells 16-24 hours after addition of the test material provides a measure of the quantity of bioavailable TLR-activating substances associated with the test material. Prior to being tested in the TLR-activation assay, NP-PN1 nanoparticles were subjected to centrifugation on a sucrose step gradient to separate the NPs from any unincorporated E. coli phospholipids and DNA to ensure that that measured TLR activation is produced exclusively by NP-associated TLR ligands. Assays were performed in triplicate. Positive controls were performed by incubating cells expressing a TLR of interest with a standardized preparation that contains an activating ligand of that TLR. For TLR2, the positive control was heat killed Listeria monocytogenes (108 cells/mL); for TLR4, the positive control was E. coli K12 LPS (100 ng/mL); and for TLR9, the positive control was CpG ODN 2006 (10 μg/mL). TLR-expressing cells that were not incubated with NP-PN1 were used to establish the baseline signal in the assay. Cells that did not express exogenous TLR were also incubated with NP-PN1, which demonstrated that NP-PN1 did not produce non-specific, TLR-independent activation of the NF-κB/AP-1 reporter.

NP-PN1 Activates TLR4 and TLR9

[0788] The data presented in **FIG. 1F** depict the fold change in reporter expression induced by exposure of cells to NP-PN1 as compared to that detected with control cells that were not exposed to NP-PN1. For each of the TLRs examined, the data depicted correspond to the fold induction of reporter expression observed at the dilution of NP-PN1 that produced maximal induction (1:100 for TLR2 and TLR4, 1:10 for TLR9). NP-PN1 activated each of TLR2, TLR4, and TLR9 significantly over control cells not exposed to NP-PN1.

Discussion

[0789] These results demonstrate that the sheared bacterial DNA and bacterial lipid extract incorporated within NP-PN1 and adhered to the surface, respectively, are bioavailable and able to activate their target TLR in the context of a cell-based bioassay.

Conclusions

[0790] NP-PN1 delivers a peanut protein payload in association with bioactive bacterial adjuvants that facilitate production of desired immunological responses.

Example 16: Basophil Activation

[0791] The present example illustrates that encapsulation of crude peanut protein in PLG nanoparticles as described in Example reduces activation of basophils from peanut allergic patients. The investigational product is NP-PN1, a therapeutic agent that includes an *Arachis hypogaea* peanut extract and sheared *E. coli* DNA encapsulated in poly(lactic-co-glycolic acid) (PLG) nanoparticles coated with *E. coli* lipid extract containing lipopolysaccharide ("LPS").The particles are formulated in a vehicle containing phosphate buffer and trehalose for oral administration.

[0792] The concept behind NP-PN1 is that encapsulating the peanut extract within PLG nanoparticles "hides" the peanut allergens present in the extract from the recipient's immune system, therefore reducing risk of anaphylaxis by premature engagement of an allergen with IgE-bearing effector cells, e.g. mast cells and basophils relative to, for example, systemic administration of peanut allergens as is done in standard immunotherapy. *See,* for example Gernez Y, Nowak-Wegrzyn A. Immunotherapy for food allergy: are we there yet? J Allergy Clin Immunol. (2017) 5:250–72. Doi: 10.1016/j.jaip.2016.12.004. Furthermore, inclusion of sheared E. coli DNA (a TLR9 agonist) within the nanoparticles, and E; coli lipid extract that includes LPS (a TLR 4 agonist) on the nanoparticle surfaces is expected to encourage the recipient's immune system to respond to the nanoparticles, and their contents, as it would to a bacterial agent – i.e., with a Th1-type response. *See,* for example, Srivastava, K. D.; Siefert, A.; Fahmy, T. M.; Caplan, M. J.; Li, X.-M.; Sampson, H. A., Investigation of peanut oral immunotherapy with CpG/peanut nanoparticles in a murine model of peanut allergy. J Allergy Clin Immunol.2016, 138 (2), 536-543. E4. Such a strategy can shift a recipient's immune response to peanut allergens away from a Th2-type response, with its attendant anaphylaxis risk, toward a Th1-type response, which is expected to suppress future Th2-type reactions.

[0793] Non-clinical studies by Sampson et al. established the principle that encapsulating peanut allergens in a PLG nanoparticle together with bacterial DNA (either CpG or sheared *E. coli* DNA) can achieve significant and long-lasting protection from anaphylaxis compared to unmasked peanut when administered to peanut-sensitive mice (Ref. 2). The codelivery of encapsulated peanut with bacterial DNA (e.g., CpG) was necessary for successful immune modulation dominated by the induction of IFN-γ producing T cells and the reduction of Th2 responses associated with allergy. These studies demonstrated the power of such a nanoparticle delivery system both to prevent anaphylaxis by encapsulating the allergen and to direct and

optimize the induction of immune responses that inhibit allergic symptoms. Although the proof of concept was successfully established, the process by which the peanut extract was encapsulated, and the nanoparticles were fabricated, was not scalable.

[0794] The development of NP-PN1, in addition to providing a scalable manufacturing process for allergen-encapsulating PLG nanoparticles from verified components, involved further pre-clinical exploration of the principle that such encapsulation of food allergens reduces activation of basophils from allergic patients as compared to unencapsulated peanut extract.

Test Substance, Vehicle, and Reagents

[0795] The Active agent in NP-PN1 is a PLG nanoparticle that encapsulates an allergenic peanut extract, together with sheared bacterial DNA. The nanoparticle is coated with a bacterial lipid extract containing lipopolysaccharide (LPS). Each of the peanut extract, sheared DNA, and lipid extract and PLG components is sourced from third-party vendors.

Dosing Amount(s) of Test Substance

[0796] 10-fold serial dilutions of the peanut extract 0.001 to 10 ug/mL, or NP-PN1 (nanoparticle-encapsulated peanut extract) 0.001 to 10ug/mL.

Study Design

[0797] Varying dilution of NP-PN1 or peanut extract were incubated with whole blood from peanut allergic patients. Flow cytometry was used to evaluate activation of basophils in the whole blood.

Basophil Activation

[0798] Whole blood samples were collected into heparinized tubes from patients recruited under the FARE protocol, which was reviewed and approved by IRB of Mount Sinai Medical Center. For basophil activation, 50 µL of whole blood was incubated (in the presence of IL-3 at 1 ng/mL) with 10-fold serial dilutions of the peanut extract (0.001 to 10 µg/mL), NP-PN1 (0.001 to 10 µg/mL), or the empty nanoparticles formulation at concentrations calculated based on the ratio of peanut extract protein to the nanoparticles material in the NP-PN1 formulation, with no stimulant, with polyclonal rabbit anti-human IgE antibody (1 µg/mL, Bethyl Laboratories, Montgomery, TX), or with formyl-methionyl-leucylphenylalanine (fMLP, 1 µM, Sigma-Aldrich, St. Louis, MO). Antibody cocktail of anti-human CD63-BV421, CD123-PE-Cy5, HLADR-PE-

Cy7, CD3-AF488, CD41a-APC, CD14-AF700, CD19-APC-H7 (all by BD Bioscience, San Jose, CA) and CD203c-PE (Beckman Coulter, Indianapolis, IN) for cell staining was also included in the stimulation mixture of final volume of 125µL. Flow cytometry analysis was performed within 96 hours using Cytoflex S flow cytometer (4 lasers, 13 colors, RUO, Beckman Coulter). Flow cytometry data were analyzed using Kaluza Analysis Software, v. 2.1 (Beckman Coulter). The basophil population was gated as CD123+, HLADR-, (CD3, CD19, CD14 –all negative), CD41aall. Basophil activation phenotype was expressed as percentage of CD63+ positive basophil.

NP-PN1 Encapsulation Reduces Activation of Basophils From Peanut Allergic Patients

[0799] As seen in **Fig. 2A**, human basophils from one of the peanut allergic patients showed greater %CD63+ expression at a lower dose of unencapsulated (unmasked) peanut extract than did human basophils exposed to NP-PN1. **FIG. 2B** shows basophil activation with treatment of CD63+ basophils with positive controls, fMLP and anti-IgE. Compilation of the analysis of all 7 individuals plotted in **FIG. 2C** as the concentration of peanut extract needed to elicit a half maximal basophil response, shows that the dose of NP-PN1 required to induce basophil activation is considerably greater (~10 fold) than the dose required by unencapsulated peanut extract.

Discussion

[0800] Basophil activation and histamine release play a crucial role in anaphylactic reactions to allergens. These data illustrate that human basophils were approximately 10 times less responsive to NP-PN1 than to the unencapsulated peanut extract or the empty vehicle control, similar to basophil responsiveness reported in the murine model.

Conclusions

[0801] These data provide ex vivo evidence that NP-PN1 substantially reduces the level of IgE mediated basophil activation, which suggests that encapsulation will increase the safety of oral immunotherapy.

Example 17: In Vitro T cell

[0802] The present example illustrates how encapsulated antigens activate antigen-specific T Cell responses in vitro. The agent assessed is NP-PN1, a therapeutic agent that includes an *Arachis hypogaea* peanut extract and sheared *E. coli* DNA encapsulated in poly(lactic-co-glycolic acid) (PLG) nanoparticles coated with *E. coli* lipid extract containing lipopolysaccharide ("LPS").The particles are formulated in a vehicle containing phosphate buffer and trehalose for oral administration.

[0803] The concept behind NP-PN1 is that encapsulating the peanut extract within PLG nanoparticles "hides" the peanut allergens present in the extract from the recipient's immune system, therefore reducing risk of anaphylaxis relative to, for example, systemic administration of peanut allergens as is done in standard immunotherapy. *See,* for example, Gernez Y, Nowak-Wegrzyn A. Immunotherapy for food allergy: are we there yet? J Allergy Clin Immunol. (2017) 5:250–72. Doi: 10.1016/j.jaip.2016.12.004 Furthermore, inclusion of sheared *E. coli* DNA (a TLR9 agonist) within the nanoparticles, and *E. coli* lipid extract that includes LPS (a TLR 4 agonist) on the nanoparticle surfaces is expected to encourage the recipient's immune system to respond to the nanoparticles, and their contents, as it would to a bacterial agent – i.e., with a Th1-type response. *See,* for example, Srivastava, K. D.; Siefert, A.; Fahmy, T. M.; Caplan, M. J.; Li, X.-M.; Sampson, H. A., Investigation of peanut oral immunotherapy with CpG/peanut nanoparticles in a murine model of peanut allergy. J Allergy Clin Immunol.2016, 138 (2), 536-543. E4. Such a strategy can shift a recipient's immune response to peanut allergens away from a Th2-type response, with its attendant anaphylaxis risk, toward a Th1-type response, which is expected to suppress future Th2-type reactions.

[0804] Non-clinical studies by Sampson et al. established the principle that encapsulating peanut allergens in a PLG nanoparticle together with bacterial DNA (either CpG or sheared *E. coli* DNA) can achieve significant and long-lasting protection from anaphylaxis compared to unmasked peanut when administered to peanut-sensitive mice (Ref. 3). The codelivery of encapsulated peanut with bacterial DNA (e.g., CpG) was necessary for successful immune modulation dominated by the induction of IFN-γ producing T cells and the reduction of Th2 responses associated with allergy. These studies demonstrated the power of such a nanoparticle delivery system both to prevent anaphylaxis by encapsulating the allergen and to direct and optimize the induction of immune responses that inhibit allergic symptoms. Although the proof

of concept was successfully established, the process by which the peanut extract was encapsulated, and the nanoparticles were fabricated, was not scalable.

[0805] The development of NP-PN1, in addition to providing a scalable manufacturing process for allergen-encapsulating PLG nanoparticles from verified components, involved further pre-clinical exploration of the principle that such encapsulation of food allergens can activate Th1-type T-cell responses. Specifically, the study described below demonstrated that encapsulation of a different food allergen (namely, ovalbumin; "OVA")), successfully activates T cell subset responses in vitro. Specifically, PLG nanoparticles analogous to NP-PN1 except for the substitution of OVA for peanut extract, were contacted with OVA-specific T cells from transgenic mice. Proliferation and cytokine secretion were evaluated as markers of T-cell activation.

Test Substance, Vehicle, and Reagents

[0806] The test substance used in this study is a PLG nanoparticle that encapsulates ovalbumin, together with sheared bacterial DNA. The nanoparticle is coated with an *E. coli* lipid extract containing lipopolysaccharide (LPS). Each of the OVA, fragmented DNA, lipid extract and PLG components is sourced from third-party vendors. OVA-containing nanoparticles were compared to control nanoparticles lacking antigen but including sheared *E. coli* DNA encapsulated in PLG nanoparticles coated with E. coli lipid extract and unencapsulated OVA.

Experimental Animals and Care

[0807] C57BL/6 WT mice and congenic C57BL/6-Ly5.1 [B6.SJL-PtprcaPepcb/BoyCrl, "CD45.1"] WT mice were purchased from Charles River Laboratories (Wilmington, MA). OT-1 [C57BL/6 Tg(TcraTcrb)1100Mjb/J] and OT-2 [B6.Cg-Tg(TcraTcrb)425Cbn/J] mice were purchased from Jackson Laboratories (Bar Harbor, ME).

[0808] OT-1 mice contain transgenic inserts for mouse Tcra-V2 and Tcrb-V5 genes. The transgenic T cell receptor is designed to recognize ovalbumin residues 257-264 in the context of H2Kb to study the response of CD8+ T cells to OVA.

[0809] OT-II transgenic mice express the mouse alpha-chain and beta-chain T cell receptor that pairs with the CD4 co-receptor and is specific for chicken ovalbumin 323-339 peptide in the context of I-Ab (CD4 co-receptor interaction with MHC class II). This results in CD4+ T cells

that primarily recognize ovalbumin peptide residues 323-339 when presented by the MHC class II molecule.

[0810] OT-1 and OT-2 mice were crossed onto the CD45.1 mice. Age- and sex-matched (male and female) mice that were between 6 and 16 weeks of age were used in all experiments. All protocols used in this study were approved by the Institutional Animal Care and Use Committee at the Yale University School of Medicine.

Study Design

[0811] In vitro T cell activation by food allergen was tested using an OVA antigen model system. Bone marrow derived dendritic cells were pulsed with unencapsulated OVA or nanoparticles comprising the OVA antigen and sheared *E. coli* DNA encapsulated in encapsulated in PLG nanoparticles coated with E. coli lipid extract LPS as in NP-PN1. These dendritic cells were cultured with OVA specific CD8+ and CD4+ T cells from OTI and OTII mice, respectively. This design allowed for testing T cell activation by the nanoparticle components in an antigen-T cell matched system. Accordingly, whether T cells were activated by the encapsulated antigen or other components could be assessed.

Table 19: Test Conditions for Proliferation and Cytokine Release

Test Substance	**Concentration (μg/ml)**	**Cell Type Assayed**
Control	100	CD4+
Unencapsulated OVA	100	CD4+
OVA Nanoparticle	100	CD4+
Control	10	CD4+
Unencapsulated OVA	10	CD4+
OVA Nanoparticle	10	CD4+
Control	1	CD4+
Unencapsulated OVA	1	CD4+
OVA Nanoparticle	1	CD4+
Control	100	CD8+
Unencapsulated OVA	100	CD8+
OVA Nanoparticle	100	CD8+
Control	10	CD8+
Unencapsulated OVA	10	CD8+
OVA Nanoparticle	10	CD8+
Control	1	CD8+
Unencapsulated OVA	1	CD8+
OVA Nanoparticle	1	CD8+

Evaluation of Antigen-Specific T Cell Responses in vitro

[0812] Bone marrow (BM) cells were prepared (as described in Naik, S. H. et al. Cutting edge: generation of splenic CD8+ and CD8- dendritic cell equivalents in Fms-like tyrosine kinase 3 ligand bone marrow cultures. *J Immunol* 174, 6592-6597, doi:10.4049/jimmunol.174.11.6592 (2005).) by flushing femurs and tibias with RPMI media using a 27-gauge needle followed by RBC lysis and filtering through a sterile 70 mm cell strainer. BM cells were cultured for 10 days at 8x106 cells/4 mL in RPMI 1640 culture medium containing 200 ng/ml murine FLT3L (Peprotech, 250-31L). Bone marrow derived dendritic cells (BMDC) were pulsed with OVA PLG nanoparticles or unencapsulated OVA for 2 hours and then cultured with ovalbumin-specific CD8+ and CD4+ T cells derived from T cell receptor transgenic mice (OT-I and OT-II).

[0813] For analysis of in vitro T cell proliferation, OVA-specific CD4+ or CD8+ T cells were prepared from spleens of OT-2 or OT-1 TCR transgenic mice using the EasySepTM CD4 or CD8 T Cell Isolation Kit (StemCell Technologies, 19852 or 19853) according to the manufacturer's instructions. The CD4+ or CD8+ T cells were labelled with CFSE (Thermofisher, C34554). FLT3L-stimulated DC were pulsed with OVA or different nanoparticle preparations as indicated in **Table 19** for 1 hour. Then DC and T cells were cocultured at 1:10 ratio and OVA specific CD4+ and CD8+ T cell proliferation were analyzed 3 days later. For cytokine stimulation, cocultured cells were harvested at Day 3, and single-cell suspensions were prepared and restimulated with 1× eBioscience Cell Stimulation Cocktail (Thermo Fisher Scientific, 00-4970-93) for 5 hours with 10 mg/ml Brefeldin A (Biolegend, 420601) for the last 4 hours. The cells were surface stained and fixed, and then permeabilized for intracellular cytokine staining with BD Cytofix/Cytoperm™ Fixation/Permeabilization Kit (BD biosciences, 554714).

NP-PN1 Activates Antigen-Specific T Cell Responses in vitro

[0814] To assess the ability of T cells to respond to encapsulated antigen, nanoparticles encapsulating ovalbumin (OVA) were prepared with a PLG matrix encapsulating both OVA proteins as well as sheared *E. coli* DNA as an adjuvant to activate TLR9. On the outside of the particles was a mixture of *E. coli* phospholipids to activate TLR4 on dendritic cells.

[0815] As can be seen in **FIGS. 3A-G**, both CD4+ and CD8+ T cells responded with proliferation to the OVA PLG nanoparticles at all doses tested. These proliferation assays are summarized in **FIG. 3A**. In addition, the OVA PLG nanoparticles induced a more robust T cell response at lower doses of OVA than did unencapsulated OVA alone. The presence of the OVA protein itself was required for the nanoparticle-induced proliferative response; empty

nanoparticles prepared with the adjuvants (encapsulated DNA and *E. coli* lipid extract only) did not drive T cell proliferation (**FIGS. 5A-B**).

[0816] These assays also show that a substantial number of OVA specific CD4+ T cells express IFN-γ and to a lesser extent, IL-4, IL-17, and IL-10, but not IL-4 (**FIGS. 3I-3L**). These cytokine production assays are summarized in **FIG. 3H**. Unencapsulated OVA, by comparison, produces little if any cytokine response. Upon stimulation, CD8+ T cells show IFN-γ/granzyme expression that is considerably higher than unencapsulated OVA (**FIGS. 3M-3N**).

Discussion

[0817] Successful allergen immune therapy (AIT) reduces Th2 response associated with allergy. These data show that OVA PLG nanoparticle-pulsed dendritic cells induce a skewed Th1 response, inducing primarily IFN-γ producing CD4+ T cells. IFN-□ was produced by both CD4+ and CD8+ T cells, which downregulate allergy-associated Th2 immunity.

Conclusions

[0818] In this *in vitro* model, OVA nanoparticles generated the specific type of T cell response necessary for successful AIT.

Example 18: In Vivo T Cells

[0819] The investigational product is NP-PN1, a therapeutic agent that includes an *Arachis hypogaea* peanut extract and sheared *E. coli* DNA encapsulated in poly(lactic-co-glycolic acid) (PLG) nanoparticles coated with *E. coli* lipid extract containing lipopolysaccharide ("LPS").The particles are formulated in a vehicle containing phosphate buffer and trehalose for oral administration.

[0820] The concept behind NP-PN1 is that encapsulating the peanut extract within PLG nanoparticles "hides" the peanut allergens present in the extract from the recipient's immune system, therefore reducing risk of anaphylaxis relative to, for example, systemic administration of peanut allergens as is done in standard immunotherapy. *See,* for example, Gernez Y, Nowak-Wegrzyn A. Immunotherapy for food allergy: are we there yet? J Allergy Clin Immunol. (2017) 5:250–72. Doi: 10.1016/j.jaip.2016.12.004. Furthermore, inclusion of sheared *E. coli* DNA (a TLR9 agonist) within the nanoparticles, and *E. coli* lipid extract that includes LPS (a TLR 4

agonist) on the nanoparticle surfaces is expected to encourage the recipient's immune system to respond to the nanoparticles, and their contents, as it would to a bacterial agent – i.e., with a Th1-type response. *See,* for example, Srivastava, K. D.; Siefert, A.; Fahmy, T. M.; Caplan, M. J.; Li, X.-M.; Sampson, H. A., Investigation of peanut oral immunotherapy with CpG/peanut nanoparticles in a murine model of peanut allergy. J Allergy Clin Immunol.2016, 138 (2), 536-543. E4. Such a strategy can shift a recipient's immune response to peanut allergens away from a Th2-type response, with its attendant anaphylaxis risk, toward a Th1-type response, which is expected to suppress future Th2-type reactions.

[0821] Previous non-clinical studies by Sampson et al. established the principle that encapsulating peanut allergens in a PLG nanoparticle together with bacterial DNA (either CpG or sheared *E. coli* DNA) can achieve significant and long-lasting protection from anaphylaxis compared to unmasked peanut when administered to peanut-sensitive mice (Ref. 2). The codelivery of encapsulated peanut with bacterial DNA (e.g., CpG) was necessary for successful immune modulation dominated by the induction of IFN-γ producing T cells and the reduction of Th2 responses associated with allergy. These studies demonstrated the power of such a nanoparticle delivery system both to prevent anaphylaxis by encapsulating the allergen and to direct and optimize the induction of immune responses that inhibit allergic symptoms. Although the proof of concept was successfully established, the process by which the peanut extract was encapsulated, and the nanoparticles were fabricated, was not scalable.

[0822] The development of NP-PN1, in addition to providing a scalable manufacturing process for allergen-encapsulating PLG nanoparticles from verified components, involved further pre-clinical exploration of the principle that such encapsulation of food allergens can activate Th1-type T-cell responses. The study described below demonstrated that encapsulation of a different food allergen (namely, ovalbumin; "OVA")), successfully activates T cell subset proliferation in vivo. Specifically, PLG nanoparticles analogous to NP-PN1 except for the substitution of OVA for peanut extract, were administered to transgenic mice with OVA-specific T cells.

Test Substance, Vehicle, and Reagents

[0823] The test substance used in this study is a PLG nanoparticle that encapsulates ovalbumin, together with sheared bacterial DNA. The nanoparticle is coated with an *E. coli* lipid extract containing lipopolysaccharide (LPS). Each of the OVA, fragmented DNA, lipid extract and PLG components is sourced from third-party vendors. OVA-containing nanoparticles were compared

to control nanoparticles lacking antigen but including sheared *E. coli* DNA encapsulated in PLG nanoparticles coated with *E. coli* lipid extract and unencapsulated OVA.

Study Design

[0824] The ability of NP-PN1 to activate T Cells in vivo was evaluated using an OVA mouse model. OVA-specific CD4 or CD8 T cells were labelled with CFSE (Thermofisher, C34554). Two million labeled cells were transferred intravenously into OT1 and OT2recipient mice. 18h later, the mice were immunized with nanoparticle preparations via oral gavage. T cell proliferation in mesenteric lymph nodes were analyzed 3 days after immunization.

Evaluation of Antigen-Specific T Cell Responses in vivo

[0825] Isolated OVA-specific CD4+ (OT2) and CD8+ (OT1) T cells (labeled with CFSE) were adoptively transferred to wild type (WT) mice. The next day, the mice were treated orally with OVA nanoparticles, empty nanoparticles, or unencapsulated OVA. 3 days after immunization, T cell proliferative responses to OVA were assessed.

NP-PN1 Activates Antigen-Specific T Cell Responses in vivo

[0826] As seen in **FIGS. 4A-B**, the OVA encapsulated nanoparticles were capable of inducing substantially greater numbers of CD4+ and CD8+ T cells to proliferate compared to nanoparticles without encapsulated OVA.

Discussion

[0827] These data indicate that PLG-encapsulated OVA activates CD4+ and CD8+ T cells in vivo to a greater extent than nanoparticles without OVA.

Conclusions

[0828] The nanoparticle construct described herein yield generated antigen specific T-cell proliferation

Example 19: Encapsulation of RNA payloads.

[0829] The present example describes PLG nanoparticles encapsulating mRNA.

[0830] Those skilled in the art will appreciate the significance of mRNA therapeutics, including as vaccines (e.g., to protect against infectious agents and/or tumors), as providing replacement polypeptides (e.g., wild type polypeptides such as enzymes, etc), etc., and furthermore will

appreciate the need for technologies that can encapsulate and/or delivery such mRNAs (i.e., beyond lipid nanoparticle technologies that include sterol, phospholipid, cationic and PEGylated lipids.

[0831] Lipid nanoparticles for encapsulation of RNA payloads were produced using a modified version of a protocol described herein (e.g., as described at Example 10) Nanoprecipitation of LNPs was done by dropwise addition in a stirred non-solvent matrix. 33mg of PLG was dissolved in 0.9 mL of DMSO. 30m/mL of RNA solution was prepared in an acetate buffer (100µL = 3mg). 30 mg/mL of PEI solution was incorporated in an acetate buffer (10 µL => 1:10 ratio PEI:RNA by mass). 12.5mL of PVA solution (5mg/mL) was used as an anti-solvent. Elements were incorporated in a dropwise manner under stirring at 150 rpm.

[0832] The particles that resulted from the above conditions were 240nm large with a polydispersity index of 0.06. Whereas literature recommends an N:P ratio of 6:1, theoretical calculations suggested an N:P ration of 0.4-1:1. When using DODMA as an RNA stabilizer, the 4:1 ratio of N:P is equivalent to 10:1 DODMA:RNA by mass. Each 1/10x reaction requires 13.2 mg of RNA which is 132 mg with respect to DODMA at 10:1 ratio (10 wt% of the PLG). Resulting properties of nanoparticles encapsulating RNA are shown in **Table 20**.

Table 20: Physical properties of Nanoparticles encapsulating RNA as produced by the present example.

	1/10x				
Sample	Z-ave (nm)	Numb. (nm)	Int. (nm)	Vol. (nm)	PDI
DODMA:RNA (1:10)	379	217	594	2196	0.26
DODMA:RNA (1:5)	270	177	411	1156	0.24
DODMA:RNA (1:2)	273	138	368	746	0.25
DODMA:RNA (1:1)	350	158	459	992	0.25

[0833] The DODMA:RNA samples was encapsulated around 60-90% efficiency based on measurement by Nanodrop, as shown in **Table 21**.

Table 21: Encapsulation of RNA into nanoparticles as measured by Nanodrop.

	First 1/10x	
Sample	RNA Encapsulation (%)	RNA in nanos (wt %)
DODMA:RNA (1:10)	88	10.5
DODMA:RNA (1:5)	63.5	7.6
DODMA:RNA (1:2)	82	9.8
DODMA:RNA (1:1)	80	9.6

[0834] The DODMA:RNA samples were encapsulated around 90-99% efficiency when measured using RiboGreen, as shown in **Table 22**.

Table 22: Encapsulation of RNA into nanoparticles as measured by RiboGreen.

	First 1/10x	
Sample	RNA Encapsulated (%)	RNA in nanos (wt %)
DODMA:RNA (1:10)	99	11.8
DODMA:RNA (1:5)	93	11.2
DODMA:RNA (1:2)	97	11.7
DODMA:RNA (1:1)	96	11.6

[0835] In some embodiments the RNA/acetate solution could be used to make nanoparticles when DODMA is added into the PLG/DMSO solution. In some embodiments the encapsulation efficiency is high (>90% according to RiboGreen). In some embodiments loading capacity is around 12% by mass.

[0836] In some embodiments, 1-propranol may be incorporated to reduce particles in size and/or improve PDI. In some embodiments the nanoparticles may be in water. In some embodiments,

there may not be a stabilizer use. In some embodiments the stabilizer may be a charged stabilizer, such as deoxycholate. In some embodiments the stabilizer may be a steric stabilizer such as hydroxypropyl methylcellulose.

Testing mRNA Encapsulation

[0837] Additional experiments are performed to test the introduction of the encapsulated mRNA into a subject through delivery of the LNP. Testing will be performed with an LNP encapsulating an mRNA encoding GFP. Analysis is performed on the following groups with 6 mice per group:

Group i. Soluble GFP

Group ii. LNP (without mRNA encoding GFP)

Group iii. LNP (with mRNA encoding GFP

Group iv. S.c. GFP (or alum GFP)

[0838] LNPs and/or control protein (e.g., GFP)is administered to each mouse by sublingual/gavage, and either subcutaneous or oral + alum as a control. Two forms of analysis will be performed. First, LNPs encapsulating mRNA encoding LNPs will be labeled (e.g., fluorescently) and tracked by visual methods. For example, cervical and mesenteric lymph nodes plus the spleen will be removed. The presenece of labelled LNPs and/or expressed GFP in the removed tissues will be determined (e.g., by flow cytometry). More specifically, with additional cellular labeling (e.g., CD80; HLA-DR; CD40) flow cytometry can be used to determine if the labelled LNPs associate with dendritic cells present in the draining lymph nodes.

[0839] Detection of an immune response to GFP encoded by the mRNA encapsulated in the LNP is a second form of analysis to be performed. Each mouse will be dosed as described in the grouping above at Day 0 and Day 14. Blood will be collected at day 0 (prior to dose), day 14 (prior to dose), day 28, and day 35. Blood samples will be analyzed for anti-GFP IgG1, IgG2a/c, IgA.

Example 20: A Phase 1/2, Randomized, Double-Blind, Placebo-Controlled, Dose Escalation and Expansion Study of NP-PN1 in Participants with Known Peanut Allergies

[0840] This present Example describes a Phase 1/2 study to evaluate the safety and clinical activity of NP-PN1 in participants with established peanut allergy. The study will be conducted in two parts.

[0841] NP-PN1 is a therapeutic agent comprised of extract of common peanut (*Arachis hypogaea*), encapsulated along with sheared *E. coli* DNA within PLG nanoparticles coated with commercially available *E. coli* lipid extract containing lipopolysaccharide (LPS). The particles are formulated in a vehicle containing phosphate buffer and trehalose for oral administration via buccal mucosa, packaged in single use ampules at 3 different dose strengths (125 µg, 500 µg, and 2000 µg), and stored at -20° C.

[0842] NP-PN1 via buccal immunotherapy (BIT) is being developed for the treatment of peanut allergy.

[0843] The *E. coli* lipid extract on the surface of the nanoparticles acts as a Toll-like Receptor 4 (TLR4) ligand, and the sheared *E. coli* DNA within the nanoparticles acts as a Toll-like Receptor 9 (TLR9) ligand, which together promote a Th1-type immune response, and reduce Th2-type reactions that are associated with allergy. Thus, presenting peanut allergens in the context of these nanoparticles is expected to shift the recipient's immune response to the peanut allergens away from an allergic response and toward tolerance. Furthermore, encapsulation within the nanoparticles effectively hides the peanut allergens from basophils and mast cells already primed for allergic response, significantly reducing the risk associated with traditional allergy immunotherapy, which involves direct exposure of subjects to the allergen(s) to which they are allergic. Preclinical studies have confirmed that incorporation of peanut allergens in PLG nanoparticles coated with *E. coli* lipid extract and incorporating sheared *E. coli* DNA can achieve significant and long-lasting protection from anaphylaxis compared to unmasked peanut. *See,* for example, Srivastava *et al., J. Allergy Clin. Immunol.* 138:536, 2016. The codelivery of encapsulated peanut with bacterial DNA was necessary for successful immune modulation dominated by the induction of IFN-γ producing T cells and the reduction of Th2 responses associated with allergy.

[0844] The present Phase 1/2 Study is performed in two parts. An exemplary schedule of events for Part I and Part I is shown in **Tables 23** and **24**.

[0845] Part 1 is a Phase 1, randomized, double-blind, placebo-controlled, dose escalation study to evaluate the safety and tolerability of NP-PN1 compared to placebo in peanut-allergic

participants. Fifteen peanut-allergic participants, ages 12 to 50 years, will be randomized at a ratio of 2:1 active to placebo, with 10 participants receiving NP-PN1 and 5 receiving placebo. Participants will receive escalating doses of NP-PN1 or placebo at three study visits over the course of 6 -14 weeks to a maximum NP-PN1 dose of 2000 µg. Escalating doses will be administered in the study site on Days 1, 15, and 29, with all maintenance doses taken buccally by the participant at home. Participants will receive Investigational Product (IP; NP-PN1 or matching placebo) at the target NP-PN1 dose of 2000 µg for 2 weeks, followed by an end-of study visit 4 weeks after the last dose of IP.

[0846] A Safety Review Committee (SRC) will convene after completion of Part 1 to review all available safety and clinical activity data from the participants enrolled in the Phase 1 portion of the study. If the benefit/risk profile appears favorable, the study will proceed to Part 2.

[0847] Part 2 is a Phase 2, randomized, double-blind, placebo-controlled study to evaluate the clinical activity and further evaluate the safety and tolerability of NP-PN1 compared to placebo in peanut-allergic participants. Forty five peanut-allergic participants, ages 12-50 years, will be randomized at a ratio of 2:1 active to placebo, with 30 participants receiving NP-PN1 and 15 receiving placebo. A similar dose escalation scheme will be utilized in Part 2 as in Part 1; participants not achieving the target dose of 2000 µg will be discontinued from study. Participants achieving the target dose will receive IP at the target NP-PN1 dose for an additional Week 52, followed by a 4-week Safety Follow-up Period and an EOS visit 4 weeks after the last dose of IP.

[0848] Written informed consent for study participation will be obtained before any study-related procedures or assessments are performed. All potential participants will be screened for participation, and those meeting all eligibility criteria will be offered participation in the study.

[0849] Subject participation in the study will be conducted in the following 3 defined periods:

Screening Period

[0850] The Screening Period begins when the informed consent form (ICF) is signed. During this period, participants will undergo baseline assessments to determine eligibility for study participation in accordance with the Schedule of Event (SOE) tables. The Screening Period duration will be up to 28 days; it will end after all evaluations required to meet eligibility have been completed. If a subject meets all eligibility criteria, they will be offered enrollment into the study.

Treatment Period

[0851] The Treatment Period will begin on Day 1 with randomization and administration of the first dose of Investigational Product (IP; NP-PN1 or placebo) and have a duration of 6-14 weeks of dose escalation for Parts 1 and 2 of the study; Part 2 will include an additional 52 weeks of administration of IP at the target NP-PN1 dose of 2000 µg. During the Treatment Period, daily doses of the IP will be administered by the oral route to the buccal mucosa.

[0852] The initial 6-14 weeks in both parts of the study will be comprised of dose escalation to the target dose of 2000 µg. The anticipated dose escalation scheme is as follows:

[0853] Day 1: Sequential administration of 31.25, 62.5, and 125 µg NP-PN1 or matching placebo every 30 minutes as tolerated, followed by a 2-hour safety observation period prior to discharge from the study site. Any participant who is unable to complete escalation to 125 µg during the Day 1 escalation study visit will be terminated from the study. Participants successfully achieving the 125 µg dose level will self-administer 125 µg of NP-PN1 or matching placebo daily by the oral route to the buccal mucosa at home on Days 2-14.

[0854] Day 15: Sequential administration of 250 and 500 µg NP-PN1 or matching placebo every 30 minutes as tolerated, followed by a 2-hour safety observation period prior to discharge from the study site. Any participant who is unable to complete escalation to 500 µg during the Day 15 escalation study visit will be terminated from the study (see below for exception). Participants successfully achieving the 500 µg dose level will self-administer 500 µg of NP-PN1 or matching placebo daily by the oral route to the buccal mucosa at home on Days 16-28.

[0855] Day 29: Sequential administration of 1000 and 2000 µg NP-PN1 or matching placebo every 30 minutes as tolerated, followed by a 2-hour safety observation period prior to discharge from the study site. Any participant who is unable to complete escalation to 2000 µg during the Day 29 escalation study visit will be terminated from the study (see below for exception). Participants successfully achieving the 2000 µg dose level will self-administer 2000 µg of NP-PN1 or matching placebo daily by the oral route to the buccal mucosa at home on Days 30-43 for Part 1 or for an additional 52 weeks for Part 2.

[0856] If dose escalation is not tolerated during a dose escalation visit, the participant will remain on their prior dose for an additional 2 weeks. After the additional 2 weeks of daily dosing, the participant will be brought back to the study site to undergo a second trial of dose escalation. If dose escalation is tolerated, the participant will self-administer that dose for the

subsequent 2 weeks according to the dose escalation scheme. If dose escalation is not tolerated on that second attempt, the participant will remain on their prior dose for another 2 weeks. After this additional 2 weeks of daily dosing, the participant will be brought back to the study site to undergo a third trial of dose escalation. If dose escalation is tolerated, the participant will self-administer that dose for the subsequent 2 weeks according to the dose escalation scheme. If dose escalation is not tolerated on that third attempt, the participant will be discontinued from the study.

[0857] Instructions on the identification and treatment of systemic reactions, including indications for self-injectable epinephrine, will be reviewed with each participant at each study visit; participants will be provided with epinephrine autoinjectors and instructions for their use as well as a 24-hour emergency telephone number and instructions for accessing emergency medical services as needed. A phone interview will be conducted with each participant on the day after each dose escalation visit to assess for any adverse events in the prior 24 hours since IP administration.

[0858] The last dose of IP in Part 1 is anticipated to be administered after 6-14 weeks of daily dosing; the last dose of IP in Part 2 is anticipated to be administered after 52 weeks of daily maintenance dosing. Participants will return to the study site for follow-up evaluations according to the SOE tables during the Treatment Period. Following the Treatment Period, participants will enter the Safety Follow-up Period.

Safety Follow-up Period

[0859] The Safety Follow-up Period will have a duration of 28 days, culminating with an end-of-study (EOS) visit.

[0860] Safety will be assessed at each study visit, and assessments of clinical activity will be assessed at specific timepoints according to the SOE tables.

[0861] If a participant does not complete all study visits or terminates early from the study, they will be asked to return to the study site for an Early Termination (ET) Visit within 7 days of withdrawal from the study.

[0862] If a participant has a DLT (see below) during the Treatment Period, IP dosing will be discontinued for that participant. Participants experiencing a DLT will be asked to remain in the study and complete the study visits through the EOS visit. These participants will not be replaced.

[0863] If a subject terminates early for a reason other than toxicity during the Treatment Period, the subject may be replaced. IP dose level modifications or dosing administration deviations outside of the protocol-specified windows are not permitted during the Treatment Period.

[0864] The SRC will periodically review all available clinical and laboratory data during the study and will be convened on an ad hoc basis to review all DLT events. Following these reviews, the SRC will make recommendations regarding cohort advancement. The SRC may recommend study continuation (with or without modification), study termination, or termination of target dose escalation.

[0865] A dose limiting toxicity (DLT) is defined as any related AE with an NCI CTCAE 5.0 Grade ≥ 3 which also represents a shift from baseline clinical status of ≥ 1 NCI CTCAE grade.

[0866] The maximum tolerated dose (MTD) is defined as the highest target NP-PN1 dose below the dose at which 3 or more of 10 participants receiving EMP-501 in Part 1 or 10 or more of 30 participants receiving EMP-501 in Part 2 experience a DLT, as confirmed by the SRC.

[0867] The optimal biologic target dose (OBD) is defined as the target NP-PN1 dose demonstrating an appropriate safety and clinical activity profile to advance into late phase development.

[0868] Dose escalation stopping rules will be used to determine whether or not investigation of higher target dose levels will proceed per protocol. If a participant is unable to escalate their target dose on 3 successive study site visits separated by 2 weeks of continued dosing, that participant will be terminated from the study. Participants experiencing a DLT during self-administration will proceed immediately to the closest Emergency Department for care, whereupon the Principal Investigator at the relevant study site will be contacted. The participant will return to the study site, and, at the discretion of the Principal Investigator, will receive the next lower dose. If the next lower dose is tolerated, the participant will remain on that next lower dose for 2 weeks. If well tolerated, then dose escalation may be attempted one additional time at the discretion of the Principal Investigator in consultation with the SRC.

[0869] The study may be stopped at the discretion of the Sponsor based on recommendations of the SRC. In all cases, all necessary measures will be taken to ensure appropriate safety follow-up of all participants in the trial.

[0870] Dosing of NP-PN1 will be permanently discontinued in a participant if any of the following occurs:

Participant withdraws consent.

Participant becomes pregnant.

Participant is unable to comply with the protocol requirements.

Sponsor terminates the study.

Study dosing cessation is mandated by a regulatory authority.

[0871] Enrollment to the study will be paused pending SRC review of all available safety and clinical activity data if:

There is any death of a study participant.

There is a severe anaphylactic reaction .

There is any case of confirmed Eosinophilic Esophagitis (EoE).

[0872] The study will be conducted at up to 10 study sites in the United States.

[0873] Part 1 of the study will enroll 15 participants (10 active/5 placebo) and will last up to 24 weeks; part 2 of the study will enroll 45 participants (30 active/15 placebo), and will last up to 74 weeks.

[0874] Participants will be required to meet all of the following inclusion criteria and none of the exclusion criteria in order to be eligible for study enrollment. In the event that a participant's screening laboratory values are outside the acceptable range, the laboratory test(s) can be repeated once, and if the repeat value(s) is/are within the acceptable range, the participant can be considered eligible for the study. In the event that a participant fails to meet the overall screening criteria, the participant may repeat the overall screening process once, and if the participant then meets all eligibility criteria, the participant will be considered eligible to enter the study.

Inclusion Criteria:

Part 1:

(1) 12-50 years of age (inclusive).

(2) A documented clinical history of peanut allergy, which includes the development of symptoms (e.g., urticaria, flushing, rhinorrhea and sneezing, throat tightness or hoarseness, wheezing, vomiting) verified by a physician.

(3) At least two of the three following requirements:

(a) Positive Skin Prick Test (SPT) (wheal diameter of ≥ 8 mm) to peanut .

(b) An elevated serum peanut specific IgE level ≥ 5 kUA/L (ImmunoCAP®) within 1 year of enrollment (including at Screening).

(c) An Ara-h2 IgE level of > 2 kUA/L (ImmunoCAP®) within 1 year of enrollment (including at Screening).

(3) Otherwise medically healthy and able to participate in the study.

(4) Able to perform spirometry testing in accordance with the American Thoracic Society (AST) guidelines (2005).

(5) All women of child-bearing potential, agree to either abstain from sexual activity or agree to use an adequate method of contraception for the duration of the study and for 30 days after the last dose of investigational product.

(6) Signed and dated written informed consent from the participant and/or parent or guardian.

(7) Signed and dated assent from participant under 18 in accordance with local IRB regulations.

Part 2:

(1) 12 to 50 years of age (inclusive).

(2) A documented clinical history of peanut allergy, which includes the development of symptoms (e.g., urticaria, flushing, rhinorrhea and sneezing, throat tightness or hoarseness, wheezing, vomiting) verified by a physician.

(3) At least two of the three following requirements within 1 year of enrollment (including at screening):

(a) Positive SPT (wheal diameter of ≥ 3 mm) to peanut .

(b) Serum peanut-specific IgE level ≥ 0.7 kUA/L (ImmunoCAP®).

(c) An Ara-h2 IgE level of > 0.7 kUA/L (ImmunoCAP®) within 1 year of enrollment (including at Screening).

(4) Participant must be willing and able to complete a DBPCFC at screening and end of study (24-hours after last dose) and experience dose-limiting symptoms at or before the 300 mg challenge dose of peanut protein during a Double Blind, Placebo Controlled Food Challenge (DBPCFC) conducted in accordance with Practical Issues in Allergology, Joint United States/European Union Initiative (PRACTALL) guidelines.

(5) Otherwise medically healthy and able to participate in the study.

(6) Able to perform spirometry testing in accordance with the American Thoracic Society (AST) guidelines (2005).

(7) All women of child-bearing potential, agree to either abstain from sexual activity or agree to use an adequate method of contraception for the duration of the study and for 30 days after the last dose of investigational product.

(8) Signed and dated written informed consent from the participant and/or parent or guardian.

(9) Signed and dated assent from participant under 18 in accordance with local IRB regulations.

Exclusion Criteria (both Parts):

(1) History of severe anaphylactic event requiring mechanical ventilation or use of intravenous (IV) vasopressor drugs.

(2) Clinically significant screening electrocardiogram (ECG) abnormality or corrected QTcF > 450 msec at Screening.

(3) FEV1 value < 80% predicted at Screening.

(4) Any hospitalization in the past year for asthma, >1 course of oral steroids for asthma, or any emergency room visit in the past 6 months for asthma.

(5) Poorly controlled atopic dermatitis.

(6) Eosinophilic gastrointestinal disease.

(7) Use of oral or IV corticosteroids within 30 days of Screening.

(8) Inability to discontinue antihistamines for skin testing.

(9) Use of omalizumab or other immunomodulatory therapy (not including corticosteroids) or biologic therapy within one year of Screening.

(10) Use of any food allergen-specific or other non-traditional form of allergen immunotherapy within one year of Screening.

(11) Use of immunosuppressive drugs within 30 days of Screening.

(12) Use of ß-blockers (oral).

(13) Evidence of clinically significant neurologic, cardiac, pulmonary, hepatic, rheumatologic, autoimmune, or renal disease by history, physical examination, and/or laboratory studies including urinalysis.

(14) Pregnant or breast-feeding (if female).

(15) Behavioral, cognitive, or psychiatric disease that in the opinion of the Investigator affects the ability of the participant to understand and cooperate with the study protocol.

(16) Known allergy to inactive ingredients of investigational product (active or placebo).

(17) Participation in another interventional clinical trial within 30 days of Screening or within 5 half-lives of the other IP.

(18) Residing at the same address as another participant in this or any peanut immunotherapy study.

Active Pharmaceutical Ingredient

[0875] As noted above, NP-PN1 is a therapeutic agent comprised of the allergenic extract of common peanut (*Arachis hypogaea*), encapsulated along with sheared *E. coli* DNA within PLG nanoparticles coated with commercially available *E. coli* lipid extract containing lipopolysaccharide (LPS). The particles are formulated in a vehicle containing phosphate buffer and trehalose for oral administration via buccal mucosa, packaged in single use ampules at 3 different dose strengths (125 µg, 500 µg, and 2000 µg), and stored at -20° C.

[0876] The placebo to be used in this trial will be the vehicle supplied in identical single use ampules for oral administration via buccal mucosa.*Statistical Analysis*

[0877] Safety analysis will describe the incidence of adverse events (including DLTs) and laboratory abnormalities. Adverse events will be coded according to system organ class and preferred term using the Medical Dictionary for Regulatory Activities (version 26.1, or the current version). Their severity will be graded using the National Cancer Institute Common Terminology Criteria for Adverse Events (NCI CTCAE) version 5.0 or the current version. Adverse events will be organized by system organ class and preferred term. Vital signs, ECG findings, physical examination data, and clinical laboratory parameters will be summarized for each post-baseline visit. Hematologic and chemistry parameters will be categorized as low, normal, or high based on laboratory normal ranges and presented as shifts from baseline.

[0878] Clinical activity analyses will present the number of study participants able to tolerate the 2000 µg dose of NP-PN1 without experiencing dose-limiting toxicity (DLT) as a proportion of the active v placebo dose cohorts. Changes in biomarkers of clinical activity will be presented as mean, median, range, and standard error for continuous data and frequency for categorical data in the active v placebo dose cohorts for the individual Parts of the study and for the pooled Parts of the study.

[0879] Changes from Baseline in wheal diameter on SPT to peanut and challenge dose outcomes during DBPCFC will be presented as mean, median, range, and standard error for continuous data and frequency for categorical data in the active v placebo dose cohorts.

[0880] Further details will be provided in the Statistical Analysis Plan (SAP) prior to database lock.

Table 23: SCHEDULE OF EVENTS FOR PART 1

See Appendix 1

SCHEDULE OF EVENTS FOR PHASE 1 – Footnotes

a. ICF and assent if appropriate must be signed prior to performing any other Screening evaluations.

b. Comprehensive Physical Examination includes the following organ or body system assessments: skin; head, eyes, ears, nose, and throat; lungs; cardiovascular; abdomen (liver, spleen); lymph nodes; and extremities, as well as an abbreviated neurological exam

c. Targeted Physical Examination of any areas of concern from the medical history or noted on the prior physical examination or indicated by participant symptoms or other findings as determined by the Investigator.

d. Vital Signs: Including blood pressure, pulse rate, pulse oximetry, respiration rate, and temperature. On those visits where investigational product is administered, the vital signs will be taken before investigational product administration, 15 minutes after each dose, and 30 minutes, 1 hour, and 2 hours after the last dose for that visit.

e. Spirometry: Forced expiratory volume in 1 second (FEV1), forced vital capacity (FVC), and peak expiratory flow (PEF) at Screening and End of Treatment Visit; PEF only at all subsequent visits.

f. ECGs will be conducted at Screening and only as indicated at subsequent visits.

g. Safety Labs include CBC with differential and serum chemistry; see Table 01 and Section 6.7.1 for list of tests.

h. Serologies / Urinalysis: See Table 01 for list of tests.

i. SPT to include peanut only.

j. Eligibility: Participants will not be enrolled until they complete all Screening evaluations, all eligibility criteria are met based on Screening evaluations, and the eligibility criteria continue to be met following all baseline assessments on Day 1.

k. IP Administration: Participants will be randomized 2:1 to receive buccal administrations of NP-PN1 or placebo daily for 6-10 weeks. See Section 5.5.1 and 5.5.2 for further investigational product administration details.

l. Patient Diary: Participants will be given a diary to record the details of investigational product self-administered at home and any adverse events or concomitant medications taken between visits. The participants will be instructed to return the completed diary at each visit.

m. All Study Day designations assume dose escalation occurs without the need to repeat any dose levels during dose escalation.

Table 24: SCHEDULE OF EVENTS FOR PART 2

See Appendix 2

SCHEDULE OF EVENTS FOR PHASE 2 – Footnotes

a. ICF and assent if appropriate must be signed prior to performing any other Screening evaluations.

b. Comprehensive Physical Examination includes the following organ or body system assessments: skin; head, eyes, ears, nose, and throat; lungs; cardiovascular; abdomen (liver, spleen); lymph nodes; and extremities, as well as an abbreviated neurological exam.

c. Targeted Physical Examination of any areas of concern from the medical history or noted on the prior physical examination or indicated by participant symptoms or other findings as determined by the Investigator.

d. Vital Signs: Including blood pressure, pulse rate, pulse oximetry, respiration rate, and temperature. On those visits where investigational product is administered, the vital signs will be taken before investigational product administration, 15 minutes after each dose, and 30 minutes, 1 hour, and 2 hours after the last dose for that visit.

e. Spirometry: Forced expiratory volume in 1 second (FEV1), forced vital capacity (FVC), and peak expiratory flow (PEF) at Screening and End of Treatment Visit; PEF only at all other visits.

f. ECGs will be conducted at Screening and only as indicated at subsequent visits.

g. Safety Labs include CBC with differential and serum chemistry; see Table 01 and Section 6.7.1 for list of tests.

h. See Serologies / Urinalysis: See Table 01 for list of tests.

i. SPT to include peanut only.

j. Eligibility: Participants will not be enrolled until they complete all Screening evaluations, all eligibility criteria are met based on Screening evaluations, and the eligibility criteria continue to be met following all baseline assessments on Day 1.

k. IP Administration: Participants will be randomized 2:1 to receive buccal administrations of NP-PN1 or placebo daily for 52 weeks following achieving the 2000 µg target dose. See Section 5.5.1 and 5.5.2 for further investigational product administration details.

l. Patient Diary: Participants will be given a diary to record the details of investigational product self-administered at home and any adverse events or concomitant medications taken between visits. The participants will be instructed to return the completed diary at each visit.

m. Maintenance Visits will occur every 4 months with bi-monthly phone calls to monitor adverse events.

n. All Study Day designations assume dose escalation occurs without the need to repeat any dose level during escalation.

Study Objectives and Endpoints

[0881] The Study Objectives and Endpoints are as set forth below:

Part 1: Primary Objective

[0882] To evaluate the safety and tolerability of buccal administrations of NP-PN1.

Part 1: Secondary Objective

[0883] To evaluate the clinical activity of buccal administrations of NP-PN1.

Part 2: Primary Objective

[0884] To evaluate the clinical activity of buccal administrations of NP-PN1.

Part 2: Secondary Objectives

[0885] To further evaluate the clinical activity of buccal administrations of NP-PN1.

[0886] To further evaluate the safety and tolerability of buccal administrations of NP-PN1.

Study Endpoints

Part 1: Primary Endpoint

[0887] Safety and tolerability of NP-PN1 as assessed by:

[0888] Type and frequency of treatment-emergent adverse events (TEAEs).

[0889] Type and frequency of treatment-emergent serious adverse events (TESAEs) .

[0890] Type and frequency of dose limiting toxicities (DLTs) and adverse events of special interest (AESIs; number and severity of systemic reactions, use of epinephrine).

[0891] Type and frequency of changes in clinical laboratory values, physical examinations, and vital signs.

Part 1: Secondary Endpoints

[0892] The number of study participants able to tolerate the 2000 µg dose of NP-PN1 without experiencing dose-limiting toxicity (DLT).

[0893] The change in biomarkers of clinical activity including but not limited to:

- total IgE levels
- peanut-specific and Ara h 1-, 2- and 3-specific IgE levels
- peanut-specific and Ara h 1-, 2- and 3-specific IgG4 levels
- IgE and IgG4 peanut allergenic epitope profiles

[0894] Changes in wheal diameter on the Skin Prick Test (SPT) to peanut.

Part 2: Primary Endpoint

[0895] The proportion of participants treated with NP-PN1 who tolerate at least a 1043 mg cumulative dose of peanut protein without experiencing dose limiting symptoms at the End of Treatment Visit DBPCFC compared to those receiving placebo

Part 2: Secondary Endpoints-Clinical Activity

[0896] The proportion of participants who tolerate at least a 443 mg cumulative dose of peanut protein without experiencing dose limiting symptoms at the End of Treatment Visit DBPCFC

[0897] The proportion of participants who tolerate at least a 2043 mg cumulative dose of peanut protein without experiencing dose limiting symptoms at the End of Treatment Visit DBPCFC

[0898] The proportion of participants who tolerate the 4043 mg cumulative dose of peanut protein without experiencing dose limiting symptoms at the End of Treatment Visit DBPCFC

[0899] The maximum severity of symptoms occurring at each challenge dose of peanut protein during the Exit DBPCFC

[0900] The number of study participants able to tolerate the 2000 μg dose of NP-PN1 without experiencing dose-limiting toxicity (DLT)

[0901] The change in biomarkers of clinical activity including but not limited to:

- total IgE levels
- peanut-specific and Ara h 1-, 2- and 3-specific IgE levels
- peanut-specific and Ara h 1-, 2- and 3-specific IgG4 levels
- IgE and IgG4 peanut allergenic epitope profiles

[0902] Changes in wheal diameter on the Skin Prick Test (SPT) to peanut

Part 2: Secondary Endpoints-Safety

[0903] Safety and tolerability of NP-PN1 as assessed by:

- Type and frequency of treatment-emergent adverse events (TEAEs)
- Type and frequency of treatment-emergent serious adverse events (TESAEs)
- Type and frequency of dose limiting toxicities (DLTs) and adverse events of special interest (AESIs; number and severity of systemic reactions, use of epinephrine)
- Type and frequency of changes in clinical laboratory values, physical examinations, and vital signs

Additional Considerations

[0904] Additional assessment of NP-PN1 may be performed, if desired. For example, dose-escalation effects of administering NP-PN1 to non-allergic individuals may be assessed and/or compared to results of administering NP-PN1 to allergic individuals (e.g., as

described in the Phase 1/2 Randomized, Double-Blind, Placebo-Controlled, Dose Escalation and Expansion Study). Such assessment can be performed using the same or substantially similar treatment scheduled as described in the phase 1 or phase 2 of the trial for participants with known peanut allergies.

References

Abbas, A. K., Lichtman, A. H. & Pillai S. Basic Immunology: Functions and Disorders of the Immune System. (Elsevier, 2016).

Blumchen, K., et al., Oral peanut immunotherapy in children with peanut anaphylaxis. J Allergy Clin Immunol, 2010. 126(1): p. 83-91 e1.

Bousquet, J., Primary and secondary prevention of allergy and asthma by allergen therapeutic vaccines. Clin Allergy Immunol, 2004. 18: p. 105-14.

Boyce JA, Assa'ad A, Burks AW, Jones SM, Sampson HA, Wood RA, Plaut M, Cooper SF, Fenton MJ, Arshad SH, Bahna SL, Beck LA, Byrd-Bredbenner C, Camargo CA Jr, Eichenfield L, Furuta GT, Hanifin JM, Jones C, Kraft M, Levy BD, Lieberman P, Luccioli S, McCall KM, Schneider LC, Simon RA, Simons FE, Teach SJ, Yawn BP, Schwaninger JM. Guidelines for the diagnosis and management of food allergy in the United States: report of the NIAID-sponsored expert panel. J Allergy Clin Immunol. 2010 Dec;126(6 Suppl):S1-58.

Bunyavanich, S. et al. Peanut allergy prevalence among school-age children in a US cohort not selected for any disease. *J. Allergy Clin. Immunol.* **134**, 2011–2013 (2014).

Fleischer DM1, Burks AW et al. Sublingual immunotherapy for peanut allergy: a randomized, double-blind, placebo-controlled multicenter trial. J Allergy Clin Immunol. 2013 Jan;131(1):119-27.e1-7.

Frew, A.J., 25. Immunotherapy of allergic disease. J Allergy Clin Immunol, 2003. 111(2 Suppl): p. S712-9.

Hofmann, A.M., et al., Safety of a peanut oral immunotherapy protocol in children with peanut allergy. J Allergy Clin Immunol, 2009. 124(2): p. 286-91, 291 e1-6.

Hunter Z. et al. A biodegradable nanoparticle platform for the induction of antigen-specific immune tolerance for treatment of autoimmune disease. ACS Nano 2014;8:2148-60.

Jones, S.M., et al., Clinical efficacy and immune regulation with peanut oral immunotherapy. J Allergy Clin Immunol, 2009. 124(2): p. 292-300, 300 e1-97.

Kapsenberg, M.L., et al., The paradigm of type 1 and type 2 antigen-presenting cells. Implications for atopic allergy. Clin Exp Allergy, 1999. 29 Suppl 2: p. 33-6.

Kim, E.H., et al., Sublingual immunotherapy for peanut allergy: clinical and immunologic evidence of desensitization. J Allergy Clin Immunol, 2011. 127(3): p. 640-6 e1.

Kumar G. et al. Drug-loaded PLGA nanoparticles for oral administration: fundamental issues and challenges ahead. Crit Rev Ther Drug Carrier Syst 2012;29:149-82.

Lehrer, S.B., et al., Immunotherapy for food allergies. Past, present, future. Clin Rev Allergy Immunol, 1999. 17(3): p. 361-81.

Narisety, S.D., et al., Open-label maintenance after milk oral immunotherapy for IgE-mediated cow's milk allergy. J Allergy Clin Immunol, 2009. 124(3): p. 610-2.

Nelson, H.S., et al., Treatment of anaphylactic sensitivity to peanuts by immunotherapy with injections of aqueous peanut extract. J Allergy Clin Immunol, 1997. 99(6 Pt 1): p. 744-51

Oppenheimer, J.J., et al., Treatment of peanut allergy with rush immunotherapy. J Allergy Clin Immunol, 1992. 90(2): p. 256-62.

Palforzia (Peanut Allergen Powder-dnfp) [package insert]. Brisbane, CA: Aimmune Theraputics; 2020.

Sah H. et al. Concepts and practices used to develop functional PLGA-based nanoparticulate systems. Int J Nanomedicine 2013;8:747-65.

Secrist, H., R.H. DeKruyff, and D.T. Umetsu, Interleukin 4 production by CD4+ T cells from allergic individuals is modulated by antigen concentration and antigen-presenting cell type. J Exp Med, 1995. 181(3): p. 1081-9.

Sheikh A. et al. Oral immunotherapy for the treatment of peanut allergy: systematic review of six case series studies. PrimCare Respir J 2012;21:41-9.

Silva AL et al. Poly-(lactic-co-glycolic-acid)-based particulate vaccines: particle uptake by dendritic cells is a key parameter for immune activation. Vaccine 2015; 33:847-54.

Skripak, J.M., et al., A randomized, double-blind, placebo-controlled study of milk oral immunotherapy for cow's milk allergy. J Allergy Clin Immunol, 2008. 122(6): p. 1154-60.

Srivastava K. et al Investigation of peanut oral immunotherapy with CpG/peanut nanoparticles in a murine model of peanut allergy. J. Allergy Clin. Immunol. 138, 536-548 (2016).

Varshney, P., et al., A randomized controlled study of peanut oral immunotherapy: clinical desensitization and modulation of the allergic response. J Allergy Clin Immunol, 2011. 127(3): p. 654-60.

Wilson, D.R., M.T. Lima, and S.R. Durham, Sublingual immunotherapy for allergic rhinitis: systematic review and meta-analysis. Allergy, 2005. 60(1): p. 4-12.

Example 21 Double Blind Placebo Controlled Food Challenge

[0905] Prior to performing DBPCFC, the participant must be off antihistamines and other medications that could interfere with the assessment of the DBPCFC for an appropriate length of time (5 half-lives of the antihistamine or other medication in question). Also prior to the DBPCFC, participants will be assessed for an exacerbation of asthma as determined by active wheezing or a PEFR < 80% of predicted. Participants must be free from active wheezing, a flare of atopic disease (*e.g.*, atopic dermatitis), or suspected intercurrent illness prior to DBPCFC. Additionally, participants must be fully recovered, *i.e.*, back to their baseline state of health, from any preceding illness for at least 3 to 7 days, depending on the investigator-determined severity of the illness.

[0906] Oral food challenges will be undertaken under direct medical supervision and with emergency medications and trained staff immediately available. The DBPCFC is performed by feeding gradually increasing amounts of a suspect allergenic food (peanut, presented as defatted

peanut flour) mixed in a vehicle (matrix) food under physician observation (Bock & Atkins, 1990; Burks et al., 2012). For this study, a uniform approach to food challenge, in accordance with the PRACTALL consensus guidelines for DBPCFC, will be used. The DBPCFC dose escalation schedules used in the current study have been modified slightly from the PRACTALL recommendations, and are presented in **Table 25**.

Table 25: Modified PRACTALL DBPCFC Doses Using Peanut Flour with 50% Peanut Protein Content for Screening and Exit DBPCFC Challenge Doses

	Amount of peanut protein [[mg]	**Amount of peanut flour [mg]**	**Cumulative amount of peanut protein [mg] at screening**	**Cumulative amount of peanut protein [mg]at exit**
Screening only	1	2	1	-
Screening & Exit	3	6	4	3
Screening & Exit	10	20	14	13
Screening & Exit	30	60	44	43
Screening & Exit	100	200	144	143
Screening & Exit	300	600	444	443
Exit only	600	1200	-	1043
Exit only	1000	2000	-	2043
Exit only	2000	4000	-	4043

[0907] Vital signs (blood pressure, pulse rate, and pulse oximetry) are to be measured just prior to each challenge dose of the DBPCFC and at 15-to-20-minute intervals post-dose, if the between challenge-dosing interval is prolonged. Assessment for signs and symptoms of allergic reaction is to be performed at the time that vital signs are checked.

[0908] The DBPCFC is halted when the investigator determines that dose-limiting symptoms have occurred. Dose-limiting symptoms, in the setting of the DBPCFC, are any symptoms that, in the investigator's judgement, indicate poor tolerability of the last challenge dose administered, and preclude safe advancement to the next challenge dose.

[0909] Dose-limiting symptoms, typically objective signs/symptoms, indicate a positive reaction and termination of dosing. The criteria for determining if symptoms are dose limiting during DBPCFC are the same as for determining whether a specific dose during up-dosing is tolerated with the exception that even mild symptoms, if they require pharmacological treatment, will be considered dose-limiting. Severe symptoms will always be assessed as dose limiting; and moderate symptoms, with only rare exceptions (requiring a documented explanation), will also

be assessed as dose limiting. Mild symptoms, on the other hand, may or may not be assessed as dose-limiting.

Assessment of DBPCFC Reactions:

[0910] The severity of the reaction will be determined on the basis of the investigator's judgment. The following definitions, developed to be consistent both with the PRACTALL consensus report on DBPCFC, and with the CoFAR grading system for allergic reactions, are provided as a general guide.

Mild Symptoms:

- Skin – limited (few) or localized hives, swelling (*e.g.*, mild lip edema), skin flushing (*e.g.*, few areas of faint erythema) or pruritus (mild, *e.g.*, causing occasional scratching).
- Respiratory – rhinorrhea (*e.g.*, occasional sniffling or sneezing), nasal congestion, occasional cough, throat discomfort.
- Gastrointestinal (GI) – mild abdominal discomfort (including mild nausea), minor vomiting (typically a single episode) and/or a single episode of diarrhea.

Moderate symptoms:

- Skin – systemic hives (*e.g.*, numerous or widespread hives), swelling (*e.g.*, significant lip or face edema), pruritus causing protracted scratching, more than a few areas of erythema or pronounced erythema.
- Respiratory – throat tightness without hoarseness, persistent cough, wheezing without dyspnea.
- GI – persistent moderate abdominal pain/cramping/nausea, more than a single episode of vomiting and/or diarrhea.

Severe symptoms:

- Skin – severe generalized urticaria/angioedema/erythema.
- Respiratory – laryngeal edema, throat tightness with hoarseness, wheezing with dyspnea, stridor.
- GI – severe abdominal pain/cramping/repetitive vomiting and/or diarrhea.
- Neurological – change in mental status.
- Circulatory – clinically significant hypotension.

Example 22: Exemplary Specifications for Certain Provided Nanoparticle Compositions

[0911] In some embodiments, a nanoparticle composition is a white solution. In some embodiments a nanoparticle composition has a total protein concentration of about 1,800 to about 3,500 μg/mL. In some embodiments, a nanoparticle composition has a safety factor greater than about 10. In some embodiments, a nanoparticle composition has a Z-average of 225-450

nm. In some embodiments, a nanoparticle composition has a polydispersity index of about 0.1 to about 0.4.

Example 23: Exemplary Method to Determine if PLG Nanoparticles Can Serve as an Oral Delivery System for GFP RNA.

[0912] The present Example provides an exemplary method of analyzing in vitro loaded nanoparticle uptake by cells (e.g., by antigen presenting cells). The present Example further provides an exemplary in vitro method of evaluating translation of RNA (e.g., RNA loaded into a nanoparticle) taken up by cells with a loaded nanoparticle.

1. In vitro analysis of nanoparticle uptake and RNA translation in an appropriate cell line.
    a. Identification of cell line to be used that most closely resembles the functions of antigen presenting cells. For example, DC2.4 is a particularly useful dendritic cell line.
    b. Groups to be tested with test (polyA) RNA prior to construction of GFP RNA:
        i. NPs (fluorescent dye 1)
        ii. NPs (without fluorescent dye)
    c. Groups to be tested with GFP RNA:
        i. NPs (fluorescent dye 1 and GFP)
        ii. NPs (fluorescent dye 1)
        iii. NPs (without fluorescent dye 1 and GFP)
2. In vitro preparation:
    a. Cell lines grown as specified and exposed to NPs for 2 hours.
    b. Cell prepared for confocal microscopy and/or flow cytometry.

Example 24: Manufacturing of Batches of Exemplary Loaded Protein Nanoparticles

[0913] The present Example provides further methods of manufacturing of exemplary protein loaded nanoparticles (e.g., NP-PN1) as described herein. The present Example further provides rationale for the described methods of manufacturing.

[0914] Table 26 shows a series of steps for encapsulating protein (e.g., peanut protein) into exemplary nanoparticles (e.g., PLG nanoparticles) disclosed herein, and corresponding rationale describing relevant experimental details.

Table 26. Exemplary Encapsulation Process.

Parameter	Target Set Point	Rationale
Antisolvent Composition	10% dimethyl sulfoxide (DMSO), 90% isopropanol (IPA)	IPA alone produced particles that were too small. Incorporation of a small percentage of DMSO causes particles to swell, bringing their size into the target range. An antisolvent comprises ~80% of total reaction volume during the nanoprecipitation reaction.
Addition Rate of Solvent to Anti-Solvent	10 mL/min at start & end, 70 mL during bulk of addition	Before the addition of a solvent, a 90% IPA 10% DMSO mixture (antisolvent) is mixed in a beaker. A solvent – i.e., the rotavapped poly(lactic-co-glycolic) acid (PLGA), peanut protein and DNA in DMSO – is slowly pumped beneath an antisolvent to create two distinct layers. To minimize mixing of these layers, a slow pump rate (10 mL/min) is used; however, once a solvent layer begins to build up, a faster pump flow rate (70 mL/min) can be used to expedite mixing. Towards the end of the addition of the solvent, however, flow rate is reduced to 10 mL/min again to prevent any air bubbles from being generated, disrupting the biphasic layer.
Impeller Positioning in Reaction Vessel	impeller blade should be immersed to 2/3" of its height in a solvent/rotavapped layer	In addition to stirring rate, blades of an overhead mixer are carefully oriented between solvent and antisolvent layers. Careful positioning between these layers promotes mixing at the interface of the solvents, which is crucial for proper nanoparticle formation.
Stirring Ramp Rate & Speed	80 rpm for 2 minfollowed by 150 rpm for 1 min	Formation of nanoparticles occurs as soon as solvent and antisolvent layers begin to mix, stirring is of critical importance. A stir speed of 80 rpm has been identified to produce particles in the correct size range; however, 80 rpm is too slow to properly homogenize the entire mixture. For this reason, a stir speed of 150 rpm is implemented to form a homogeneous mixture at the end of the nanoprecipitation process.
Stirrer Type, Size & Geometry	4.5" (114 mm) 4 blade radial impeller (1" blade height)	When performing a nanoprecipitation step (e.g., when using a 1 L beaker, e.g., when using approximately 500 mL of reaction volume), an overhead mixer with an impeller with a 2" (50.8 mm) blade is used. To scale up to a 10 L beaker with a 217 mm diameter, a

Parameter	Target Set Point	Rationale
		114 mm (4.5") impeller blade is used. Final manufacturing process involves performing a 10 L nanoprecipitation reaction 3 times in the same configuration.
Reaction Vessel Type, Size & Geometry	10 L glass beaker (217 mm diameter)	As determined for stirring, a 10 L beaker size was determined scaling up a 1 L reaction . A beaker was chosen as a desired reaction vessel because it is solvent-compatible, scalable, and fit for purpose, as no temperature control is required for this reaction.

[0915] Table 27 shows a series of homogenization steps of exemplary (e.g., PLG nanoparticles) disclosed herein, and corresponding rationale describing relevant experimental details.

Table 27. Homogenization Summary for Exemplary Protein Encapsulation.

Parameter	Target Set Point	Rationale
Homogenization #1 Buffer Composition	10 mM AmBic + 0.11 mg/mL Deoxycholate	Concentration of deoxycholate was used to ensure the PLG nanoparticles were sufficiently coated to provide necessary stabilization in aqueous solutions used for subsequent processing steps. Ammonium bicarbonate (AmBic) amount was chosen to maximize protein solubility and not impacting particle stability. Basic pH of the ammonium bicarbonate solution also provides protein stability.
Ammonium Bicarbonate Addition Rate and Time	2.7 LPM over 7 minutes	The addition rate and time are set such that ammonium bicarbonate is added at an appropriate speed to avoid any particle aggregation and maximize deoxycholate coating on nanoparticle surface.
Homogenization #1 Speed	10,000 RPM	Homogenization process is started at 5,000 rpm then ramped up to 10,000 rpm over 1 minute. Maximum speed is set so that appropriate mixing is reached , and excessive foaming from potentially unbound surfactant (e.g., deoxycholate) is avoided.
Homogenization #1 Time	6 minutes + 2 minute recirculation	Homogenization time depends on homogenization speed and ammonium bicarbonate rate of addition.
Homogenization #1 Dilution Buffer Composition	10 mM AmBic + 0.08 mg/mL Deoxycholate	PLG nanoparticles are homogenized once per batch, with homogenization being performed three times according to the number of nanoprecipitations. Three batches of homogenized material are combined and diluted to aid in downstream purification by TFF. Concentrations of deoxycholate and AmBic are kept at 0.08 mg/mL and 10 mM, respectively, to match a final desired concentration.

[0916] **Table 28** shows a series of tangential flow filtration (TFF) steps for purification of a batch of an exemplary nanoparticle (e.g., a PLG nanoparticle) from excess free protein (e.g., peanut protein), and corresponding rationale describing relevant experimental details.

Table 28. TFF Summary to Purify Excess Free Protein from Exemplary Protein Loaded Nanoparticles.

Parameter*	Target Set Point	Rationale
TFF #1 and TFF #2 – Buffer pH	8.20 ± 0.05	Optimum pH for peanut protein solubility and nanoparticle dispersion in solution is 8.20. Lower pH was found to reduce protein solubility and produce particles that have a larger size and higher PDI as observed by DLS. All phosphate buffers used for the wash steps of TFF #1 and TFF #2 are titrated to pH 8.2.
TFF #1 and TFF #2 – Buffer Temperature	Chilled (~ 2-10 °C)	To limit the potential for premature peanut protein release and maintain particle stability, buffers used during TFF processing are chilled to ensure that the temperature of the particles is kept reasonably low. Temperature is logged.
TFF #1 and TFF #2 – Filter Membrane Material Type	mPES	Modified polyethersulfone (mPES) has low protein binding, is broadly commercially available, and has demonstrated desirable chemical compatibility and durability when used in various processes.
TFF #1 and TFF #2 – Filter Pore Size	750 kD	A larger the pore size of a filter results in a greater likelihood that a given molecule can travel through pores and out of the system. If the pores are too large, however, probability of fouling and/or loss of PLG nanoparticles increases. For this reason, a 750 kD pore size (10x larger than the largest peanut allergen, e.g., Ara H1) was chosen. A 750kD pore size facilitates removal of free protein and retains PLG nanoparticles.
TFF #1 only – Tubing Type	Platinum-Cured Silicone	Platinum-Cured Silicone has desirable chemical compatibility and low protein binding. This tubing does not have the highest durability of all tubing types; however, because peristaltic pumping is not used for this filtration step, durability is not of utmost importance.
TFF #1 only – Membrane Surface Area	41,000 cm^2	Volume of dilute PLG nanoparticles to surface area of membrane is kept constant at ~5 mL/cm^2 membrane area or a 41,000 cm^2 to process ~192 L of diluted, homogenized material. Using the above described volume resulted in reasonable processing times, limited fouling, and sufficient removal of unwanted residuals.

Parameter*	Target Set Point	Rationale
TFF #1 only – Diafiltration Volumes	20×	To ensure that excess peanut protein is sufficiently removed from the PLG nanoparticles, an excess of diafiltration or "wash" volumes are utilized. Clearing of free protein is thus maximized.
TFF #1 only – Wash Buffer Composition	10 mM Phosphate + 20 mM NaCl + 0.08 mg/mL Deoxycholate	TFF#1 wash buffer with a 10 mM phosphate concentration because ammonium bicarbonate is removed from PLG nanoparticles due to its unpleasant odor and taste. Concentration of 20 mM NaCl allowed for sufficient protein (e.g., peanut protein) solubility to clear the system of the protein, while maintaining nanoparticle stability. Higher salt content would increase the efficiency of the diafiltration but at the expense of increasing the size of the particles. Concentration of 0.08 mg/mL deoxycholate was kept constant in buffers to maintain an adequate concentration of capping agent throughout processing.
TFF #1 only – Shear Rate	19,000 s^{-1}	High shear is maintained during TFF of PLG nanoparticles to prevent membrane fouling and inhibition of free protein passage. At a small scale, a shear rate of 16,000 s^{-1} was utilized; however, when the production process is scaled , a shear rate of 19,000 $s^{-1}$is used.
TFF #2 only – Tubing Type	PharmaPure & Tygon 2375	During TFF #2, a peristaltic pump is used, requiring the tubing through the pump head to be durable due to compression being used to pump fluid through the system. PharmaPure tubing is used through the pump head as it provides exceptional durability and long life when subject to pressure, and provides desirable chemical compatibility and low protein binding. Tygon® 2375 tubing is used in the remaining parts of the assembly where tubing type is less critical.
TFF #2 only – Membrane Surface Area	8,500 cm^2	Volume of dilute PLG nanoparticles to membrane surface area is kept constant at ~1.6 mL/cm^2 membrane area or 8,500 cm^2 to process 13.5 L of PLG nanoparticles from TFF #1, which results in reasonable processing times, limited fouling, and sufficient removal of unwanted residuals that may not have been removed during TFF #1.
TFF #2 only – Diafiltration Volumes	3.6× with 3.4 L in TFF system	At the TFF #2 step, a modest number of washes is performed, because majority of protein (e.g., peanut protein) is removed during TFF #1. A sample is washed slightly above the final target volume.

Parameter*	Target Set Point	Rationale
TFF #2 only – Wash Buffer Composition	10 mM Phosphate + 0.08 mg/mL Deoxycholate	Unlike a TFF #1 Wash Buffer, a TFF #2 Wash Buffer does not contain any NaCl in order to maintain the particle's size in the desired range. A TFF #2 wash buffer contains the same 10 mM phosphate + 0.08 mg/mL deoxycholate concentration as the TFF #1 buffer. The pH of the phosphate buffer keeps loaded protein (e.g., peanut protein) stable, and concentration of deoxycholate helps hold the capping agent concentration constant and maintain particle stability.
TFF #2 only – Shear Rate	16,400 s^{-1}	High shear is maintained during the TFF of PLG nanoparticles to prevent membrane fouling and inhibition of free protein passage.

Example 25: Assessment of Properties of Exemplary Protein Loaded Nanoparticles

[0917] The present Example demonstrates further characterization of exemplary protein loaded nanoparticles (e.g., NP-PN1). The present Example provides methods for and results of evaluating exemplary protein loaded nanoparticles (e.g., NP-PN1) to assess appearance, identify peanut allergen protein (e.g., protein loaded into nanoparticles) by Western blot, DLS & PDI, test in vitro drug release, determine extractable volume, quantify nanoparticle loading (e.g., via SDS-PAGE, BCA, and/or qPCR), determine PLGA content, detect residual solvents, and perform microbial limit tests.

Appearance

[0918] Appearance is a general visual test procedure. Use of a visual method documents that the solution is a clear to off-white solution. Specifically, an exemplary method used to evaluate the solution formulation is outlined below.

1. Perform a visual inspection of an original sample container (i.e., an ampoule with nanoparticles) and assess for any defects including leakages or contamination, scratches or cracks on a container, etc.

a. If any of the original containers have defects or are not otherwise intact, a sample inside a container should be replaced by a suitable sample (e.g., a sample from a container without defects).

2. If original sample container is not suitable for performing visual analysis of particles (e.g., nanoparticles) due to its opacity, transfer suitable samples into a glass vial under aseptic conditions using a syringe with a needle.

a. Inspect the destination glass vial for any defects prior to transfer. Only vials with no defects should be used.

3. Prepare a view box by allowing the light to warm up until stable for at least 10 minutes.

4. Set the Lux option to f, check light intensities and adjust an angle of a light source for 2000 to 3750 Lux range.

5. Ensure sample particles (e.g., nanoparticles) are well suspended by inverting a vial, ensuring no bubbles have been introduced.

6. View each sample against a black background of a view box for 5 seconds. Swirl a vial again and view a sample against a white background for 5 seconds.

7. After viewing in front of both backgrounds, record appearance of a solution noting a presence of any visible particles (e.g., clumping nanoparticles or non-nanoparticles), suspended solids or any other visible particulate matter.

a. If no visible particles are present, record result as "no particles".

b. If visible particles are present, record result as "particles present." Describe visible particles with respect to their color, size, shape, buoyancy, and quantity, when feasible. If any particle is observed, a second operator must example the same sample within an hour of the first analyst's observation. A supervisor may also participate in confirmation of presence of visible particles in the sample.

Western Blot

[0919] An exemplary Western blotting method is outlined below.

Reagent Preparation

1. Buffer Preparation

a. Prepare a 1x Tris-Buffered Saline with TWEEN 20® (TBST), a Blocking Buffer (1x TBST, 5% blocker), a Running Buffer (1x Tris-Glycine-SDS2 buffer) and a 0.5 M (dithiothreitol) DTT stock. Expiry of buffer solutions should be noted.

2. Standards, Control and Sample Preparation

a. Buffer Blanks

i. Prepare both Reduced (75 µL 4x Laemmli sample buffer + 30 µL 0.5 M DTT + 195 µL water) and a non-reduced buffer blank (25 µL 4x Laemmli sample buffer + 75 µL water). Both should be prepared fresh for same-day use.

b. Exemplary peanut proteins (e.g., AraH1 and AraH2)

i. Dilute AraH1 and AraH2 stocks (typically at 1.0 and 1.1 mg/mL, respectively) to 0.01 mg/mL with water and pipette to mix as shown in **Tables 29** and **30**.

Table 29. Exemplary AraH1 Gel dilution

AraH1 Gel								
Label	**Stock Concentration**	**Stock Volume (µL)**	**Diluent**	**0.5M DTT (µL)**	**4x Laemmli Sample Buffer (µL)**	**Final Volume (µL)**	**Concentra-tion (ng/µL)**	**Total Amount Loaded on Gel (ng)**
AraH1 (50 ng)	0.01 mg/mL AraH1 (from Label prepared above)	30.0	9.0 µL Milli-Q Water	6.0	15.0	60.0	5.0	50
AraH1 (10 ng)	0.005 mg/mL AraH1 (from Label prepared above)	12.0	48.0 µL reduced buffer blank	0	0	60.0	1.0	10

MW Std	Invitrogen MagicMark XP Western Protein Standard	30.0	0	0	0	30.0	N/A	N/A
*PLGA Nanos AraH1	2000 µg/mL PLGA Nanos (diluted 1:10 in water before use)	2.5	36.5 µL Milli-Q Water	6.0	15.0	60.0	8.3	83.3
	500 µg/mL PLGA Nanos	1.0	38.0 µL Milli-Q Water	6.0	15.0	60.0	8.3	83.3
	125 µg/mL PLGA Nanos	4.0	35.0 µL Milli-Q Water	6.0	15.0	60.0	8.3	83.3

Table 30. Exemplary AraH2 Gel dilution

AraH2 Gel								
Label	**Stock Concentration**	**Stock Volume (µL)**	**Diluent**	**0.5M DTT (µL)**	**4x Laemmli Sample Buffer (µL)**	**Final Volume (µL)**	**Concentration (ng/µL)**	**Total Amount Loaded on Gel (ng)**
AraH2 (50 ng)	0.01 mg/mL AraH2 (from Label prepared above)	30.0	9 µL Milli-Q Water	6.0	15.0	60.0	5.0	50
AraH2 (15 ng)	0.005 mg/mL AraH2 (from Label prepared above)	18.0	42.0 µL uL reduced buffer blank	0	0	60.0	1.5	15
MW Std (1:10)	Invitrogen MagicMark XP Western Protein Standard	3.0	27.0 µL non reduced buffer blank	0	0	30.0	N/A	N/A

PLGA Nanos AraH2	2000 µg/mL PLGA Nanos (diluted 1:10 in water before use)	6.0	33.0 µL Milli-Q Water	6.0	15.0	60.0	200.0	2000.0
	500 µg/mL PLGA Nanos	24.0	15.0 µL Milli-Q Water	6.0	15.0	60.0	200.0	2000.0
	125 µg/mL PLGA Nanos	39.0	0	6.0	15.0	60.0	121.9	1218.8

ii. Heat each of the final preparations in a 70 °C heat block for 10 minutes. MW standards do not need to be heated.

iii. Cool heated final preparations to room temperature for 5 minutes before centrifuging to collect the liquid phase of preparation at the bottom of centrifuge tubes.

3. Gel Electrophoresis & Trans-Blot Turbo Transfer

a. Assemble a gel tank and install prepared gels. Place the tank on ice. Fill all chambers with an appropriate amount of running buffer.

b. Load 10 µL of each preparation in wells according to **Table 31**. Do not load any samples into wells 1, 2, 14 or 15.

Table 31. Exemplary Gel 1 and Gel 2 Loading Schemes

Gel 1 – Anti-AraH1 Ab Stain			**Gel 2 – Anti-AraH2 Ab Stain**		
Well	**Sample**	**Amount (ng)**	**Well**	**Sample**	**Amount (ng)**
3	MW Std	-	3	1:10 MW Std	-
4	AraH1	50.0	4	AraH2	50.0
5	AraH1	10.0	5	AraH2	10.0

6	Empty	-	6	Empty	-
7	PLGA Nanos Sample 1	83.3	7	PLGA Nanos Sample 1	83.3
8	PLGA Nanos Sample 2 (if necessary)	83.3	8	PLGA Nanos Sample 2 (if necessary)	83.3
9	PLGA Nanos Sample 3 (if necessary)	83.3	9	PLGA Nanos Sample 3 (if necessary)	83.3
10	PLGA Nanos Sample 4 (if necessary)	83.3	10	PLGA Nanos Sample 4 (if necessary)	83.3
11	PLGA Nanos Sample 5 (if necessary)	83.3	11	PLGA Nanos Sample 5 (if necessary)	83.3
12	Empty	-	12	Empty	-
13	MW Std	-	13	1:10 MW Std	-

c. Perform gel electrophoresis at 300 V for 14 minutes on ice. Upon completion, remove the gel from the cassette.

d. Perform a gel transfer using a Trans-Blot® Turbo™ (#1704150, BioRad) Transfer System following an appropriate manual, and using a Mini Format Transfer Pack (BioRad). Cut a notch in one of the membrane corners to ensure proper orientation is maintained during the remainder of the processes.

4. Membrane Blocking

a. Remove any adhered pieces of gel from a membrane, onto which protein has been transferred, and place the membrane into an appropriate container for Western blotting. Ensure volumes of solutions are adjusted to fully cover the membrane if alternate e.g., container types are used. Exemplary buffer volumes described herein are particularly useful for a container, which can fit a Mini Format Transfer Pack (BioRad) membrane.

b. Add 30 mL of TBST and shake for 10-30 minutes on an orbital shaker – 50 rpm is recommended.

c. Add 30 mL of Blocking Buffer and incubate either 30-60 min shaking at room temperature or overnight (for 6-24 hours) at 2-8 °C with no shaking.

5. Antibody Staining

a. Make 150 mL of Blocking Buffer on day 2. (i.e., 150 mL of TBST + 7.5 g of blocker).

b. Make primary antibody stains.

i. AraH1 primary antibody stain (1:5000): combine 30 mL of blocking buffer with 6 µL of anti-AraH1 antibody and mix well.

ii. AraH1 primary antibody stain (1:2000): combine 30 mL of blocking buffer with 15 µL of anti-AraH2 antibody and mix well.

c. Add 30 mL of primary antibody stains to containers with membranes with transferred AraH1 and AraH2 proteins. Incubate membranes with antibody solutions for 45-50 minutes and then decant.

i. During this incubation, make a secondary antibody stain – both membranes use the same secondary antibody stain (1:20,000 dilution).

ii. For each, combine 30 mL of Blocking Buffer with 3 µL of secondary antibody and mix well.

d. Rinse all membranes with 1x TBST once quickly, then wash 4 times by shaking membranes with 30 mL of 1x TBST on an orbital shaker for 5-10 minutes.

e. Add 30 mL of secondary antibody stain to each membrane. Incubate for 45-50 minutes and then decant secondary stain.

f. Rinse with 1x TBST once quickly, then wash 3 times for 5 minutes, then another time for at least 5 minutes (each rinse should happen with 30 mL of 1x TBST)

i. The membrane is then left in 1x TBST until each membrane is developed and imaged.

6. Signal Development and Imaging

a. Membranes are developed and imaged one at a time.

b. Combine 1 mL of solution A and 1 mL of solution B of the ECL Prime Western Blotting Reagents (Cytiva) in a container to make a developing solution. Mix well and cover container with the mixed solution with aluminum foil to protect from light until ready for use – the solution is made fresh and stored for < 20 minutes before use.

c. Remove each membrane from 1x TBST, drain and lightly tap on a Kimwipe.

d. Place each membrane face up in a plastic staining box and drip 2 prepared developing solution evenly over the membrane surface. Cover the staining box with aluminum foil and incubate for 5 minutes at room temperature.

e. After incubation, remove, drain, and wipe the excess solution on a Kimwipe.

f. Image the gel on an Amersham™ gel imager.

i. Place a membrane face-up on the black tray. Image in chemiluminescence mode, exposing the membranes, as follows:

1. AraH1 Membrane: 7.7 seconds

2. AraH2 Membrane: 18.2 seconds – *A double band for the AraH2 protein should be seen. Adjust the exposure time so that the doublet bands do not merge.*

ii. Exposure times can be adjusted up to 30 seconds if necessary.

7. System Suitability Acceptance Criteria

a. Molecular weight standards on each membrane should be visible.

b. No major smears that obstruct individual bands should be observed in any gel lanes.

c. On an AraH1 membrane, a 50 ng and a 10 ng AraH1 band should be visible between 60-80 kDa.

d. On an AraH2 membrane, a 50 ng and a 15 ng AraH2 band should be visible between 20-30 kDa. In the 15 ng AraH2 band, the band should appear as a doublet.

8. Test Sample Acceptance Criteria

a. On an AraH1 membrane, a 83.3 ng sample should show a band between 60-80 kDa.

b. On an AraH2 membrane, a 2000 ng sample should show a doublet band around 20 ± 5 kDa.

9. Reportable Value

a. Report if test samples Pass or Fail acceptance criteria.

BCA Assay

1. **Reagent preparation**

a. Phosphate Buffer Preparation

i. Prepare potassium phosphate buffer by weighing out 348 mg of potassium diphosphate dibasic and 49 mg of potassium phosphate monobasic, and dilute in water.

ii. Adjust pH of a prepared buffer to 8.20 ± 0.05 using 1 N sodium hydroxide (NaOH).

iii. Dilute pH-adjusted buffer with water to a final volume of 200 mL and store at 2-8 °C until use.

2. **Standards, Control, Sample Preparation and Assay Procedure**

Each experiment is a two-day process that is completed in two consecutive days. Each exemplary method may be applied to three different product concentrations as shown below.

a. **An exemplary procedure for a 2000 µg/mL product (e.g., nanoparticles as described herein)**

i. Determine density of water by weighing 1 mL of water filtered via a Milli-Q® filtration systemin a 1.5 mL tube, which had been tared prior to weighing water.

Day 1 Procedure

ii. Prepare samples by removing 6 aliquots of product ampoules from a freezer, allowing samples to equilibrate at room temperature for at least 30 minutes. Once thawed, combine all 6 ampoules into a single 15 mL conical tube and mix thoroughly.

iii. Remove previously prepared phosphate buffer from a refrigerator and allow to equilibrate for at least 30 minutes at room temperature.

iv. Prepare samples to evaluate free protein concentration in nanoparticle samples by adding 700 µL of a sample transferred from product ampoules and 700 µL of phosphate buffer to 3 different 1.5 mL tubes. Invert to mix. For all product concentrations, these samples are described herein as "free protein samples", or "FP".

v. Centrifuge all tubes at 21,100 RCF for 20 minutes at room temperature.

vi. While samples are centrifuging, prime 0.1 µm syringe filters with Milli-Q®-filtered water using a 10 mL syringe. One filter should be used for each final sample. Once rinsed, aspirate air, then reattach the filter and push air through the filter.

vii. Once centrifugation is complete, pipette out ~900 µL of supernatant from a tube without disturbing a pellet at the bottom of a tube. Use a water-primed

filter and a 1 mL syringe to filter transferred supernatant into a clean microcentrifuge tube before storing at 2-8 °C.

1. Note that each sample is to be analyzed on the following day.

viii. Prepare samples to evaluate total protein concentration in nanoparticle samples by adding 1 mL of a sample from an ampoule from step a.ii. to 8 mL of phosphate buffer, followed by adding 1 mL of 1 N NaOH into a 15 mL conical tube. Repeat this preparation 3 times to have triplicate measurements. Invert to mix before rotating overnight to degrade nanoparticles at ambient temperature for 16-22 hours. For all product concentrations, these samples are described herein as "total protein samples", or "TP".

Day 2 Procedure

ix. Remove free protein samples prepared on Day 1, lyophilized peanut protein, phosphate buffer and an E. coli total lipid extract from a freezer, and equilibrate all reagents for at least 30 minutes.

x. Prepare a 5 mg/mL peanut protein stock solution by adding 70 to 90 mg of peanut protein into a conical tube, accounting for purity of the peanut solid. Add an appropriate volume of phosphate buffer to bring concentration of a peanut protein stock solution to 5 mg/mL. Do not shake or vortex – sonicate a prepared solution for 30 to 60 seconds.

xi. Prepare an E. coli lipid extract solution diluting a thawed E. coli lipid extract with phosphate buffer a concentration to 1 mg/mL based on weighed mass of the lipid extract. Sonicate a prepared lipid solution for at least 30 minutes until solution is homogenous.

xii. Prepare 4 mL of trehalose solution at 150 mg/mL by weighing 600 mg of trehalose and 4 mL of phosphate buffer in a tared tube.

xiii. Prepare a peanut standard by adding 16 µL of a prepared stock peanut solution to 984 µL of phosphate buffer to achieve aa final concentration of 80 µg/mL. Invert a tube with the prepared sample to mix.

xiv. Prepare a total protein background sample by adding 2891 µL of phosphate buffer, 34 µL of a prepared trehalose solution and 75 µL of a prepared lipid extract solution into a tube. Invert a tube with the prepared sample to mix.

xv. Prepare free protein background samples by mixing 1445 µL of phosphate buffer, 1000 µL a prepared trehalose solution and 555 µL of a prepared lipid extract solution into a tube. Invert a tube with the prepared sample to mix.

xvi. Dilute overnight digested samples from Day 1 by adding 750 µL of phosphate buffer and 150 µL of a total protein sample aliquot into a 1.5 mL tube. Repeat this dilution for all 3 total protein sample aliquots. Do not dilute free protein samples.

b. An exemplary procedure for a 500 µg/mL product

i. Determine density of water by weighing 1 mL of water filtered via a Milli-Q® filtration systemin a 1.5 mL tube, which had been tared prior to weighing water.

Day 1 Procedure

ii. Prepare samples by removing 7 aliquots of product ampoules from a freezer, allowing samples to equilibrate at room temperature for at least 30 minutes. Once thawed, combine all 7 ampoules into a single 15 mL conical tube and mix thoroughly.

iii. Remove previously prepared phosphate buffer from a refrigerator and allow to equilibrate for at least 30 minutes at room temperature.

iv. Prepare free protein samples by adding 1100 µL of a sample transferred from product ampoules to 3 different 1.5 mL tubes. Invert to mix.

v. Centrifuge all tubes at 21,100 RCF for 20 minutes at room temperature.

vi. While samples are centrifuging, prime 0.1 µm syringe filters with Milli-Q®-filtered water using a 10 mL syringe. One filter should be used for each final sample. Once rinsed, aspirate air, then reattach the filter and push air through the filter.

vii. Once centrifugation is complete, pipette out ~900 µL of supernatant from a tube without disturbing a pellet at the bottom of a tube. Use a water-primed filter and a 1 mL syringe to filter transferred supernatant into a clean microcentrifuge tube before storing at 2-8 °C.

    1. Note that the each sample is to be analyzed the following day.

viii. Prepare the total protein samples by adding 1 mL of a sample from an ampoule from step b.ii. and 111 µL of 1 N NaOH into a 15 mL conical tube. Repeat this preparation 3 times such that there are triplicate preparations. Invert to mix before rotating overnight to degrade nanoparticles at ambient temperature for 16-22 hours.

Day 2 Procedure

ix. Remove free protein samples prepared on Day 1, lyophilized peanut protein, phosphate buffer and an E. coli total lipid extract from a freezer, and equilibrate all reagents for at least 30 minutes.

x. Prepare a 5 mg/mL peanut protein stock solution by adding 70 to 90 mg of peanut protein into a conical tube, accounting for purity of the peanut solid. Add an appropriate volume of phosphate buffer to bring concentration of a peanut protein stock solution to 5 mg/mL. Do not shake or vortex – sonicate a prepared solution for 30 to 60 seconds.

xi. Prepare an E. coli lipid extract solution diluting a thawed E. coli lipid extract with phosphate buffer a concentration to 1 mg/mL based on weighed mass

of the lipid extract. Sonicate a prepared lipid solution for at least 30 minutes until solution is homogenous.

xii. Prepare 4 mL of trehalose solution at 150 mg/mL by weighing 600 mg of trehalose and 4 mL of phosphate buffer in a tared tube.

xiii. Prepare a peanut standard by adding 16 µL of a prepared stock peanut solution to 984 µL of phosphate buffer to achieve a a final concentration of 80 µg/mL. Invert a tube with the prepared sample to mix.

xiv. Prepare total protein background samples by adding 2814 µL of phosphate buffer, 120 µL of a prepared trehalose solution and 66 µL of a prepared lipid extract solution into a tube. Invert a tube with the prepared sample to mix.

xv. Prepare free protein background samples by mixing 721 µL of phosphate buffer, 2000 µL of a prepared trehalose solution and 279 µL of a prepared lipid extract solution into a tube. Invert a tube with the prepared sample to mix.

xvi. Dilute overnight digested samples from Day 1 by adding 700 µL of phosphate buffer and 50 µL of a total protein sample aliquot into a 1.5 mL tube. Repeat this dilution for all 3 total protein sample aliquots. Do not dilute free protein samples.

c. An exemplary procedure for a 125 µg/mL product

i. Perform all steps as outlined for the 500 µg/mL product above (e.g., as described in steps b.i-b.xvi.), noting the differences in preparation of background samples, outlined below.

ii. Prepare total protein background samples by adding 2589 µL of phosphate buffer, 360 µL of a prepared trehalose solution and 51 µL of a prepared lipid extract solution into a tube before inverting to mix.

iii. Prepare free protein background samples by mixing 931 µL of phosphate buffer, 2000 µL of a prepared trehalose solution and 69 µL of a prepared lipid extract solution into a tube before inverting to mix.

iv. Dilute overnight digested samples from Day 1 by adding 800 µL of phosphate buffer and 200 µL of a total protein sample aliquot into a 1.5 mL tube. Repeat this dilution for all 3 total protein sample aliquots. Do not dilute free protein samples.

d. Procedure for Standard Curve, Sample and Background Preparations (this applies to all 3 concentrations of products)

i. Load assay plates as shown in **Table 32**. Using a 300 µL multichannel pipette, add 150 µL of phosphate buffer into each well except for wells A1:A5, A8:A12, E1:E5, E8:E12.

ii. Pipet 150 µL of 80 µg/mL peanut standard to wells A6:A7. Dilution with phosphate buffer in each well results in a 40 µg/mL peanut concentration. Pipet up and down to mix thoroughly.

iii. With a multichannel pipet, transfer 150 µL of solution in wells A6:A7 to wells directly below (B6:B7). Pipet up and down to mix. Repeat this procedure as a serial dilution until wells G6:G7. Do not transfer samples to row H, and discard 150 µL from wells G6:G7.

iv. Pipet 300 µL of total protein samples, free protein samples, total protein background samples and free protein background samples as shown in **Table 32**.

v. Using a multichannel pipette, transfer 150 µL of solution in wells A1:A5 and A8:A12 into wells directly below (e.g., B1:B5 and B8:B12, respectively). Pipette to mix, then repeat this procedure for respective wells in rows B to C, and rows C to D, to dilute samples serially. Remove 150 µL of sample from row D and discard. Repeat this process for wells E1:E5 and E8:E12 (e.g., transfer 150 µL of solution in wells E1:E5 and E8:E12 2 into

wells F1:F5 and F8:F12, respectively, then serially dilute samples into rows G and H. Discard 150 µL from row H.

e. Bicinchoninic Acid (BCA) Procedure

i. Prepare a BCA reagent by transferring 7.5 mL of Reagent A, 7.2 mL of Reagent B and 0.3 mL of Reagent C into a 15 mL conical tube. Invert the tube to ensure that the solution is thoroughly mixed.

ii. Add 150 µL of a prepared BCA reagent into each well. Pipette up and down 5-8 times to mix. Use fresh pipette tips for each row.

iii. Immediately after adding a BCA reagent to the last row, place a lid on the assay plate and incubate the plate for 2 hours at 37 °C.

iv. Turn on a microplate reader 10 minutes before use.

v. After completing assay plate incubation, insert the plate into the microplate reader, and measure light absorbance at 562 nm.

Table 32. Exemplary BCA assay plate format.

	1	2	3	4	5	6	7	8	9	10	11	12
A	FFC Prep1	FFC Prep2	FFC Prep3	TP BG		40 µg/mL Peanut		FFC Prep1	FFC Prep2	FFC Prep3	TP BG	
B						20 µg/mL Peanut						
C						10 µg/mL Peanut						
D						5 µg/mL Peanut						
E	FFC Prep1	FFC Prep2	FFC Prep3	TP BG		2.5 µg/mL Peanut		FFC Prep1	FFC Prep2	FFC Prep3	TP BG	
F						1.25 µg/mL Peanut						
G						0.625 µg/mL Peanut						
H						Phosphate Buffer						

[0920] **Table 32** shows an exemplary assay plate layout for performing a BCA assay to evaluate free protein concentration in exemplary nanoparticle samples described herein. Each sample is evaluated in triplicate (e.g., Prep1, Prep2, and Prep3). Samples loaded onto an

assay plate are designated as follows: total protein content samples (TPC), free protein samples (FPC), total protein background (TP BG), and free protein background (FP BG). Concentrations of exemplary protein standard samples (e.g., peanut protein, or "Peanut") used in a BCA assay are shown, as loaded onto an assay plate, in columns 6 and 7 (e.g., 40 µg/mL, 20 µg/mL, 10 µg/mL, 5 µg/mL, 2.5 µg/mL, 1.25 µg/mL, 0.625 µg/mL, and 0 µg/mL (e.g., Phosphate Buffer)).

f. Data Analysis

i. Follow appropriate procedures for operation of a microplate reader.

ii. Standard Curve Generation:

1. Average phosphate buffer in wells H6:H7, which are used as plate background values, and subtract averaged values from values in remaining assay plate wells used to generate a background subtracted standard curve for sample concentrations.

2. Generate a standard curve using known peanut concentrations from columns 6 and 7 as independent variables, and absorbance values corresponding to peanut concentration in rows 6 and 7 values as a dependent variable.

3. Calculate a Y-intercept and an R-squared value.

iii. Total Protein Sample:

1. Average total protein background values obtained for wells A4:A5 and E11:E12

2. Subtract the averaged values from values obtained for wells A1:A3 and E8:E10.

3. Apply the same calculation pattern wells B1:B3, C1:C3, D1:D3, F8:F10, G8:G10, and H8:H10

iv. Free Protein Sample:

1. Average free protein background values obtained for wells A11:A12 and E4:E5

2. Subtract the averaged values from values obtained for wells A8:A10 and E1:E3.

3. Apply the same calculation pattern to wells B8:10, C8:C10, D8:D10, F1:F3, G1:G3, and H1:H3

v. Sample Concentration Calculation:

1. Determine a measured peanut protein concentration of each sample by fitting each background subtracted sample to a background subtracted standard curve generated in step f.ii.

2. Back-calculate an undiluted concentration (e.g., concentration of a peanut protein after transfer from ampoules) of samples by multiplying a measured peanut protein concentration by serial dilution factors (e.g., 2X, 4X, 8X, 16X, 32X, 64X, 128X, et cetera).

vi. Safety Factor Calculation:

1. Safety factor is calculated according to Equation 2 below.

a. ***Safety Factor = Measured Free Protein Concentration / Measured Total Protein Concentration.*** (2)

g. System Suitability Acceptance Criteria

i. An R-squared value calculated for a generated standard curve is ≥ 0.98.

ii. A coefficient of variation (%CV) of the absorbance (OD) values for each standard is $\leq 20\%$.

h. Test Sample Acceptance Criteria

i. Results of each BCA assay must pass all acceptance criteria for a particular lot to be considered passing. Exemplary acceptance criteria are listed in **Table 33**.

Table 33. Exemplary Sample Acceptance Criteria

Parameter	Acceptance Criteria
Total Protein Concentration	• %CV of the OD values is ≤ 20%. • Measured concentrations are within a range of 70-130% of proposed concentrations for product at 2000, 500 or 125 µg/mL. • Results are within the range of the generated standard curve (e.g., between absorbance values for the lowest and the highest measured standard peanut protein concentrations).
Free Protein Concentration	• %CV of the absorbance (OD) values is ≤ 20%. • Result is reported.

i. **Reportable Values:**

i. Report Total Protein Concentration to one decimal place.

ii. Report Free Protein Concentration to one decimal place.

Dynamic Light Scattering (DLS) and Polydispersity Index (PDI)

[0921] An exemplary DLS and PDI method used to evaluate exemplary loaded nanoparticles (e.g., NP-PN1) is described herein..

1. Prepare a 0.9% NaCl solution, Further dilute the 0,9% NaCl solution to make a 0.06% NaCl polystyrene latex (PSL) standard prior to preparing each standard. A PSL standard solution should be made fresh and used on the same day. Filter each PSL standard solution with a 0.2 µm filter.

2. Turn on a DLS instrument and allow its laser to warm up for at least 30 minutes.

3. Create a measurement standard operating procedure (SOP) for standards and each sample, or use an existing SOP as appropriate, following measurement parameters outlined in **Table 34**:

Table 34: Exemplary DLS Instrument Parameters

Parameters for	Standard	Sample
Measurement Type	Size	
Material	Polystyrene Latex	PLGA
Sample Material Absorption	0.010	1.100
Material RI	1.59	1.460
Sample Dispersant/ Solvent	Water	
Dispersant RI	1.330	
Sample Temperature	25°C	
Equilibration Time	120 seconds	60 seconds
Stabilization Time	3-5 minutes for 200 nm	N/A
Cuvette Type	DTS0012 Disposable cuvettes (UV Transparent)	
Measurement Angle	173°C Backscatter (NIBS default) for measurement	
Measurement Settings	Positioning method: Seek for optimum position	
Measurement Duration	Set to automatic, Number of measurements= 3 and delay between measurements (sec)= 0	Set to automatic, Number of measurements= 3 and delay between measurements (sec)= 0.

4. Clean a sample cuvette appropriately by using a Kimtech™ wipe and canned air to remove any dust that may contribute to scattering. This cleaning method should be performed prior to performing each measurement.
5. **Size Standard Measurement:**
    a. Invert a bottle with a relevant standard, ensuring it is homogeneous. Discard the first drop of each standard.
    b. Dilute Prepare and measure 200 and 500 nm PSL standards by diluting appropriately with 0a diluted NaCl solution, pipetting carefully to mix.
    c. Cap the cuvette and measure. Repeat this process such that 3 times repeated measurements for 2 cuvettes are collected for each standard.
6. **Sample Preparation:**

a. Thaw an exemplary loaded nanoparticle sample (e.g., NP-PN1) at room temperature until a sample is completely thawed. Once completely thawed, transfer to a clean microcentrifuge tube.
b. Once a sample has been equilibrated unperturbed for 5 to 10 minutes, visually inspect the sample to understand if any precipitation or sedimentation is observed in the sample. Do not proceed with a measurement and immediately inform your supervisor or manager.
c. Dilute an inspected sample appropriately to 25 µg/mL in molecular biology grade water based on the dosage of the sample in the cuvette the sample is to be measured in, as outlined in **Table 35**.
    i. Do not centrifuge or vortex any samples.

Table 35: Exemplary Sample Preparation

Dose (µg/mL)	Volume of Sample (µL)	Volume of Water (µL)	Final Concentration (µg/mL)	Total Volume (µL)
2000	12.5 µL from 2000 µg/mL	987.5 µL	25 µg/mL	1000
500	50 µL from 500 µg/mL	950.0 µL	25 µg/mL	1000
125	200 µL from 125 µg/mL	800.0 µL	25 µg/mL	1000

d. Once the sample has been added to the cuvette, pipette carefully at least 5-10 times. Be careful not to introduce bubbles.
e. Cap the cuvette, select an appropriate SOP and measure light scatter.
    i. Prior to measuring nanoparticle samples with 2000 and 500 µg/mL concentrations, allow each sample to stabilize in the cuvette for 5 minutes before starting the measurement.
    ii. A nanoparticle sample with a 125 µg/mL concentration should be diluted immediately prior to beginning the measurement.
f. Repeat this process such that 3-times repeated measurements for 22 cuvettes are collected for each sample.

7. **Standard Check**
    a. Prepare one cuvette of a 200 nm standard as outlined above and measure.

i. This standard check should be prepared after every 12 cuvettes (e.g., 6 samples) and/or at the end of all measurements.

ii. An exemplary sequence of sample analysis is outlined in **Table 36**.

Table 16: Exemplary Sequence of Sample Measurements

Sample or Standard	Cuvette	Number of Measurements
200 nm Standard	A	3
200 nm Standard	B	3
500 nm Standard	A	3
500 nm Standard	B	3
Sample 1	A	3
Sample 1	B	3
Sample n	A	3
Sample n	B	3
200 nm Standard Check	A	3

8. **System Suitability**

a. **Size Standards:** System suitability should be evaluated by assessing the data obtained from the size standards.

i. A mean Z-average of each 200 nm standard is within ± 2% of the certified mean diameter of a manufacturer's Certificate of Analysis, and the PDI should be ≤ 0.1. The mean Z-average of a 500 nm size standard is within the specification for mean number diameter by disc centrifuge defined on a manufacturer's Certificate of Analysis.

ii. For all standard analyses, displayed quality parameter shows "good".

b. **Sample:** For each sample's analysis, percent difference for Z-average values between duplicate preparations is ≤ 20%. Mean PDI value is ≤ 0.6.

9. **Reporting of Results:**

a. Exemplary Calculations

i. Calculate percent differences (%Difference) for Z-average values between duplicate preparations for each sample (e.g., for N samples) according to Equation 3.

$$\%\boldsymbol{Difference} = \frac{\boldsymbol{Z\ avg\ Prep\ 1 - Z\ avg\ Prep\ 2 - (\ldots) - Z\ avg\ Prep\ N}}{(\frac{\boldsymbol{Zavg\ Prep\ 1 + Z\ avg\ Prep\ 2 + (\ldots) + Zavg\ Prep\ N}}{\boldsymbol{N}})} \boldsymbol{X\ 100\%} \qquad (3)$$

ii. Calculate mean Z-average (n=6) for each sample.

iii. Calculate mean PDI (n=6) for each sample (see, for example, Stepto. *Pure Appl. Chem.*, 2009, 81(2), 351-353, which is incorporated by reference herein in its entirety).

b. Reportable Values

i. The reportable value is defined as the mean Z-average (n=6 measurements) obtained by measuring two preparation per sample.

c. Report Results

i. Report the mean Z-average (n=6) as a whole number (X d.nm).

ii. Report the mean PDI (n=6) to 1 decimal places (X.X) for information only.

Extractable Volume

[0922] Exemplary protein loaded nanoparticles described herein (e.g., NP-PN1) are tested for extractable volume, as described in detail below.

1. Collect 6 ampules m each containing a sample, and equilibrate to room temperature. Randomly select 5 ampules to be used to determine sample mass, and use the remaining ampule for density evaluation.

2. Tare a suitable container on an analytical balance. Invert the ampule (cap side up)and lightly flick the ampule cap end 3 times.

3. Twist off the ampule cap, ensuring that no material exits the ampule. If any material is lost during this process, do not use the ampule for evaluation (e.g., discard the ampule with a sample).

4. Carefully dispense the contents of the open ampule into the tared container by squeezing an inverted he ampule 5 times (e.g., cap side down).

a. Record the mass of the dispensed material to four decimal places. This is the mass of an exemplary loaded nanoparticle sample solution extracted from a single ampule.

5. Repeat this process 4 times, such that 5 samples are measured.

6. Evaluate densitometer system suitability by performing density measurements of water for injection (WFI) at 25°C ± 2°C in triplicate.

    a. Verify that the measured value is within 0.001 g/cm^3 of the theoretical density value. If any of the measurements do not meet this criterion, clean the instrument and repeat the measurement.

7. Once system suitability has been confirmed, pool contents of all 5 samples that were examined for sample mass determination in steps 2-5. Add contents of the remaining ampule, e.g., 6th ampule, to the densitometer. Additional ampules may be added if volume of a pooled solution is insufficient to perform densitometry.

    a. Ensure that a pooled sample reaches 25°C ± 2°C by placing in a water bath for at least 30 minutes.

8. Flush the densitometer with a pooled sample solution 2 times before injecting a pooled sample for a density measurement. Record measured density to 4 decimal places.

9. Formula for calculating extractable volume for each ampule is shown in Equation 4.

    a. Extractable Volume (mL) = Mass/Density, (4)

    where,

    Mass is a measured mass of an exemplary nanoparticle sample (mg); and

    Density is a measured density of an exemplary nanoparticle sample (mg/mL).

10. Report five individual extractable volume values for each sample (five ampules per sample) with an appropriate number of significant figures.

    a. Exemplary extractable volume is not less than (NLT) 1 mL.

***In Vitro* Drug Release**

[0923] An *in vitro* method for evaluating drug release from exemplary loaded nanoparticles described herein is detailed below.

Solution Preparation:

1. Prepare a 20 mM phosphate buffer /salt buffer (e.g., PB/salt buffer): 20 mM phosphate buffer + 20 mM NaCl, pH 8.0 (for example, 1 L of buffer is prepared).
    a. Weigh out $K_2HPO_4 \cdot 3H_2O$, KH_2PO_4, and NaCl, and dissolve in an appropriate amount of water.
    b. Use 1N NaOH to adjust pH of a prepared PB/salt buffer to 8.0 ± 0.05 before filtering through a 0.2 μm filter, and store at 2-8 °C for up to 2 months.
2. Peanut protein stock solution (freshly prepared): Prepare a 5 mg/mL peanut protein stock solution with 20 mM PB/salt buffer.
    a. Weigh 50 mg of peanut protein (e.g., Peanut Arachis Hypogaea) and completely dissolve in 10 mL of PB/salt buffer.
3. Peanut protein working stock: Prepare an 80 μg/mL peanut protein working stock solution with 20 mM PB/salt buffer (freshly prepared).
    a. Mix 16 μL of freshly prepared 5 mg/mL peanut protein stock and 984 μL of 20 mM PB/salt buffer.
4. Phospholipid stock: Prepare a 1 mg/mL phospholipid stock solution with 20 mM PB/salt buffer (freshly prepared).
    a. Equilibrate a vial with an exemplary phospholipid (e.g., an *E. Coli* Total Lipid Extract) to room temperature.
    b. Based on the mass of phospholipid outlined by a manufacturer, add a necessary volume of PB/salt buffer to the vial to reach a final concentration of 1 mg/mL. Sonicate the solution until it becomes homogeneous.

5. Trehalose stock: Prepare a 20 mg/mL trehalose stock solution with 20 mM PB/salt buffer (freshly prepared).
    a. Weigh 100 mg of trehalose dihydrate in a vial, and add 5 mL of 20 mM PB/salt buffer. Vortex to dissolve trehalose dihydrate in the solution.
6. Free Background (BG): Prepare a 10 mg/mL BG solution with 20 mM PB/salt buffer (freshly prepared) .
    a. Mix 500 µL of freshly prepared 20 mg/mL trehalose stock and 500 µL of 20 mM PB/salt buffer.
7. Free Background (BG) + Phospholipids (PL): 10 mg/mL Trehalose and 50 µg/mL Phospholipid stock (freshly prepared).
    a. Mix 500 µL of 20 mg/mL Trehalose stock, 50 µL of 1 mg/mL PL stock and 4500 µL of 20 mM PB/salt buffer.

Sample Preparation:

Day 1

1. Set up and label 1.5 mL microcentrifuge tubes.
    a. Use two tubes (A and B) per time point for 3 time points (0, 4 and 24 hr) and 3 possible dosages (2000, 500 and 125 µg/mL).
2. Thaw samples in a water bath, pipette gently up and down to mix thawed samples, pipette 0.1 mL of sample into a corresponding tube and cap each tube tightly.
3. Flash freeze all samples in a liquid nitrogen (LN2) bath. Once frozen, place 0 hr-labeled tubes and 4 hr-labeled Tubes into a -20 °C freezer.
4. Thaw 24 hr-labeled Tubes only. Add 0.9 mL of PB/salt buffer to each tube and cap tightly.
5. Place 24 hr-labeled tubes onto a rotator and into a 37 °C incubator, and record time.

Day 2

6. 20 hrs after placing 24 hr-labeled samples in the incubator, thaw 4 hr-labeled sample tubes.
7. Add 0.9 mL of 20 mM PB/salt buffer to each tube and cap tightly.
8. Place these samples onto a rotator and into a 37 °C incubator, and record time.
9. 24 hours after placing 24 hr-labeled samples into an incubator, thaw 0 hr-labeled sample tubes – these samples will not be placed into an incubator.
10. Add 0.9 mL of 20 mM PB/salt buffer to each tube and cap tightly.
11. Retrieve 4 hr-labeled and 24 hr-labeled sample tubes from an incubator.

Sample Processing:

12. Centrifuge all tubes at 21000 RCF for 20 minutes. During this time, prime 0.1µm syringe filters with 10 mL of PB/salt buffer, one for each sample.by using Afterwards, push 10 mL of air through each syringe and remove as much liquid as possible.
13. After centrifugation, remove 700-800 µL of supernatant from each sample tube using a new syringe. Load each syringe with a primed filter and eject supernatant into a new 1.5 mL microcentrifuge tube. Store samples at 2-8 °C until needed.

BCA Assay:

14. Equilibrate all samples to room temperature.
15. Prepare a peanut protein stock solution, a peanut protein working stock solution, a phospholipid stock solution, a trehalose stock solution, a free BG solution, and a free BG + PL solution. Prepare peanut protein standards with 80 µg/mL peanut protein working stock solution as outlined in **Table 37**.

Table 37. Exemplary Peanut Protein Standard Preparation.

Std ID	Conc. (µg/mL)	Amount (µL)	PB Buffer Amount (µL)
1	40.000	400 µL of 80µg/mL std stock	400
2	20.000	400 µL of Std 1	400
3	10.000	400 µL of Std 2	400
4	5.000	400 µL of Std 3	400
5	2.500	400 µL of Std 4	400
6	1.250	400 µL of Std 5	400
7	0.625	400 µL of Std 6	400
8	0	0	400

16. BCA Assay

a. Prepare an assay plate by adding 150 µL/well of each of standard (free BG), control (free BG + PL) and sample solutions in duplicates, as shown in **Table 38**.

b. Prepare 25 mL of BCA working solution by mixing 12.5 mL of Buffer A, 12 mL of Buffer B, and 0.5 mL of Buffer C well.

c. Add 150 µL BCA working solution to each well and seal an assay plate with sealing tape tightly.

d. Incubate a loaded assay plate at 37 °C for 2 hours. Turn on a plate reader prior to use.

e. Read an assay plate at 562 nm after incubation 2 hours of incubation at 37 °C.

Table 38. Exemplary In Vitro Drug Release Assay Plate Layout.

	Standard		2000µg/mL		500µg/mL		125µg/mL					
BCA	1	2	3	4	5	6	7	8	9	10	11	12
A	40µg/mL		FPC 0hr - RT (A)		FPC 0hr - RT (A)		FPC 0hr - RT (A)					
B	20µg/mL		FPC 0hr - RT (B)		FPC 0hr - RT (B)		FPC 0hr - RT (B)					
C	10µg/mL		FPC 4hr - 37C (A)		FPC 4hr - 37C (A)		FPC 4hr - 37C (A)					
D	5µg/mL		FPC 4hr - 37C (B)		FPC 4hr - 37C (B)		FPC 4hr - 37C (B)					
E	2.5µg/mL		FPC 24hr - 37C (A)		FPC 24hr - 37C (A)		FPC 24hr - 37C (A)					
F	1.25µg/mL		FPC 24hr - 37C (B)		FPC 24hr - 37C (B)		FPC 24hr - 37C (B)					
G	0.625µg/mL		Free BG (no PL, 10 replicate)									
H	0µg/ml (buffer only)		Free BG (50 PL, 10 replicate)									

Data Analysis:

17. Calculate normalized absorbances of peanut protein standards (e.g., Std. Abs):

    a. *Normalized Std. Abs = Measured Std Abs – Measured Std Abs for 0 µg/mL std.*

18. Use the normalized standard absorbances to create a standard curve (e.g., a a 4-parameter logistic fit curve).

19. Calculate the normalized sample absorbances:

    a. *Normalized Sample Abs = Collected Sample Abs – Collected "free BG+PL" Abs.*

20. Use the standard curve to calculate values of all normalized samples, and graph results.

21. Adjust 0 hr protein release timepoint values to 0 µg/mL, and graph results:

    a. *Adjusted Sample Release = Calculated Sample Release – Calculated Release for a 0 hr timepoint.*

22. Calculated percent protein release (e.g., %FPC) according to Equation 5 below and graph results.

$$\%\text{FPC} = \frac{Calculated\ concentration\ of\ FPC\ in\ a\ sample}{Theoretical\ concentration\ of\ FPC\ in\ a\ sample} \times 100\% \qquad (5)$$

23. Adjust values for 0 hr-timepoint %Protein release to 0%, and graph results.:

    a. *Adjusted %Release = Calculated Sample %Release – Calculated %Release for a 0 hr timepoint.*

Exemplary System Suitability Acceptance Criteria:

24. Percent CV for duplicate absorbances of peanut protein standards should is ≤ 20%

25. R-squared calculated for an exemplary standard curve is ≥ 0.98.

Exemplary Loaded Nanoparticle Sample Acceptance Criteria:

26. Absorbance values of all samples should be within the range of a corresponding standard curve.

27. Percent CV for the duplicate absorbances of all samples should be ≤ 20%.

Reportable Values

28. Report released protein concentration to one decimal place.

29. Report percent released protein concentration to one decimal place.

Phosphatidylethanolamine (PE) Identity

[0924] An exemplary phosphatidylethanolamine (PE) identity method is detailed herein.

1. Prepare various sample controls, standards, and samples to be used in a PE identity assay. Any phosphatidylethanolamine assay kit may be used.
    a. Reagents for an exemplary PE assay comprise a PE Assay Buffer, a Converter Enzyme/PE Converter, a PE Developer, a Developer Solution/Enzyme Mix and an OxiRed/PE Probe.
2. Prepare a dilution buffer according to a kit manufacturer protocol.
3. Prepare a positive control solution using an E. Coli Extract Total solution.
4. Prepare an experimental sample of exemplary protein loaded nanoparticles (e.g., PLGA Nanos or NP-PN1) by thawing a frozen sample and diluting it to a specified concentration using a prepared dilution buffer.

5. Heat and cool a positive control sample and an experimental sample several times at 80 °C before centrifuging and retrieving a portion of supernatant from each sample.
    a. Place each supernatant sample into a drybath set to 45 °C.
6. After 10 minutes of incubating sample supernatants in a dry bath, prepare an assay plate by adding appropriate volumes of background control samples, positive control samples, experimental samples and PE Assay Buffer, as specified.
7. Dilute and add Converter Mix to all wells except those designated for background controls.
    a. Cover with a lid, mix and incubate a prepared assay plate at 45 °C for 1 hour.
8. Dilute a PE Developer solution with a PE Assay buffer,add specified amounts of a diluted Developer solution to each well of the assay plate, followed by addition of an OxiRed probe to all wells,including background controls.
    a. Cover with a lid, mix and incubate a prepared assay plate at 40 °C for 3 hours.
9. Measure fluorescence with a plate reader as outlined, with an excitation setting of 535 nm and emission of 587 nm.
10. Ensure results meet necessary criteria before data analysis – e.g.,a value for positive control relative fluorescence units (RFUs) with background RFUs subtracted is greater than a blank only value..
11. Report mean and duplicate RFU measurements for each blank, positive control, experimental sample(s), and background control. Calculate a response ratio by performing the following Equation 6:

$$\text{Response Ratio} = (\text{Mean RFU}_{\text{"Positive Control" or "Sample"}} - \text{Mean RFU}_{\text{"Background Control"}}) / \text{Mean RFU}_{\text{Blank.}} \quad (6)$$

12. PE identity is determined to be positive if a response ratio of a sample is ≥ 1.0.
13. PE Identity of a sample is negative if a response ratio of a sample is < 1.0.

Sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

[0925] An exemplary SDS-PAGE method is detailed herein.

1. **Prepare SDS-PAGE buffers.**

    a. Prepare a 1x Tris/Glycine/SDS running buffer. – For example, 1 L of a running buffer is prepared by diluting a concentrated stock buffer by a 10xdilution (e.g., 100 mL of a 10x concentrated buffer is diluted to 1 L of a 1x buffer). Use diluted buffer on the same day after preparation.

    b. Prepare a 1 M DTT solution. For example,,1 mL of 1 M DTT is prepared by mixing ~77 mg of weighed solid DTT with 1 mL of water. Use a prepared solution on the same day after preparation.

    c. Prepare a dilution buffer (10 mM K_2HPO_4, 1.8 mM $KH_2PO_4 \cdot H_2O$, pH 8.2 buffer). For example, 200 mL of buffer is prepared, and the buffer is titrated to 8.20±0.05 with 1N NaOH or 1N HCl. Store a prepared dilution buffer solution at 2-8 °C Do not use dilution buffer that has been stored for more than 7 days.

    d. Prepare a 5 mg/mL peanut protein extract (PPE) stock solution. For example, 5 mL of a PPE stock solution is prepared by adding 25 mg of weighed peanut protein solid to 5 mL of dilution buffer.

2. **Peanut Protein Extract Standard Curve**

    a. Dilute the 5 mg/mL PPE stock solution according to **Table 39**.

Table 39. Exemplary Initial Peanut Protein Extract Dilutions. Label column corresponds to wells to be loaded in an SDS-PAGE gel.

Label	Final Concentration of PPE (µg/µL)	PPE stock at 5 mg/mL (µL)	Dilution Buffer (µL)	Final Volume (µL)
A	0.05	5	495	500
B	0.2	20	480	500
C	0.6	60	440	500

b. Dilute PPE further according to **Table 40** in labels to be further loaded onto the gel.

Table 40. Exemplary Peanut Protein Extract Dilutions and Gel Running Preparation. Label column corresponds to wells to be loaded in an SDS-PAGE gel.

Label	PPE Label Stock	PPE Stock Volume Added (Final Concentration (µL)	Dilution Buffer Volume (µL)	DTT Volume (µL)	4x Loading Buffer Volume (µL)	Final Volume (µL)	Final PPE Concentration (µg/µL)	Total Mass Loaded onto the gel (µg)	Loading Volume onto the gel (µL)
D	-	-	58.5	9.0	22.5	90.0	0.000	0.000	30.0
E	A	18.7	39.8	9.0	22.5	90.0	0.010	0.312	30.0
F	A	37.5	21.0	9.0	22.5	90.0	0.021	0.625	30.0
G	B	18.8	39.8	9.0	22.5	90.1	0.042	1.250	30.0
H	B	37.5	21.0	9.0	22.5	90.0	0.083	2.500	30.0
I	C	25.0	33.5	9.0	22.5	90.0	0.167	5.000	30.0

3. **Free Protein Content (FPC)**

a. Aliquot 1 mL of an exemplary loaded nanoparticle sample (e.g., PLGA Nanos or NP-PN1) into a tube and centrifuge at 21100 RCF for 20 minutes.

b. Meanwhile, prime a 0.1 µm syringe filter with 10 mL of water.

c. After centrifugation, remove an exemplary loaded nanoparticle sample (e.g., PLGA Nanos or NP-PN1)supernatant from the tube. Discard the pellet and the remaining 200 µL of supernatant collected. Filter the remaining supernatant into a new tube.

d. Dilute filtered supernatant further, as shown in **Table 41**.

Table 41. Exemplary Peanut Protein Extract Dilutions and Gel Running Preparation. Label column corresponds to wells to be loaded in an SDS-PAGE gel.

:

Label	Volume of FPC Stock (µL)	Volume of Dilution Buffer Added (µL)	DTT (µL)	4x LB (µL)	Final Volume (µL)	Loading Volume (µL)
O	58.5	0.0	9.0	22.5	90.0	30.0

4. **Total Protein Content (TPC)**

   a. Dilute a 2000 µg/mL sample according to **Table 42**.

Table 42. Exemplary Sample Dilutions for Total Protein Content Evaluation. Label column corresponds to wells to be loaded in an SDS-PAGE gel.

Label	Label Stock	Volume of Stock Solution (µL)	Volume of Dilution Buffer Added (µL)	DTT Volume (µL)	4x LB Added (µL)	Final Volume (µL)	Target Final Concentration (µg/µL)	Total Mass to be loaded onto the gel (µg)	Loading Volume (µL)
J	--	2.5	97.5	--	--	100.0	0.05	--	--
K	--	10.0	90.0	--	--	100.0	0.20	--	--
M	J	37.5	21.0	9.0	22.5	90.0	0.021	0.625	30.0
N	K	37.5	21.0	9.0	22.5	90.0	0.036	2.500	30.0

5. **Final PPE, FPC, TPC and Gel Preparation**

   a. After gentle mixing, incubate tubes labeled D-I, and M-O for samples prepared according to **Tables 40-42** at 95 °C for 10 minutes.

   b. Remove Precision Plus Protein Dual Color Standards from 2-8 °C, and equilibrate to room temperature.

   c. Load gel(s) into a gel chamber and fill inner and outer chambers to an appropriate operating volume with 1x running buffer.

      i. In the first lane of each gel, load 8 µL of Precision Plus Protein Dual Color Standard.

      ii. In subsequent lanes, load 30 µL of samples from tubes labeled D-I, M-O.

6. **Running and Staining of the Gel**

   a. Run each gel at 110 V for 70 minutes.

b. Remove each gel from a gel chamber and cassette, and transfer to a gel staining box filled with instant Coomassie protein staining solution. Stain each gel in a box on an orbital shaker for 1 hour, set to a rotation speed of 50 rpm.

c. Decant the Coomassie protein staining solution, add MilliQ water and continue shaking for 30 minutes at 50 rpm.

d. Decant the MilliQ water, add another wash of MilliQ water and continue shaking overnight at 50 rpm.

7. **Gel Analysis**

a. Next day, decant the water and image each gel appropriately. Capture all detected bands with imaging software on an automatic imaging mode and exposure. If faint bands are observed, they can be manually selected and imaged.

b. An Integrated Optical Density (IOD) of three densest bands of a TPC sample (e.g., bands between 50-75 kD, ~75 kD and 20-25 kD) are recorded. An estimated MW is calculated for each band based on a molecular weight standard that was concurrently run on the same gel.

c. Calculate and report the following for each TPC lane:

$$\%\ of\ AraH\ Proteins = 100 * \frac{IOD\ of\ Band\ 1 + IOD\ of\ Band\ 2 + IOD\ of\ Band\ 3}{IOD\ of\ All\ Bands}$$

qPCR

[0926] An exemplary qPCR method is detailed herein.

Procedure:

1. **Exemplary Preparation of Standards, Controls and Samples**

Thaw all materials on ice for at least 30 min. Clean bench surfaces and pipettes with 70% isopropanol and/or DNA Zap.

a. Preparation of DNA standard:

i. Thaw (e.g.., DNA standard), briefly centrifuge a thawed DNA control to collect, and vortex to mix.

ii. Prepare standards by serial dilution 30 ng/µL E. Coli DNA control according to **Table 43**. Vortex to mix and briefly centrifuge each prepared standard to collect after each dilution. A 30 ng/µL E. Coli DNA control is not used to generate a standard curve for DNA concentration analysis.

Table 43. Exemplary Dilutions of Standard Solution

E. coli DNA Standard		Dilution	
Tube Label	pg/µL	H2O (µL)	DNA Standard
*STD A	3000	90	10 µL *E. coli* 30ng/µL DNA control
STD 1	300	450	50 µL STDA
STD 2	30	450	50 µL STD1
STD 3	3	450	50 µL STD2
STD 4	0.3	450	50 µL STD3
STD 5	0.03	450	50 µL STD4

b. Total Sample Preparation

i. Set up tubes for each Total and Soluble sample to be assayed in triplicate. Label each tube with a sample number, a replicate letter, and a dilution number, e.g., 1A1 - 1C3, 2A1-2C3, et cetera.

ii. Add 90 µL of DMSO to all "A" tubes and 90 µL of H_2O to all "B" and "C" tubes. If adding a spike-in control DNA to "C" tubes, subtract volume of added spike-in DNA from volume of water in a sample. Vortex al samples for approx. 5 seconds at maximum speed.

iii. Add 10 µL of each vortexed sample into a corresponding "A" tube and vortex to mix.

iv. Add 10 µL of each sample from an "A" tube into a corresponding "B" tube, vortex to mix, and centrifuge each "B" tube to collect the sample.

v. Dilute 10 µL of each "B" tube into a corresponding "C" tube, vortex to mix, and centrifuge each "C" to collect the sample.

vi. Store tubes with diluted samples at -20 °C for up to 1 week or proceed with PCR.

c. Soluble Sample Preparation

i. Label tubes as outlined in step b.i. above, and add one additional tube labeled "P" for each soluble analyte. Add DMSO to "A", "B", and "C" tubes.

ii. Vortex corresponding samples for approximately 5 seconds on maximum speed. Transfer 100 µL of each vortexed sample to a corresponding "P" tube.

iii. Centrifuge all "P" tubes at 10,000 rpm for 5 mins. After centrifugation, without disturbing the pellet, transfer 10 µL of supernatant from each "P" tube into all three corresponding "A" tubes containing DMSO.

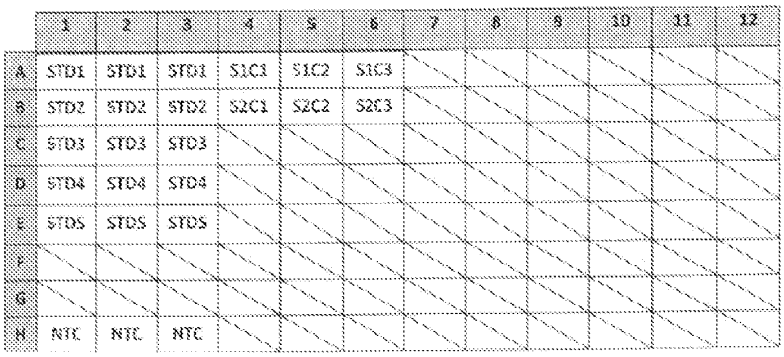
iv. Vortex all "A" tubes to mix, and briefly centrifuge to collect samples. Process the "A" tubes as outlined in step c.iii.

v. Store tubes at -20 °C for up to 1 week or proceed with PCR.

2. **PCR Plate Assay Procedure**

a. Determine the number of wells needed to run all samples. **Table 44** shows an exemplary assay plate for evaluating two samples.

Table 44. Exemplary PCR Plate Layout.



b. Dilute 50 µM ROX dye to 0.333 µM by adding 1 µL of the dye into 149 µL of water. Add 3 µL of diluted dye to each 20 µL reaction for a final dye concentration of 50 nM.

c. Prepare a master mix solution according to **Table 45**. Add 15 µL of prepared master mix to each well of a PCR plate, e.g., as shown in **Table 44**.

Table 45. Exemplary PCR Reaction Master Mix.

Reagents	µL per well
0.333 µM ROX dye	3
10 × *E. coli* Assay mix	2
2 × Environmental Master Mix	10
Total	15

d. Add 5 µL of each prepared standard to wells, e.g., as shown in **Table 44**.

e. Add 5 µL of water to negative template control (NTC) wells, e.g., as shown in **Table 44**.

f. Add 5 µL of each sample to a corresponding well, e.g., as shown in **Table 44**.

g. Seal a prepared assay plate with an optical adhesive film. Centrifuge a sealed assay plate for 30 seconds before inserting it into a PCR machine.

h. Perform PCR using cycling parameters shown in **Table 46**.

Table 46. Exemplary PCR Cycling Conditions.

Stage	Repetitions	Step	Condition
1	1	1	Hold at 95.0°C for 2:00 (min:sec)
2	40	1	Hold at 95.0°C for 00:15 (min:sec)
		2	Hold at 60.0°C for 01:00 (min:sec)

3. **Calculations and Reporting of Results**

a. System Suitability

i. System suitability is assessed using a standard curve generated with concentrations obtained from values in diluted E. Coli DNA control (e.g., standard) wells.

b. Calculate and report the following:

i. A coefficient of determination (R-squared) for a generated standard curve.

ii. A percent relative standard deviation (e.g., %RSD) of a cycle threshold value (e.g., Ct) for each standard.

c. System Suitability Acceptance Criteria

i. R-squared for is each standard curve is $\geq 0.98/$

ii. Relative standard deviation calculated from triplicate values for each standard is $\leq 15\%$.

iii. Results meet additional specifications, if any are provided.

d. Reportable Value

i. Report each test sample result (e.g., DNA concentration) in "ng/mL" to a number of decimal places provided in a specification. If a specification is not provided, DNA concentration is provided to three decimal places.

PLGA Content

[0927] An exemplary PLGA content method is detailed herein.

1. Prepare stock and standard solutions as outlined below:

    a. **Diluent:** Prepare a diluent by mixing 7 parts of water with three parts of acetonitrile.

    b. **1 mg/mL Stock Standard Preparation:** Weigh out PLGA in a glass tube and add an appropriate volume of tetrahydrofuran (THF) to obtain a final concentration of 1 mg/mL. Sonicate a prepared PLGA stock solution for 30 minutes, vortex and filter through a 0.2 µm filter.

2. Prepare diluted stock and spiking standards as outlined below:

    a. **100 µg/mL Diluted Stock:** Add 100 µL of a 1 mg/mL PLGA stock standard (e.g., as prepared in step 2.b.) to 900 µL of a diluent solution in a microcentrifuge tube. Vortex to mix.

    b. **10 µg/mL Spiking Standard:** Add 100 µL of 100 µg/mL diluted stock solution to 900 µL of diluent solution in a microcentrifuge tube. Vortex to mix.

3. Prepare PLGA linearity standards as shown in **Table 47**.

Table 47. Exemplary PLGA Linearity Standard Preparation

Standard Concentration (µg/mL)	Volume of Stock (µL)	Stock Used	Volume of Diluent (µL)
0 (Diluent Blank)	0	N/A	1000
1	100	10 µg/mL	900
5	50	100 µg/mL	950
10	100		900
25	25	1000 µg/mL	975
50	50		950
75	75		925
100	100		900

    a. Prepare additional standards with 1, 10, 50, and 100 µg/mL concentrations in a similar manner as linearity standards (e.g., as shown in **Table 47**) with a separate set of stock and diluted stock solutions for use as QC Standards.

4. Preparate loaded nanoparticle samples for analysis.

a. Dilute samples to a target PLGA concentration of 10 µg/mL for analysis. If initial results are outside of a standard (e.g., calibration) curve range, additional dilutions may be prepared beyond those specified below.

b. Place 50, 200, or 800 µL for 2000, 500, and 125 µg/mL loaded nanoparticle samples, respectively, of each loaded nanoparticle sample into a glass tube and add an appropriate amount of THF a final volume of 5 mL as shown in **Table 48**. Sonicate each sample solution for 30 minutes. Pass each sonicated sample solution through a 0.2 µm PTFE syringe filter and dilute a filtered solution 1:10 in diluent.

Table 48. Exemplary Sample Preparation

Sample Concentration (µg/mL)	Volume of Sample (µL)	Volume of THF (mL)
2000	50	4.95
500	200	4.80
125	800	4.20

5. Prepare column conditioning standard:

a. A 25 µg/mL linearity standard is used as a column conditioning standard.

6. Prepare blank controls:

a. Diluent blank: transfer an aliquot of diluent solution to an autosampler vial.

7. Set liquid chromatography (LC) parameters according to **Table 49**.

Table 49. Exemplary Liquid Chromatography Parameters.

Column	Agilent Poroshell 120 EC-C18 2.7µm 4.6 x 150 mm
Mobile phase A	0.1% Formic Acid in Water

Mobile phase B	Acetonitrile
Gradient	time (min): 0 15 25 25.1 35 %B: 30 95 95 30 30
Flow rate	0.6 mL/min
Injection volume	10 µL
Column temperature	40°C
Autosampler array	15°C

8. Set MSD Parameters according to **Table 50**. All transitions monitored are summed into a single channel for analysis.

Table 50. Exemplary MSD parameters.

Ion Mode	APCI+/MRM
Transitions Monitored	*m/z* 709.9→507.2 CE 30 eV *m/z* 709.9→579.3 CE 30 eV *m/z* 767.9→565.2 CE 30 eV *m/z* 767.9→637.1 CE 30 eV *m/z* 825.9→623.1 CE 35 eV
Divert to Waste	0 to 5 min and 15-35 min
Collision Energy	30-35 eV
DP	100V
CAD	9
Curtain gases	20
NC	3
TEM	450 °C
UV/Vis	Wavelength range: 200 to 400 nm
Calibration compound	Pos PPG

9. Perform mass spectrometry (MS): calibration:

   a. Ensure that a mass spectrometer is calibrated in a positive electrospray ionization mode: +Q1 and +Q3 using a Positive PPG Solution or another suitable standard prior to sample set analysis. Exemplary mass accuracy is equal to or greater than ± 0.3 Da for the reference ions.

   b. Change source to APCI after calibration check and back to ESI for post calibration check.

10. Clean mass spectrometer:

a. If necessary, clean the instrument and other equipment components prior to beginning LC/MS analysis.

11. Perform column conditioning:

a. Equilibrate a column with a starting mobile phase composition for at least 20 minutes. Subsequently, condition the column with at least 2 injections of a 25 µg/mL PLGA column conditioning standard followed by at least 2 injections of a diluent blank solution.

12. Sample stability and storage:

a. Standards, 2000 µg/mL loaded nanoparticle samples, and 500 µg/mL loaded nanoparticle samples may be stored for up to 24 hours in an autosampler at 18°C or a refrigerator at 2-8 °C prior to analysis. Standards and samples are prepared on the same date and stored together. Analysis of freshly prepared standards or samples should not be performed together with stored standards or samples for generating quantitative results. Before analysis, any stored samples or standards should be gently mixed. Stability of stored 125 µg/mL loaded nanoparticle samples has not been evaluated, therefore, these samples are analyzed on the day of preparation.

13. Evaluate system suitability:

a. Conditioning: Condition a column with the mobile phase as described in step 11.a. Verify there are no discreet chromatographic peaks at an expected retention time of an analyte in the last injection of a diluent blank.

b. Analysis sequence: Analyze all samples according to step 14 outlined below.

c. Exemplary acceptance criteria:

I. A diluent blank shows no significant discreet peaks (NMT 10% of peak area of a 1 µg/mL standard) by visual inspection of a chromatography output at an expected retention time of an analyte.

II. A generated calibration curve has an R-squared value of at least 0.99.

III. A percent recovery for linearity standards is within ± 50% for a 1 µg/mL standard and within ± 25% for all other standards.

IV. A percent recovery for all injections of QC standards is within ± 50% for a 1 µg/mL standard and within ± 25% for all other standards.

V. A 1 µg/mL PLGA standard has a signal-to-noise ratio of at least 10.

14. Exemplary sample analysis procedure:

a. The following is an example sample analysis sequence including both column conditioning and system suitability injections.

1) Column Conditioning (25 µg/mL PLGA Standard): minimum 2 injections;
2) Diluent Blank(s): minimum 2 injections;
3) Linearity Standards – 1 injection per standard;
4) Diluent Blank;
5) QC Standards – 1 injection per standard;
6) Diluent Blank;
7) Sample #1;
8) Diluent Blank ;
9) Sample #2;
10) Diluent Blank;
11) Sample #3;
12) Diluent Blank;
13) QC Standards – 1 injection per standard.

b. Samples at multiple concentrations are analyzed in the same sequence. Time between preparation and injection of each sample should be minimized as possible. If more than 3 samples are to be analyzed, a separate sequence is run to ensure suitability of all prepared samples throughout the sequence.

c. Ensure all samples are bracketed by a set of QC Standards. Any series of injections of a sample are followed by a diluent blank injection to minimize possible carryover. All series of standards are followed by a diluent blank injection to minimize possible carryover.

d. All blanks and standards are analyzed in a single run unless otherwise noted. Samples are analyzed with a single injection and are bracketed by a diluent blank and a set of QC Standards.

e. System suitability is determined based on 14.a.2)-6) , and separate injections of these solutions are not necessary.

15. Data analysis and reporting:

a. Perform data analysis as follows:

I. Plot, smooth, and integrate a PLGA peak in each chromatogram using the Analyst instrument software (Sciex), ensuring that all 5 multiple reaction monitoring (MRM) transitions are being summed and displayed as a single peak. Peak integration should is visually inspected and adjusted manually if necessary.

II. Create a calibration curve using linearity standards and the Analyst instrument software and set linear weighting to $1/x^2$. A generated calibration curve is used to calculate recovery of standards and determine a concentration of PLGA in each test sample.

III. Determine peak area for PLGA in loaded nanoparticle samples. Determine concentration of PLGA in these samples using the Analyst software and a calibration curve, as generated in step 15.a.II. Report results according to step 15.b., as outlined below.

IV. Estimate limit of detection of an assay measuring an average signal-to-noise ratio for a 1 µg/mL calibration standard and use this ratio to estimate concentration of PLGA, wherein signal-to-noise ratio is equal to 3. This value is considered as an estimate only, because linearity is not known for sample concentrations below 1 µg/mL. Integrated chromatograms are printed and included in a report.

b. Reporting of results:

I. This test is a quantitative test with a range of 1-100 µg/mL PLGA exemplary loaded nanoparticles described herein. Results should be reported as a measured concentration of PLGA and a calculated undiluted sample concentration.

Residual Solvents

[0928] Exemplary loaded nanoparticle samples (e.g., NP-PN1) are tested for residual solvents. Samples are tested at least for residual isopropyl alcohol (IPA) and dimethyl sulfoxide (DMSO) by headspace gas chromatography. Exemplary methods for evaluating loaded nanoparticle samples for presence and/or amount of residual solvents are detailed herein.

Residual Solvents – IPA

1. Prepare standard solutions:
    a. Stock standard solution: weigh 50 mg IPA in a tared 50 mL volumetric flask. Dilute to a 50 mL volume with filtered water and mix well. Concentration of this solution is 250 µg/mL or 2500 ppm with respect to a loaded nanoparticle sample.
    b. Working standard: transfer 5.0 mL of a prepared stock standard solution (e.g., as described in 1.a.) into a 100 mL volumetric flask, dilute to a 100 mL volume with filtered water and mix well. Concentration of this solution is 500 µg/mL or 5000 ppm with respect to a loaded nanoparticle sample.
2. Prepared a loaded nanoparticle sample: weigh 100 mg of loaded nanoparticle sample in a tared 10 mL headspace vial. Add 1.0 mL of filtered water. Crimp the vial closed.
3. Prepare a blank sample: Add 1.0 mL of filtered water into a 10 mL headspace vial. Crimp the vial closed.
4. Prepare a working standard solution: Transfer 1.0 mL of a prepared working standard (e.g., as described in 1.b.) into 10 mL headspace vial and crimp the vial closed.
5. Set gas chromatograph (GC) parameters as follows:
    a. Headspace Sampler: Agilent Model 7694 or Model G1888A Headspace Sampler equipped for 10-mL headspace vials or equivalent.

b. Gas Chromatograph: Agilent Model 6890 or 7890 with split/splitless injection port, electronic pressure control, and flame-ionization detection.
c. GC Column: Restek Rxi-624SilMS, 30 m x 0.32 mm x 1.8 µm.
d. Oven Temperature: 40°C for 5 minutes, ramp 10°C/ minute to 240°C and maintain at 240°C for 5 minutes (total run time 30 minutes).
e. Flow: 2.1 mL/minute, Helium.
f. Injection Technique: Split.
g. Type of liner: 2 mm Focus liner.
h. Inlet Temperature: 140°C.
i. Split Flow: 1.05 mL/min.
j. Split ratio: 0.5:1 for G1888A headspace sampler (5:1 if using Agilent Model 7694 headspace sampler).
k. Detector Temperature: FID at 250°C.
l. Hydrogen flow: 30.0 mL/min.
m. Air flow: 300 mL/min.
n. Detector Makeup Gas: Nitrogen 28 mL/minute.

6. Set Agilent G1888 Headspace Sampler parameters as follows:

a. Oven Equilibration Time: 45 minutes.
b. Cycle Time: 43 minutes.
c. Injection Time: 0.2 minute.
d. Oven Temperature: 80°C.
e. Loop Temperature: 100°C.
f. Transfer Line Temperature: 140°C.
g. Pressurization Time: 0.17 minutes.
h. Loop fill time: 0.30 minutes.
i. Loop Equilibration Time: 0.05 minute.
j. Vial Pressure: 10 psi.
k. Transfer Line Flow: 15 mL/minute.
l. Injection Volume: 1 µL.
m. Shake Speed: Low.

7. Set Agilent 7694 Headspace Autosampler parameters as follows:

a. Oven Equilibration Time: 45 minutes
b. Cycle Time: 43 minutes.
c. Injection Time: 0.2 minute.
d. Oven Temperature: 80°C.
e. Loop Temperature: 100°C.
f. Transfer Line Temperature: 140°C.
g. Pressurization Time: 0.17 minutes.
h. Loop Equilibration Time: 0.05 minute.
i. Vial Pressure: 10 psi.
j. Injection Volume: 1 mL (fixed loop).
k. Shake Speed: 5 (low).

8. Exemplary data system used is Waters® Empower™ 3 software.

9. Calculations and reporting are performed as follows:

a. System Suitability:

i. Make one injection of a blank sample into the system.

ii. Make six replicate injections of a working standard solution.

iii. Inject the loaded nanoparticle samples. If more than 10 loaded nanoparticle samples are to be tested, inject a working standard solution at an intermediate point so that each segment of a sequence of analyzed samples contains not more than 10 loaded nanoparticle samples.

iv. Each sequence should conclude with a working standard solution injection followed by a blank preparation injection.

v. Acceptance Criteria:

1. Percent relative standard deviation of a DMSO peak area response for six injections of Working Standard is no more than 15.0%.

2. Overall percent relative standard deviation for all working standards is no more 15.0%.

b. Sample Calculations:

i. Calculated concentration of IPA in µg/g (ppm) in each sample using Empower software, as shown below in Equation 7:

$$\frac{\mu g}{g} = Cstd \times \frac{ACSpl}{ACStd} \times \frac{1.0mL}{g\ of\ Sample}, \quad (7)$$

Wherein:

Cstd = concentration of IPA in a working standard in µg/mL;

AC spl = peak area of IPA peak in a loaded nanoparticle sample; and

AC std = peak area of IPA peak in a working standard (average of initial 6 injections).

ii. If no IPA is detected in a loaded nanoparticle sample, this result is reported as less than a limit of quantification (LOQ).

c. Reporting

i. Report an amount of IPA in a sample rounded up to an integer if above LOQ. If amount of IPA in a sample below LOQ, report "<50 ppm", wherein 50 ppm with respect to a sample is an LOQ of a residual solvent evaluation for IPA.

Residual Solvents – DMSO

1. Standard solutions:

a. Prepare a diluent solution: dilute acetonitrile (ACN) in water to an 80% concentration (v/v).

b. Prepare a DMSO stock standard solution: weigh 50 mg of DMSO in a tared 100 mL volumetric flask and bring up to a final volume to obtained a 500 µg/mL DMSO stock standard solution.

c. Working standard solution: Transfer 1 mL of a 500 µg/mL prepared DMSO stock standard solution (e.g., as prepared in step 1.b.) into a 50 mL volumetric flask and dilute to a 50 mL volume with prepared diluent to obtain a solution with a 10 µg/mL (e.g., 5000 ppm) concentration with respect to a loaded nanoparticle sample. Transfer to a gas chromatography vial and crimp to close.

2. Loaded nanoparticle sample preparation: weigh 100 mg of a loaded nanoparticle sample in a tared 50 mL volumetric flask. Add 30 mL of ACN diluent and swirl to dissolve. Add 10 mL of highly pure filtered water, swirl to dissolve and dilute to a 50 mL volume with diluent. Transfer to a gas chromatography vial and crimp the vial to close.

3. Prepare a blank sample: add diluent to a gas chromatography vial and crimp the vial closed.

4. GC Chromatograph Parameters:

a. Gas Chromatograph: Agilent Model 6890 with split/splitless injection port, electronic pressure control, and flame-ionization detection or equivalent
b. GC Column: Restek Rxi-624SilMS, 30 m x 0.32 mm x 1.8 µm
c. Oven Temperature: 50°C for 10 minutes, ramp 10°C/ minute to 240°C and maintain at 240°C for 5 minutes (total run time 34 minutes)
d. Flow: 2.1 mL/minute, Helium
e. Injection Technique: Split
f. Type of Liner: 4 mm Siltek liner

g. Inlet Temperature: 140°C
h. Split Flow: 2.1 mL/min
i. Split ratio: 1:1
j. Detector Temperature: FID at 250°C
k. Hydrogen flow: 30.0 mL/ min
l. Air flow: 300 mL/ min
m. Detector Makeup Gas: Nitrogen 28 mL/minute

5. Exemplary data system used is Waters® Empower™ 3 software.

6. Calculations and Reporting:
   a. System Suitability
      i. Make one injection of a blank sample into the system.
      ii. Make six replicate injections of a working standard solution.
      iii. Inject the loaded nanoparticle samples. If more than 10 loaded nanoparticle samples are to be tested, inject a working standard solution at an intermediate point so that each segment of a sequence of analyzed samples contains not more than 10 loaded nanoparticle samples.
      iv. Each sequence should conclude with a working standard solution injection followed by a blank preparation injection.
      v. Acceptance Criteria
         1. Percent relative standard deviation of a DMSO peak area response for six injections of Working Standard is no more than 15.0%.
         2. Overall percent relative standard deviation for all working standards is no more 15.0%.
   b. Sample Calculations
      i. Calculated concentration of DMSO in µg/g (ppm) in each smaple using Empower software, as shown in Equation 8:

$$\frac{\mu g}{g} = Cstd \times \frac{ACSpl}{ACStd} \times \frac{50.0mL}{g\ of\ Sample} \quad , \qquad (8)$$

Wherein:

Cstd = concentration of DMSO in a working standard in µg/mL;

AC spl = peak area of DMSO peak in a loaded nanoparticle sample; and

AC std = peak area of DMSO peak in a working standard (average of initial 6 injections)

ii. If no DMSO is detected in a loaded nanoparticle sample, this result is reported as less than LOQ.

c. Reporting

i. Report an amount of DMSO in a sample rounded up to an integer if above LOQ. If amount of DMSO in a sample is below LOQ, report "<1250 ppm", wherein 1250 ppm with respect to sample is an LOQ of a residual solvent evaluation for DMSO.

Microbial Limits Test

[0929] Exemplary loaded nanoparticles (e.g., NP-PN1)are tested for microbial limits according to sections 61 and 62 of United States Pharmacopeia, which are incorporated by reference herein in their entirety.

Example 26: Characterization of Exemplary Protein Loaded Nanoparticles

[0930] The present Example demonstrates characterization of certain protein loaded nanoparticles (e.g., PLGA Nanos or PN-NP1), e.g., nanoparticle size, zeta potential, allergen (e..g., peanut protein) distribution within a nanoparticle, and/or overall nanoparticle appearance.

[0931] **Table 51** shows concentration of various components of exemplary protein loaded nanoparticles (e.g., PN-NP1) described herein.

Table 51: Exemplary NP-PN1 Composition

Ingredient	Concentration (mg/mL)			Function
	0.125	**0.500**	**2.000**	
Peanut Allergen Extract, Lyophilized	0.125	0.500	2.000	Allergen
PLGA	2.264	9.057	36.24	Matrix
E. Coli Lipid Extract	0.098	0.393	1.572	TLR4 agonist
Sheared *E. Coli* DNA	0.009	0.036	0.145	TLR9 agonist
Potassium Phosphate, Monobasic	0.25	0.25	0.25	Buffer

Potassium Phosphate, Dibasic, Anhydrous	1.59	1.59	1.59	Buffer
Trehalose Dihydrate	94.3	94.3	94.3	Cryoprotecant
Purified Water	q.s. to 1 mL	q.s. to 1 mL	q.s. to 1 mL	Solvent

[0932] A Dynamic Light Scattering (DLS) measurement was performed to determine a z-average value of exemplary protein loaded nanoparticles (e.g., NP-PN1) described herein (e.g., as described in Example 25). A diameter determined by DLS is a hydrodynamic diameter - a measurement of the size of a particle plus its functional layer of molecules, ligands or other agents on the surface, when dispersed in a specific solution. An average diameter of exemplary protein loaded nanoparticle samples was 394 nm, ranging from 308 nm to 407 nm. Additionally, zeta potential was measured for multiple samples, yielding values ranging from -34 to -40 mV.

[0933] Free protein and total protein content were measured as described in Example 25. Encapsulated protein content ranged from 92% to 98% of total protein as measured exemplary protein loaded nanoparticle samples (e.g., 2-8% of free protein was found). Having no more than 20% of total protein content as free protein ensures that encapsulated protein is always above 80% of the total protein. Thus, no more than 20% of total protein is exposed on the surface of the nanoparticle.

[0934] Additionally, the exemplary protein loaded nanoparticles (e.g., NP-PN1) were tested for activation of basophil histamine release to assess surface exposed allergen. Basophils from human peanut allergic subjects were more reactive to a free peanut extract (e.g., peanut protein) than to NP-PN1. Specifically, human basophils were approximately 10 times less responsive to NP-PN1 than to an unencapsulated peanut extract (e.g., peanut protein).

[0935] Structure of exemplary protein loaded nanoparticles (e.g., NP-PN1) was evaluated by scanning electron microscopy (SEM). SEM allows for assessment of surface topography and size distributions of components of a sample. A representative image of a nanoparticle sample is shown in **FIG. 7**. **FIG. 7** depicts spherical nanoparticles of relatively uniform size with a few larger clusters observed in a sample.

Example 27: Batch Analysis of Exemplary Protein Loaded Nanoparticles

[0936] The present Example demonstrates analysis of multiple batches of exemplary protein loaded nanoparticles (e.g., NP-PN1) according to methods described in Example 25. The present Example shows total protein content, free protein content, average diameter, polydispersity indices, in vitro drug release and/or E. coli DNA content.

[0937] Exemplary protein loaded nanoparticles were manufactured as a bulk solution (e.g., a batch) and tested for total protein content (e.g., concentration) by BCA analysis. Each batch was loaded into ampoules. Volume of a solution comprising exemplary protein loaded nanoparticles was measured to ensure uniformity and potency of each sample.

[0938] A pooled sample of 6 to 7 ampoules from each batch was used to perform a BCA assay as described in Example 25, and number of ampoules used depended on concentration of a sample (e.g., 6 ampoules were used to analyze highly concentrated samples, whereas 7 ampoules were used to analyze less concentrated samples). Multiple samples and replicates were analyzed from pooled samples across multiple dilutions. Pooling multiple (e.g., 6 or 7) ampoule samples provided support for uniformity of protein content in exemplary protein loaded nanoparticle samples described herein. Ampoules for quality control testing described herein were randomized and selected for analysis from each batch containing 100 ampoules. Random selection of ampoules to performed to ensure representative sampling. Additionally, multiple batches with exemplary protein loaded nanoparticle samples were evaluated for uniformity between measurements.

[0939] Total protein content and free protein content were measured independently by two laboratories (e.g., Laboratory 1 and Laboratory 2). **FIG. 8** shows that total protein content measurements made were consistent between two laboratories. **FIG. 9** shows that free protein content measurements were consistent between two laboratories. Furthermore, batches 1 and 2 with lower concentrations (e.g., 0.125 mg/mL and 0.5 mg/mL, respectively) had proportionally less total and free protein (**FIGS. 8** and **9**).

[0940] Additional batches 12-20 of exemplary protein loaded nanoparticles were tested for total protein content (**FIG. 10**) and free protein content (**FIG. 11**) with a BCA assay. **FIG. 10** shows that, for all batches, measured total protein content was within a desired (e.g., target) concentration range. Safety factor, an exemplary calculation for which has been

shown in Example 25, was calculated based on the total and free protein contents. **FIG. 12** shows that safety factor was above 10 (e.g., above a desired threshold) for all batches.

[0941] Further quality control analysis comprised DLS analysis, which was performed for batches 1-20, for examples, as described in Example 25. **FIG. 13** shows that exemplary loaded nanoparticles from batches 12-20 were within a desired z-average diameter range between 225 and 400 nm. **FIG. 14** shows that PDIs of exemplary loaded nanoparticles from batches 12-20 were within a desired range from 0.1-0.4. **FIG. 15** shows similar z-average diameter results for batches 1-11.

[0942] PLGA concentration determination, as described in Example 25, was additionally performed for quality control analysis. **FIG. 16** shows that PLGA concentration corresponded to total protein and free protein content measurements for the same batches, e.g., as shown in **FIGS. 8** and **9** (e.g., PLGA concentration was proportional to protein loaded nanoparticle concentration in respective batches).

[0943] Acceptance criteria for a percent ratio of measured free to total protein content for batches 1-11 were met for all batches (**FIG. 17**). Additionally, acceptance criteria for a percent ratio of measured total protein content to target total protein content were also met (**FIG. 18**). These findings suggest particular suitability of exemplary protein loaded nanoparticles to be used in further pre-clinical and clinical studies.

[0944] Percent protein release from protein loaded nanoparticles in batches 1-10 was additionally evaluated, as described in Example 25. Percent protein release was greater for Batches 1 and 2, which contained lower concentrations of protein loaded nanoparticles than batches 3-10 (**FIG. 19**). **FIG. 19** further demonstrates that elease of protein from batches 3-10, which contained the same 2.0 mg/mL concentration of protein loaded nanoparticles, was consistent across all batches.

[0945] Final component of exemplary quality control analysis was assessment of E. coli DNA content in batches 1-11 via PCR, as described in Example 25. Total and free E. coli DNA content were both largely commensurate with protein loaded nanoparticle concentration, as shown in **FIG. 20**. **Table 52** shows values of measured total and free E. Coli DNA content in protein loaded nanoparticles.

Table 52. E. coli DNA Analysis for Total Protein in the Drug Product Batches

Test	Acceptance Criteria	Dose	0.125 mg/mL	0.5 mg/mL	2 mg/mL								
		Batch Number	Batch 1	Batch 2	Batch 3	Batch 4	Batch 5	Batch 6	Batch 7	Batch 8	Batch 9	Batch 10	Batch 11
Total E. Coli DNA (ng/mL)	Report Result : Total	Total	796	3,687	6,862	4,540	8,268	9,457	11,383	11,652	4,452	6,115	17,010
	Report Result : Free	Free	680	3,011	6,340	4,042	7,388	8,638	9,775	11,772	3,914	5,602	15,330

[0946] The present Example demonstrated that batches with exemplary protein loaded nanoparticles described herein had been manufactured consistently, and complied with established acceptance criteria (e.g., according to desired total and free protein concentrations, nanoparticle diameter, polydispersity indices, etc.), for example, to warrant use in a study (e.g., pre-clinical study, a clinical trial, a clinical study, etc.). The present Example further demonstrates favorable manufacturability of exemplary protein loaded nanoparticles described herein.

Example 28: Good Laboratory Practice (GLP)-compliant 4-week Repeat-Dose Toxicology Study with a 2-week Recovery Period with Daily Buccal Administration to Conscious Göttingen Minipigs

[0947] The present Example provides exemplary methods and results for a GLP mini-pig toxicology study performed to assess treatment-related effects of exemplary protein loaded nanoparticles (e.g., NP-PN1).

[0948] In a GLP toxicology study, either NP-PN1 in a formulation carrier (phosphate buffer, pH 9.0, and trehalose at 100 mg/mL) or vehicle (formulation carrier alone) was administered once daily by buccal administration. Specifically, NP-PN1 or vehicle was administered via a syringe injection into a cheek pouch, which was held for up to 5-10 seconds by an individual administering a sample to conscious naïve young male and female Göttingen Minipigs. Minipigs weighed between 10 and12 kg at the beginning of the study. The study was performed for four weeks. NP-PN1 was administered in doses of either 125 µg or 2000 µg. An objective of this study was to safety of NP-PN1, and/or potential reversibility of any observed toxicities after a 2-week-long dose free recovery period. Minipigs were terminated on Days 29 and 43 for treatment

(e.g., main study) and treatment and recovery (e.g., recovery) groups, respectively. Study design is shown in **Table 53**.

Table 53. Experimental Design for 4-weeks of Daily Buccal Administration in Minipigs

Test Material	Dose Level (µg/day)	Dose Volume (mL)	Concentration (µg/mL)	Number of Animals	
				Necropsy Day 29	Necropsy Day 43
				Males/Females	Males/Females
Placebo	0	1	0	3/3	2/2
NP-PN1	125	1	125	3/3	-
NP-PN1	2000	1	2000	3/3	2/2

[0949] The following parameters and end points were evaluated in minipigs: mortality, moribundity, clinical observations, body weights, ophthalmology, clinical pathology (hematology, clinical chemistry, coagulation, and urinalysis), gross necropsy, organ weights, a limited histopathological examination.

[0950] Minipigs were a chosen animal model, because minipigs have comparable oral mucosa and comparable oral immune subpopulations to humans (see, e.g., Thirion-Delande et al. 2017. PLoS One. 12(9):e0183398, which is incorporated by reference herein in its entirety).

[0951] In this minipig study, 125 and 2000 µg doses were administered once daily for 4-weeks. These dosing regiments comprised a 2-fold longer duration of dosing at the low (e.g., 125 µg) and high (e.g., 2000 µg) doses, a 1.5-fold higher total cumulative dose in minipigs , and a 7-fold higher cumulative dose in minipigs when factoring in species weight differences shown in **Table 54** (e.g., compared to the highest clinically administered dose level in the exemplary human clinical study described in Example 20). NP-PN1 and vehicle were administered to minipigs by buccal administration (1mL) via an NP-PN1 compatible syringe (e.g., a new Baxter Baxa Exacta-Med® Dispenser 1 mL syringe) to match an intended clinical route of administration via buccal administration (1 mL). During administration, each minipig was held by one technician, while a second technician administered an appropriate dose of NP-PN1 or a vehicle, and held the animal's mouth closed after administration for up to 10 seconds to ensure adequate oral absorption.

Table 54. Exemplary Clinical and Nonclinical Dose Regimens.

Species	First 2-weeks	Second 2-weeks	Third 2-weeks	Cumulative Dose Administered (µg)	Cumulative Dose Administered (µg/kg)[1]
Human	125 µg	500 µg	2000 µg	36,750 µg	525 µg/kg
Minipig	125 µg	125 µg	-	3,500 µg	233 µg/kg
Minipig	2000 µg	2000 ug	-	56,000 µg	3,733 µg/kg

[0952] Animals from the main study cohort were necropsied on Day 29, whereas animals from the recovery cohort were necropsied on Day 43. A full set of standard GLP systemic tissues were collected, weighed, and stored in fixative. Histopathology sectioning and staining with hematoxylin and eosin (H&E) was performed on a limited panel of tissues consistent with the buccal route of administration, e.g., dosed and contralateral cheeks and surrounding tissue, thymus, heart, lung, liver, kidneys, spleen, stomach, small intestine (incl. Peyer's patches) and large intestine, and lymph nodes (mandibular and mesenteric lymph nodes).

[0953] There was no placebo or NP-PN1-related mortality or moribundity, and no effect on animal body weight or food consumption was observed. There were no placebo- or NP-PN1-related changes in any ophthalmology, clinical pathology (e.g., hematology, clinical chemistry, coagulation, and urinalysis) parameters, or in organ weights.

[0954] Non-adverse observations were made during the gross necropsy. However, the nondiverse observations, and macroscopic and microscopic findings in examines tissues were considered procedure-related, and not a direct effect of the placebo or NP-PN1. Specifically, procedure-related effects of buccal administration and forced holding of minipigs' check to ensure absorption were considered. These observations included brown discoloration in the left dosed cheek of one low dose male and one high dose male. For a male minipig that received the high (e.g., 2000 µg) dose, this finding corresponded to a focal mild ulceration, associated with subacute inflammation of the subjacent submucosa and muscle tissue. A focal minimal erosion was also observed in the left cheek and the administration site of one placebo-arm female. In the right cheek (contralateral to the site of administration), focal but minimal perivascular infiltration of mixed inflammatory cells was seen in the muscle tissue in a female that had been administered a vehicle control, and focal but minimal degeneration and/or regeneration of muscle fibers was

observed in a low(e.g., 125 µg) dose female. These findings were considered to not be NP-PN1-related.

[0955] Vehicle and NP-PN1-related changes were limited to clinical observations of lip smacking and salivation and were not considered adverse.

[0956] There were no vehicle- or NP-PN1-related observations or changes in any parameter evaluated after the 2-week dose free recovery period. Furthermore, no brown discoloration or associated microscopic changes were observed in these animals. One female in a vehicle control arm had focal minimal infiltration of mixed inflammatory cells in the buccal salivary gland in the right contralateral cheek, which was considered incidental.

[0957] No vehicle control or NP-PN1 treatment-related adverse effects were identified after daily oral administration via the buccal mucosa to minipigs for 4 weeks of NP-PN1 at dose levels 0 (vehicle control), 125 and 2000 µg. Therefore, the no observed adverse effect level (NOAEL) in this study was considered to be 2000 µg/day.

[0958] The present Example demonstrated that there were no identified NP-PN1 treatment-related adverse effects (or vehicle control- related adverse effects) after oral administration via the buccal mucosa to minipigs for 4 weeks of NP-PN1 at dose levels 0 µg per animal (control), 125 µg per animal (low) and 2000 µg per animal (high). The present Example demonstrated administration of NP-PN1, including its sheared Escherichia coli (E. coli) DNA and E. coli lipid extract containing lipopolysaccharide (LPS) at doses proposed in a clinical trial protocol (e.g., as described in Example 20) did not show an adverse safety profile in minipigs.

Appendix 1

Table 23: SCHEDULE OF EVENTS FOR PART 1

	Screening	Baseline	Treatment Period		End of Treatment/Early Termination Visit	End of Study Follow-up
	Within 28 Days Prior to Day 1	Pre-Dose on Day 1	Day 1	Bi-Weekly Dose Escalation Visits (Days 15 ± 3 day and 29 ± 3 days)[m]	24 Hours After Last Dose (Day 43 ± 5 days)	4 Weeks After Last Dose (Day 71 ± 5 days) – via phone call
Informed consent form	X[a]					
Record demographic data	X					
Serologies[h]	X					
Complete medical/surgical history	X					
Update medical/surgical history		X				
Record medications taken within 30 days prior of Screening	X					
Record concomitant medications		X	X	X	X	X
Comprehensive physical examination[b]	X				X	
Targeted physical examination[c]		X	X	X		
Height	X					
Weight	X				X	
Vital signs[d]	X	X	X	X	X	
Spirometry/PEF[e]	X	X		X	X	
Electrocardiogram[f]	X					
Safety labs[g]	X	X		X	X	

	Screening	Baseline	Treatment Period		End of Treatment/Early Termination Visit	End of Study Follow-up
	Within 28 Days Prior to Day 1	**Pre-Dose on Day 1**	**Day 1**	**Bi-Weekly Dose Escalation Visits (Days 15 ± 3 day and 29 ± 3 days)[m]**	**24 Hours After Last Dose (Day 43 ± 5 days)**	**4 Weeks After Last Dose (Day 71 ± 5 days) – via phone call**
Urinalysis [h]	X	X			X	
Urine β-hCG (WCBP only)	X				X	
Total IgE & peanut-specific, peanut component-specific, and allergenic peanut epitope-specific IgE & peanut-specific and peanut allergenic epitope-specific IgG_4	X				X	
SPT[i]	X				X	
Assessment of inclusion/exclusion criteria[j]	X	X				
Randomize To Treatment			X			
Investigational product administration[k]			X	X		
Provide participant with investigational product for self-administration at home & instruct on buccal administration			X	X		
Provide participant diary[l]			X	X		
Review participant diary				X	X	
Collect investigational product and count				X	X	
AE monitoring		X	X	X	X	X

	Screening	Baseline	Treatment Period		End of Treatment/Early Termination Visit	End of Study Follow-up
	Within 28 Days Prior to Day 1	**Pre-Dose on Day 1**	**Day 1**	**Bi-Weekly Dose Escalation Visits (Days 15 ± 3 day and 29 ± 3 days)[m]**	**24 Hours After Last Dose (Day 43 ± 5 days)**	**4 Weeks After Last Dose (Day 71 ± 5 days) – via phone call**
Phone interview the day after each weekly dose escalation visit to assess for any AEs			X	X		
Instruct on identification & treatment of systemic reactions, including indications for self-injectable epinephrine; confirm epinephrine autoinjector and instruct on use			X	X		
Provide 24-hour emergency telephone number			X	X		

β-hCG = beta-chorionic gonadotropin; AE = adverse event; Ig = immunoglobulin; SPT = skin prick test; WCBP = women of childbearing potential

Appendix 2.

Table 24: SCHEDULE OF EVENTS FOR PART 2

	Screening	Baseline	Treatment Period			End of Treatment/Early Termination Visit	End of Study Follow-up
	Within 30 Days Prior to Day 1	Pre-Dose on Day 1	Day 1	Biweekly Dose Escalation Visits (Days 15 ± 3 days and 29 ± 3 days)[j,n]	Maintenance Visits[m] – Every 4 Months (Days 30 to 393 ± 5 Days)	24 Hours After Last Dose (Day 394 ± 5 Days)	4 Weeks After Last Dose – via Phone call (Day 421 ± 5 Days)
Informed consent form	X[a]						
Record demographic data	X						
Serologies[h]	X						
Complete medical/surgical history	X						
Update medical/surgical history		X					
Record medications taken within 30 days prior to Screening	X						
Record concomitant medications		X	X	X	X	X	X
Comprehensive physical examination[b]	X					X	
Targeted physical examination[c]		X	X	X	X		
Height	X						
Weight	X				X	X	
Vital signs[d]	X	X	X	X	X	X	
Spirometry/PEF[e]	X	X		X	X	X	
Electrocardiogram[f]	X					X	
Safety labs[g]	X	X		X	X	X	

	Screening	Baseline	Treatment Period			End of Treatment/Early Termination Visit	End of Study Follow-up
	Within 30 Days Prior to Day 1	**Pre-Dose on Day 1**	**Day 1**	**Biweekly Dose Escalation Visits (Days 15 ± 3 days and 29 ± 3 days)[j,n]**	**Maintenance Visits[m] – Every 4 Months (Days 30 to 393 ± 5 Days)**	**24 Hours After Last Dose (Day 394 ± 5 Days)**	**4 Weeks After Last Dose – via Phone call (Day 421 ± 5 Days)**
Urinalysis[h]	X	X					
Urine β-hCG (WCBP only)	X					X	
Total IgE & antigen-specific IgE & IgG_4	X				X	X	
SPT[i]	X					X	
Assessment of inclusion/exclusion criteria[j]	X	X					
Randomize to treatment			X				
Investigational product administration[k]			X	X	X		
DBPCFC	X					X	
Provide participant with investigational product for self-administration at home & instruct on buccal administration			X	X	X		
Provide participant diary[l]			X	X	X		
Review participant diary				X	X	X	
Collect investigational product and count				X	X	X	
AE monitoring		X	X	X	X	X	X

	Screening	Baseline	Treatment Period			End of Treatment/Early Termination Visit	End of Study Follow-up
	Within 30 Days Prior to Day 1	**Pre-Dose on Day 1**	**Day 1**	**Biweekly Dose Escalation Visits (Days 15 ± 3 days and 29 ± 3 days)[j,n]**	**Maintenance Visits[m] – Every 4 Months (Days 30 to 393 ± 5 Days)**	**24 Hours After Last Dose (Day 394 ± 5 Days)**	**4 Weeks After Last Dose – via Phone call (Day 421 ± 5 Days)**
Phone interview the day after each weekly dose escalation visit to assess for any AEs				X			
Instruct on identification & treatment of systemic reactions, including indications for self-injectable epinephrine; confirm epinephrine autoinjector and instruct on use			X	X	X		
Provide 24-hour emergency #			X	X	X		

β-hCG = beta-chorionic gonadotropin; AE = adverse event; Ig = immunoglobulin; SPT = skin prick test; WCBP = women of childbearing potential.

CLAIMS

What is claimed is:

1. A method of manufacturing a population of nanoparticles comprising steps of:
providing a first preparation, which comprises a hydrophilic payload in a first aqueous solvent system and a second preparation, which comprises a hydrophobic polymer in a second solvent system, wherein:
the second solvent system is non-aqueous, and
the hydrophobic polymer is not soluble in the first aqueous solvent system;
combining the first and second preparations to form a mixture that comprises the hydrophilic payload and the hydrophobic polymer in a combined solvent system; and
adding a non-solvent system to the mixture, so that a population of nanoparticles comprising the hydrophilic payload and the hydrophobic polymer is formed in a nanoparticle suspension
(*e.g.*, wherein the non-solvent system is a non-solvent of the hydrophobic polymer and the hydrophilic payload)
(*e.g.*, wherein the non-solvent system precipitates the hydrophilic payload and the hydrophobic polymer, so that each of the nanoparticles comprises the hydrophilic payload and the hydrophobic polymer).

2. The method of claim 1, wherein a ratio of the hydrophilic payload and the hydrophobic polymer in the nanoparticles is between 0.1 to 0.9 of a ratio of the hydrophilic payload and the hydrophobic polymer in the mixture.

3. The method of any preceding claims, further comprising removing a portion of the combined solvent system from the mixture.

4. The method of any preceding claims, further comprising adding a stabilizing agent solution to the nanoparticle suspension.

5. The method of claim 4, wherein the stabilizing agent solution comprises PVA.

6. The method of any preceding claims, further comprising purifying the population of nanoparticles (*e.g.*, filtration, (*e.g.*, tangential flow filtration), sonication, dilution).

7. The method of any preceding claims, further comprising drying the nanoparticle suspension.

8. The method of any preceding claims, wherein the population of nanoparticles has a mean size within a range of approximately 100-500 nm.

9. The method of any preceding claims, wherein the hydrophilic payload is selected from the group consisting of polypeptides, nucleic acids, and combinations thereof.

10. The method of claim 9, wherein the hydrophilic payload comprises a RNA.

11. The method of claim 10, wherein the hydrophilic payload comprises mRNA.

12. The method of claims 10-11, wherein the RNA comprises 200 to 1000000 residues.

13. The method of any preceding claims, wherein the hydrophilic payload is or comprises a crude preparation.

14. The method of any preceding claims, wherein the hydrophilic payload comprises antigen.

15. The method of claim 14, wherein the antigen is allergic antigen, infectious antigen, and/or disease-associated antigen.

16. The method of any preceding claims, wherein the combined solvent system comprises water and DMSO.

17. The method of claim 16, wherein a volume ratio of water and DMSO is within a range of 1:99 to 20:80, or 1:99 to 10:90.

18. The method of any preceding claims, wherein the non-solvent system comprises alcohol.

19. The method of claim 18, wherein the non-solvent system comprises propanol, ethanol, methanol, or combinations thereof.

20. The method of any preceding claims, wherein the hydrophobic polymer comprises PLG.

21. The method of any preceding claims, wherein the hydrophobic polymer has a molecular weight within a range of 5,000 – 5,000,000 Daltons.

22. A method comprising steps of:

providing a first liquid preparation, which comprises a fragile payload in a first aqueous solvent system and a second liquid preparation, which comprises a hydrophobic polymer in a second solvent system;

combining the first and second preparations to form a mixture that comprises the fragile payload and the hydrophobic polymer in a combined solvent system; and

adding a liquid non-solvent system to the mixture, so that a population of nanoparticles comprising the hydrophilic payload and the polymer is formed

(*e.g.*, wherein the method does not involve energy input)

(*e.g.*, wherein the non-solvent system does not degrade the fragile payload, or decrease one or more biological or pharmaceutical activities of the fragile payload)

(*e.g.*, wherein one or more biological or pharmaceutical activities of fragile payload are substantially same before and after the step of adding).

23. The method of claim 22, wherein the fragile payload is selected from the group consisting of polypeptides, nucleic acids, and combinations thereof.

24. The method of claim 23, wherein the fragile payload comprises a RNA.

25. The method of claim 24, wherein the fragile payload comprises mRNA.

26. The method of claims 24-25, wherein the RNA comprises 200 to 100000 residues.

27. The method of claim 23, wherein the fragile payload further comprises one or more structural proteins.

28. The method of claims 22-27, further comprising removing a portion of the combined solvent system from the mixture.

29. The method of claims 22-28, wherein a ratio of the fragile payload and the hydrophobic polymer in the nanoparticles is between 0.1 to 0.9 of a ratio of the fragile payload and the hydrophobic polymer in the mixture.

30. The method of claims 22-29, further comprising removing a portion of the combined solvent system from the mixture.

31. The method of claims 22-30, further comprising adding a stabilizing agent solution to a nanoparticle suspension comprising the population of nanoparticles.

32. The method of claim 31, wherein the stabilizing agent solution comprises PVA.

33. The method of claims 22-32, further comprising purifying the population of nanoparticles (*e.g.*, filtration, (*e.g.*, tangential flow filtration), sonication, dilution).

34. The method of claims 22-33, further comprising drying the nanoparticle suspension.

35. The method of claims 22-34, wherein the population of nanoparticles has a mean size within a range of approximately 100-500 nm.

36. The method of claims 22-35, wherein the fragile payload comprises antigen.

37. The method of claim 36, wherein the antigen is allergic antigen, infectious antigen, and/or disease-associated antigen.

38. The method of claims 22-37, wherein the combined solvent system comprises water and DMSO.

39. The method of claim 38, wherein a volume ratio of water and DMSO is within a range of 1:99 to 20:80, or 1:99 to 10:90.

40. The method of claims 22-39, wherein the non-solvent system comprises alcohol.

41. The method of claim 40, wherein the non-solvent system comprises propanol, ethanol, methanol, or combinations thereof.

42. The method of claims 22-41, wherein the hydrophobic polymer has a molecular weight within a range of 5,000 – 5,000,000 Daltons.

43. The method of claims 22-42, wherein the hydrophobic polymer comprises PLG.

44. A vaccine composition comprising a nanoparticle population comprising:
one or more payloads, or precursor(s) thereof, that activate antigen-specific T cells,
wherein the one or more payloads is/are displayed by an MHC class I complex or an MHC class II complex.

45. The vaccine composition of claim 44, further comprising an immune adjuvant.

46. The vaccine composition of claim 44, wherein the immune adjuvant is provided from one or more bacterial sources.

47. The vaccine composition of claims 45-46, wherein the immune adjuvant comprises cellular lysate (e.g., microbial lysate) or cellular lysate fractions.

48. The vaccine composition of claims 45-47, wherein the immune adjuvant is a mucosal immune adjuvant.

49. The vaccine composition of claims 44-48, wherein the nanoparticle population has a mean particle diameter of from 50 nm to 150 nm.

50. The vaccine composition of claims 44-49, wherein the one or more payloads are attached to a surface of nanoparticles of the nanoparticle population.

51. The vaccine composition of claims 44-50, wherein the hydrophobic polymer comprises PLG.

52. The vaccine composition of claims 44-51, further comprising a pore forming toxin.

53. A vaccine comprising first and second nanoparticle populations, wherein:
the first nanoparticle population comprises a first payload, or precursor(s) thereof, that activate first antigen-specific T cells, wherein the first payload is displayed by an MHC class I complex; and
the second nanoparticle population comprises a second payload, or precursor(s) thereof, that activate second antigen-specific T cells, wherein the second payload is displayed by an MHC class II complex.

54. The vaccine composition of claim 53, wherein the first and second nanoparticle populations are included in a same composition.

55. The vaccine composition of claims 53-55, wherein the vaccine further comprises an immune adjuvant.

56. The vaccine composition of claim 55, wherein the immune adjuvant is provided from one or more bacterial sources.

57. The vaccine composition of claims 55-56, wherein the immune adjuvant comprises cellular lysate (e.g., microbial lysate) or cellular lysate fractions.

58. The vaccine composition of claims 55-57, wherein the immune adjuvant is a mucosal immune adjuvant.

59. The vaccine composition of claims 53-58, wherein the first and/or second nanoparticle populations have a mean particle diameter of from 50 nm to 100 nm.

60. The vaccine composition of claims 53-59, wherein the first and/or second payloads are attached to a surface of nanoparticles of the first and/or second nanoparticle populations.

61. The vaccine composition of claims 53-60, wherein the hydrophobic polymer comprises PLG.

62. The vaccine composition of claims 53-61, further comprising a pore forming toxin.

63. A method comprising steps of:
administering to a subject in need thereof a nanoparticle composition comprising a nanoparticle population having one or more payloads, or precursor(s) thereof, that activate antigen-specific T cells, wherein the nanoparticle composition is administered orally, sublingually or buccally.

64. The method of claim 63, wherein the one or more payloads is/are displayed by an MHC class I complex.

65. The method of claim 63, wherein the one or more payloads is/are displayed by an MHC class II complex.

66. The method of claim 63, wherein the one or more payloads is/are displayed by an MHC class I complex and an MHC class II complex.

67. The method of claims 63-66, wherein the nanoparticle composition further comprises an immune adjuvant.

68. The method of claim 67, wherein the immune adjuvant is provided from one or more bacterial sources.

69. The method of claim 68, wherein the immune adjuvant comprises cellular lysate (e.g., microbial lysate) or cellular lysate fractions.

70. The method of claims 67-69, wherein the immune adjuvant is a mucosal immune adjuvant.

71. The method of claims 63-70, wherein the nanoparticle population has a mean particle diameter of from 50 nm to 100 nm.

72. The method of claims 63-71, wherein the one or more payloads are attached to a surface of nanoparticles in the nanoparticle population.

73. The method of claims 63-72, wherein the hydrophobic polymer comprises PLG.

74. The method of claims 63-73, wherein the nanoparticle composition comprises a pore forming toxin.

75. A method comprising steps of:

administering to a subject in need thereof a nanoparticle composition comprising a first nanoparticle population having a first payload, or precursor(s) thereof, that activate a first antigen-specific T cell, and a second nanoparticle population having a second

payload, or precursor(s) thereof, that activate a second antigen-specific T cell, wherein the nanoparticle composition is administered orally, sublingually or buccally.

76. The method of claim 75, wherein the first payload is displayed by an MHC class I complex, and the second payload is displayed by an MHC class II complex.

77. The method of claims 75-76, wherein the nanoparticle composition further comprises an immune adjuvant.

78. The method of claim 77, wherein the immune adjuvant is provided from one or more bacterial sources.

79. The method of claims 77-78, wherein the immune adjuvant comprises cellular lysate (e.g., microbial lysate) or cellular lysate fractions.

80. The method of claims 77-79, wherein the immune adjuvant is a mucosal immune adjuvant.

81. The method of claims 75-80, wherein the first and/or second nanoparticle populations have a mean particle diameter of from 50 nm to 100 nm.

82. The method of claims 75-81, wherein the first and/or second payloads are attached to a surface of nanoparticles in the first and/or second nanoparticle populations.

83. The method of claims 75-82, wherein the hydrophobic polymer comprises PLG.

84. The method of claims 75-83, the nanoparticle composition comprises a pore forming toxin.

85. A method comprising steps of:

administering to a subject in need thereof a first nanoparticle composition comprising a first nanoparticle population having a first payload, or precursor(s) thereof, that activate a first antigen-specific T cell;

administering to the subject a second nanoparticle composition comprising a second nanoparticle population having a second payload, or precursor(s) thereof, that activate a second antigen-specific T cell,

wherein the first and/or second nanoparticle compositions are administered orally, sublingually or buccally.

86. The method of claim 85, wherein the first payload is displayed by an MHC class I complex, and the second payload is displayed by an MHC class II complex.

87. The method of claims 85-86, wherein the first nanoparticle composition and/or the second nanoparticle composition further comprise an immune adjuvant.

88. The method of claims 85-87, wherein the immune adjuvant is provided from one or more bacterial sources.

89. The method of claim 88, wherein the immune adjuvant comprises cellular lysate (e.g., microbial lysate) or cellular lysate fractions.

90. The method of claims 87-88, wherein the immune adjuvant is a mucosal immune adjuvant.

91. The method of claims 85-90, wherein the first and/or second nanoparticle populations have a mean particle diameter of from 50 nm to 100 nm.

92. The method of claims 85-91, wherein the first and/or second payloads are attached to a surface of nanoparticles in the first and/or second nanoparticle populations.

93. The method of claims 85-92, wherein the hydrophobic polymer comprises PLG.

94. The method of claims 85-93, the first and/or nanoparticle composition(s) comprises a pore forming toxin.

95. The method of any one of claims 1-43, wherein the mixture comprising the hydrophilic payload and the hydrophobic polymer in a combined solvent system is layered under the non-solvent system.

96. The method of claim 95, wherein a stirring paddle is placed at the interface of the solvent system and the non-solvent system and the stirring paddle is operated to perform gentle stirring.

97. A nanoparticle preparation prepared by the method of claims 1-43, the nanoparticle preparation comprising a plurality of nanoparticles, each of which comprises a hydrophilic payload in a polymer.