

IMPROVED FORMULATION PROVIDING INCREASED STABILITY FOR VACCINES COMPOSITION

[0001] This application claims benefit of and priority to U.S. Provisional Application No. 63/617,736 filed on January 4, 2024, and is incorporated by reference in its entirety.

FIELD

[0002] The disclosure is generally related to the field of vaccine compositions and formulations. In one embodiment, the disclosure provides formulations for storage-stable liquid vaccine compositions.

BACKGROUND

[0003] Vaccine stabilization has been a long-lasting challenge and large amounts of vaccines have been wasted due to improper storage. Although global immunization currently saves the lives of 2-3 million children every year, of the 10.5 million child deaths that occur annually, 2.5 million are due to diseases that are preventable by vaccines.

[0004] Vaccines are biological substances that may lose their effectiveness quickly if they become too hot or too cold, especially during transport and storage. Inadvertent freezing, heating above 8° C, or other breaks in the cold chain may result in either failure of efficacy or vaccine wastage.

[0005] In 2020, the global COVID-19 pandemic intensified the demand for global cold chain and distribution networks as the first COVID-19 vaccines became available. According to the National Institutes of Health (NIH), the US government invested at least \$31.9bn to develop, produce, and purchase mRNA covid-19 vaccines, including sizeable investments in the three decades before the pandemic through March 2022 (Lalani HS, *et al.*, BMJ. 2023 Mar 14;380:p587). Despite the promising efficacy and safety of the mRNA COVID-19 vaccines, maintaining ultra-cold storage conditions is expensive and difficult to arrange in areas of the world with limited resources. Due to the short shelf life of these vaccines and vaccine hesitancy among the U.S. population, many expired COVID-19 vaccines end up discarded (Uddin MN, Roni MA., Vaccines (Basel). 2021 Sep 17;9(9):1033.). Globally, about half of the vaccines are wasted due to improper temperature control (World Health Organization. *Monitoring Vaccine Wastage at Country Level: Guidelines for Programme Managers*. World Health Organization; Geneva, Switzerland: 2005). One of the challenges is to develop a clinically effective

thermostable mRNA vaccine, which can be stored for a longer period without high storage costs.

[0006] With the considerable amount spent on vaccine development, even 1% vaccine wastage because of cold chain failure is a considerable sum. Indeed, for five U.S. states, the average wastage of 1% to 5% cost approximately \$6-\$31 million. In other parts of the world, vaccine wastage can reach 10%. The two most common forms of wastage relate to heat stability and shelf life, with inadvertent freezing remaining another key problem. The global COVID-19 pandemic highlighted the need for better cold storage solutions and a great need for storage-stable active agents, e.g., storage-stable vaccines, with longer shelf life that can maintain efficacy under various robust environmental conditions, e.g., without requiring cold chain compliance.

[0007] The present disclosure provides compositions and method of making liquid formulations for storage-stable vaccines. In one embodiment, the formulation of the present invention provides a vaccine composition wherein the vaccine potency drops less than 0.5 Log₁₀ when maintained at 4°C for at least 4 months.

SUMMARY

In accordance with the purpose(s) of this invention, as embodied and broadly described herein, this invention, in one aspect, relates to a liquid vaccine formulation for administering to a recipient comprising: an immunogen; at least 1X of a stabilizing agent, and at least 20-400 mM of a buffer; wherein the immunogen is capable of eliciting a protective immune response in the recipient; wherein the liquid formulation has a pH of about pH 6.0 and pH 8.0; and wherein the liquid formulation maintains storage-stable immunogenicity at 4°C for at least 4 months with a vaccine potency loss of less than 0.5 Log₁₀.

[0008] In one embodiment, the vaccine is against an enveloped virus selected from a group consisting of herpesviruses, cytoviruses, poxviruses, arenaviruses, arteriviruses, hepadnaviruses, flaviviruses, togaviruses, coronaviruses, orthomyxoviruses, paramyxoviruses, rhabdoviruses, bunyaviruses, filoviruses, baculoviruses, iridoviruses, and retroviruses. In another embodiment, the vaccine is against a coronavirus wherein the vaccine comprises a viral expression vector expressing a heterologous polypeptide comprising a spike (S) and/or nucleocapsid (N) proteins of a coronavirus. In yet another embodiment, the coronavirus is a severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), a variant of interest or a variant of concern of SARS-CoV-2, wherein the variant of interest or a variant of concern of SARS-CoV-2 is a SARS-CoV-2 beta variant,

a SARS-CoV-2 gamma variant, a SARS-CoV-2 delta variant, a SARS-CoV-2 omicron variant, or other variants SARS-CoV-2.

[0009] In one other embodiment, the stabilizing agent is selected from a group consisting of sucrose phosphate glutamate (SPG), carbohydrates, sodium glutamate, proteins such as peptone, albumin or casein, protein containing agents such as skimmed milk, an amino acid, buffers and combinations thereof. In some embodiment, the carbohydrate is selected from a group consisting of sorbitol, mannitol, lactose, sucrose, glucose, dextran, and trehalose. In other embodiments, the amino acid is selected from a group of consisting of arginine, histidine, lysine, proline, glycine, methionine, and glutamic acid and combinations thereof. In yet another embodiment, the stabilizing agent is the combination of sucrose phosphate glutamate and arginine.

[0010] In other embodiments, the buffer is selected from a group consisting of a phosphate buffer, citrate buffer, citrate phosphate buffer, borate buffer, tris(hydroxymethyl) aminomethane (Tris) containing buffer, succinate buffer, buffers containing glycine or histidine as one of the buffering agents. The buffer may be at a concentration of about 20, 30, 40, 50 mM, 60 mM, 70 mM, 80 mM, 90 mM, 100 mM, 110 mM, 120 mM, 130 mM, 140 mM, 150 mM, 160 mM, 170 mM, 180 mM, 190 mM, 200 mM, 210 mM, 220 mM, 230 mM, 240 mM, 250 mM, 260 mM, 270 mM, 280 mM, 290 mM, 300 mM, 310 mM, 320 mM, 330 mM, 340 mM, 350 mM, 360 mM, 370 mM, 380 mM, 390 mM, or 400 mM.

[0011] In yet another embodiment, the pH of the liquid formulation is about pH 6.0, pH 6.2, pH 6.4, pH 6.8, pH 7.0, pH 7.2, pH 7.4, pH 7.6, pH 7.8, pH 8.0, pH 8.2, pH 8.4, pH 8.6, pH 8.8, or pH 9.0. The liquid formulation maintains storage-stable immunogenicity for at least 1 month, 2 months, 3 months, 4 months, 5 months, 6 months or longer with a 0 Log₁₀, 0.1 Log₁₀, 0.2 Log₁₀, 0.3 Log₁₀, 0.4 Log₁₀, or 0.5 Log₁₀ loss in vaccine potency.

[0012] Additional advantages of the invention will be set forth in part in the description which follows, and in part will be obvious from the description, or may be learned by practice of the invention. The advantages of the invention will be realized and attained by means of the elements and combinations particularly pointed out in the appended claims. It is to be understood that both the foregoing general description and the following detailed description are exemplary and explanatory only and are not restrictive of the invention, as claimed.

BRIEF DESCRIPTIONS OF THE DRAWINGS

[0013] The accompanying drawings, which are incorporated in and constitute a part of this specification, illustrate (one) several embodiment(s) of the invention and together with the description, serve to explain the principles of the invention.

[0014] Figure 1 is a line graph showing the stability of CVXGA1 in 1xSPG. The vials of CVXGA1-PP3 MVS stock formulated in 1xSPG were placed at -80, -20 and 4°C, potency assay was performed at the indicated time points.

[0015] Figures 2A -2C are line graphs showing virus titer loss at pH from 6.6 to 7.8. CVXGA1 virus was formulated in 1xSPG+1% arginine buffer. The pH was adjusted to pH 6.6 to 7.8. then put at 34°C. Virus titers were tested. Log10 titer loss was calculated and shown.

[0016] Figures 3A-3D are line graphs showing the stability of CVXGA1 in citrate phosphate formulation. Fig. 3A shows CVXGA1 formulated in 20 or 200mM citrate phosphate with or without 1% Arginine and placed at 4°C. Fig. 3B shows CVXGA1 formulated in 50-400mM citrate phosphate with 1% Arginine and placed at 4°C. Fig. 3C shows CVXGA1 formulated in 50-200mM citrate phosphate with 1% Arginine were placed at 4°C. Fig. 3D shows CVXGA1 formulated in 20-50mM citrate phosphate with 1% Arginine were placed at 4°C. Potency assay was performed at the indicated time points. Log10 titer loss was calculated and shown. Potency assay was performed at the indicated time points. Log10 titer loss was calculated and shown.

[0017] Figures 4A-4F are line graphs showing anti-S IgG antibodies induced by CVXGA1 in mice. Figs. 4A-4C show that mice (Fig. 4A – All, Fig. 4B – female and Fig. 4C - male) were vaccinated with 10^6 PFU of CVXGA1 in frozen or liquid formulation. Figs. 4D-4F show that mice (Fig. 4D – All, Fig. 4E – female and Fig. 4F - male) were vaccinated with 10^5 PFU of CVXGA1 in frozen or liquid formulation.

DETAILED DESCRIPTION

[0018] The present invention may be understood more readily by reference to the following detailed description of preferred embodiments of the invention and the Examples included therein and to the Figures and their previous and following description.

I. Definitions

[0019] To facilitate an understanding of the principles and features of the various embodiments of the disclosure, various illustrative embodiments are explained herein. Although exemplary embodiments of the disclosure are explained in detail, it is to be

understood that other embodiments are contemplated. Accordingly, it is not intended that the disclosure is limited in its scope to the details of construction and arrangement of components set forth in the description or examples. The disclosure is capable of other embodiments and of being practiced or carried out in various ways.

[0020] In describing the exemplary embodiments, specific terminology will be resorted to for the sake of clarity. As used in the specification and the appended claims, the singular forms “a,” “an” and “the” include plural references unless the context clearly dictates otherwise. For example, reference to a component is intended also to include composition of a plurality of components. References to a composition containing “a” constituent is intended to include other constituents in addition to the one named.

[0021] Ranges may be expressed herein as from “about” or “approximately” or “substantially” one particular value and/or to “about” or “approximately” or “substantially” another particular value. When such a range is expressed, other exemplary embodiments include from the one particular value and/or to the other particular value.

[0022] Similarly, as used herein, “substantially free” of something, or “substantially pure”, and like characterizations, can include both being “at least substantially free” of something, or “at least substantially pure”, and being “completely free” of something, or “completely pure.”

[0023] The terms “patient”, “individual”, “subject”, and “animal” are used interchangeably herein and refer to mammals, including, without limitation, human and veterinary animals (e.g., cats, dogs, cows, horses, sheep, pigs, etc.) and experimental animal models. In a preferred embodiment, the subject is a human.

[0024] As used herein, the term “combination” of a PIV5-based composition as described herein and at least a second pharmaceutically active ingredient means at least two, but can mean any desired combination of compounds that can be delivered simultaneously or sequentially (e.g., within a 24-hour period). It is contemplated that when used to treat various diseases, the compositions and methods of the present disclosure can be utilized with other therapeutic methods/agents suitable for the same or similar diseases. Such other therapeutic methods/agents can be co-administered (simultaneously or sequentially, in any order) to generate additive or synergistic effects. Suitable therapeutically effective dosages for each agent may be lowered due to the additive action or synergy. Also, two or more embodiments of the disclosure may be also co-administered to generate additive or synergistic effects.

[0025] As used herein, the term “virus” refers to an infectious agent composed of a nucleic acid encapsulated in a protein. Such infectious agents are incapable of autonomous replication (i.e., replication requires the use of the host cell's machinery). Viral genomes can be single-stranded (ss) or double-stranded (ds), RNA or DNA, and can or cannot use reverse transcriptase (RT). Additionally, ssRNA viruses can be either sense (+) or antisense (−). Exemplary viruses include, but are not limited to, dsDNA viruses (e.g. Adenoviruses, Herpesviruses, Poxviruses), ssDNA viruses (e.g. Parvoviruses), dsRNA viruses (e.g. Reoviruses), (+)ssRNA viruses (e.g. Picornaviruses, Togaviruses), (−)ssRNA viruses (e.g. Orthomyxoviruses, Rhabdoviruses), ssRNA-RT viruses, i.e., (+)sense RNA with DNA intermediate in life-cycle (e.g. Retroviruses), and dsDNA-RT viruses (e.g. Hepadnaviruses). In some embodiments, viruses can also include wild-type (natural) viruses, killed viruses, live attenuated viruses, modified viruses, recombinant viruses or any combinations thereof. Other examples of viruses include, but are not limited to, enveloped viruses, respiratory syncytial viruses, non-enveloped viruses, bacteriophages, recombinant viruses, and viral vectors. The term “bacteriophages” as used herein refers to viruses that infect bacteria. In one embodiment, the disclosure provides liquid formulations for storage-stable vaccines against viruses.

[0026] The term “enveloped virus” refers to any of the viruses with a lipoprotein envelope surrounding the nucleoprotein core of the virus for example to Herpesviruses, Cytoviruses, Poxviruses, Arenaviruses, Arteriviruses, Hepadnaviruses, Flaviviruses, Togaviruses, Coronaviruses, Orthomyxoviruses, Paramyxoviruses, Rhabdoviruses, Bunyaviruses, Filoviruses, Baculoviruses, Iridoviruses, and Retroviruses, including human pathogens and the model virus Murine Leukemia Virus (MuLV). These viral envelopes can be derived from portions of the host cell membranes (phospholipids and proteins), but include some viral glycoproteins. Functionally, viral envelopes can be used to help viruses enter host cells. For example, glycoproteins on the surface of the envelope serve to identify and bind to receptor sites on the host's membrane. The viral envelope then fuses with the host's membrane, allowing the capsid and viral genome to enter and infect the host. However, as the viral envelope is relatively sensitive to desiccation, heat and detergents, these enveloped viruses can be sterilized more easily than non-enveloped viruses, and thus have limited survival outside host environments. In one embodiment, the disclosure provides liquid formulations for storage-stable vaccines against enveloped viruses.

[0027] The term “coronavirus” refers to a group of related RNA viruses that cause diseases in mammals and birds. In humans, these viruses cause respiratory tract infections that can range from mild to lethal. Mild illnesses include some cases of the common cold (which is caused also by certain other viruses, predominantly rhinoviruses), while more lethal varieties can cause SARS, MERS, and COVID-19.

[0028] The term “SARS” or “severe acute respiratory syndrome” refers to a viral respiratory disease of zoonotic origin that surfaced in the early 2000s caused by severe acute respiratory syndrome coronavirus (SARS-CoV or SARS-CoV-1), the first-identified strain of the SARS coronavirus species severe acute respiratory syndrome-related coronavirus (SARS-CoV). The syndrome caused the 2002-2004 SARS outbreak. In 2019, its successor, the related virus strain Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2), was discovered.

[0029] The term “Covid-19” or “Coronavirus disease 2019” refers to a severe acute respiratory syndrome (SARS) caused by a virus known as SARS-Coronavirus 2 (SARS-CoV2).

[0030] The term “immunogen” or “antigen” can be used interchangeably and refers to any substance, e.g., vaccines, capable of eliciting an immune response in a recipient. An “immunogen” or “antigen” is capable of inducing an immunological response against itself on administration to a subject. The term “immunological” or “antigenic” as used herein with respect to an immunological response, refers to the development of a humoral (antibody mediated) and/or a cellular (mediated by antigen-specific T cells or their secretion products) response directed against an immunogen in a recipient subject. Such a response can be an active response induced by administration of an immunogen or immunogenic peptide to a subject or a passive response induced by administration of antibody or primed T-cells that are directed towards the immunogen. A cellular immune response is elicited by the presentation of polypeptide epitopes in association with Class I or Class II MHC molecules to activate antigen-specific CD4⁺T helper cells and/or CD8⁺ cytotoxic T cells. Such a response can also involve activation of monocytes, macrophages, NK cells, basophils, dendritic cells, astrocytes, microglia cells, eosinophils or other components of innate immunity.

[0031] The term “vaccine” or “vaccine formulation” or “vaccine composition” as used herein refers to any preparation of killed microorganisms, live attenuated organisms, subunit antigens, toxoid antigens, conjugate antigens or other type of antigenic molecule that when introduced into a subjects body produces immunity to a specific disease by

causing the activation of the immune system, antibody formation, and/or creating of a T-cell and/or B-cell response. Generally, vaccines against microorganisms are directed toward at least part of a virus, bacteria, parasite, mycoplasma, or other infectious agent.

[0032] The term “immunogenicity” refers to the ability of a substance, such as an antigen or epitope, to provoke humoral and/or cell-mediated immunological response in a subject. The term “original immunogenicity” refers to the ability of the immunogen to retain its original potency or original ability to provoke humoral and/or cell-mediated immunological response in a subject. A skilled artisan can readily measure immunogenicity of a substance. The presence of a cell-mediated immunological response can be determined by any art-recognized methods, e.g., proliferation assays (CD4+ T cells), CTL (cytotoxic T lymphocyte) assays (see Burke, supra; Tigges, supra), or immunohistochemistry with tissue section of a subject to determine the presence of activated cells such as monocytes and macrophages after the administration of an immunogen. One of skill in the art can readily determine the presence of humoral-mediated immunological response in a subject by any well-established methods. For example, the level of antibodies produced in a biological sample such as blood can be measured by Western blot, ELISA or other methods known for antibody detection.

[0033] The formulations provided herein reduce the degradation rate of an immunogen (e.g., vaccine) at about 4° C for at least 4 months and maintain the original immunogenicity with potency loss less than 0.5 Log₁₀.

[0034] As described herein, the term “vaccinating” designates typically the sequential administration of one or more antigens to a subject, to produce and/or enhance an immune response against the antigen(s). The sequential administration includes a priming immunization followed by one or several boosting immunizations.

[0035] Within the context of the present invention, the term “pathogen” refers to any agent that can cause a pathological condition. Examples of “pathogens” include, without limitation, cells (e.g., bacteria cells, diseased mammal cells, cancer mammal cells), fungus, parasites, viruses, prions or toxins. Preferred pathogens are infectious pathogens. In a particular embodiment, the infectious pathogen is a virus, such as the coronaviruses.

[0036] An antigen, as used therein, can be used interchangeably with “immunogen” and generally designates any molecule or fragment which can cause a T-cell or B-cell immune response in a subject. An “antigen” or “immunogen” specific for a pathogen is, typically, an element obtained or derived from said pathogen, which contains an epitope,

and which can cause an immune response against the pathogen. Depending on the pathogenic agent, the “antigen” or “immunogen” may be a (poly)peptide, protein, nucleic acid, lipid, cell, and the like. Live weakened forms of pathogens (e.g., bacteria, viruses), or killed or inactivated forms thereof or purified material therefrom such as proteins, peptides, lipids, and the like may be used as well. The “antigen” or “immunogen” may be naturally occurring or artificially created. It may be exogenous to the treated mammal, or endogenous (e.g., tumor antigens). The antigen may be produced by techniques known per se in the art, such as, for example, by synthetic or recombinant technologies, or enzymatic approaches.

[0037] In a particular embodiment, the “antigen” or “immunogen” is a protein, polypeptide and/or peptide. The terms “polypeptide”, “peptide” and “protein” are used interchangeably herein to refer to a polymer of amino acid residues. The terms also apply to amino acid polymers in which one or more amino acid residues may be modified or non-naturally occurring residues, such as an artificial chemical mimetic of a corresponding naturally occurring amino acid. It should be understood that the term “protein” also includes fragments or variants of different antigens, such as epitope-containing fragments, or proteins obtained from a pathogen and subsequently enzymatically, chemically, mechanically or thermally modified.

[0038] A “therapeutically effective amount” means the amount of a vaccine composition or formulation (e.g., a PIV5-based vaccine composition as described herein) that, when administered to a subject effects an immunological response sufficient, for example, to elicit a protective response against a specific pathogen in a subject. The “therapeutically effective amount” will vary depending on a number of factors including but not limited to the vaccine composition administered as well as the protective response desired; the disease or pathogen against which the protective response is targeted; the age; weight; physical condition and responsiveness of the recipient in which the immune response is to be elicited.

[0039] The phrase “pharmaceutically acceptable”, as used in connection with compositions of the disclosure, refers to molecular entities and other ingredients of such compositions that are physiologically tolerable and do not typically produce untoward reactions when administered to a mammal (e.g., a human). Preferably, as used herein, the term “pharmaceutically acceptable” means approved by a regulatory agency of the Federal or a state government or listed in the U.S. Pharmacopeia or other generally recognized pharmacopeia for use in mammals, and more particularly in humans.

[0040] The term “pharmaceutically acceptable composition” as used herein refers to a composition comprising at least one compound as disclosed herein formulated together with one or more pharmaceutically acceptable carriers.

[0041] The term “administration” refers to the introduction of an amount of a predetermined substance into a patient by a certain suitable method. The composition disclosed herein may be administered via any of the common routes, as long as it is able to reach a desired tissue, for example, but is not limited to, inhaling, intraperitoneal, intravenous, intramuscular, subcutaneous, intradermal, oral, topical, intranasal, intrapulmonary, or intrarectal administration. However, since peptides are digested upon oral administration, active ingredients of a composition for oral administration should be coated or formulated for protection against degradation in the stomach.

[0042] The term “dose” means a single amount of a compound or an agent that is being administered thereto; and/or “regimen” which means a plurality of pre-determined doses that can be different in amounts or similar, given at various time intervals, which can be different or similar in terms of duration. In some embodiments, a regimen also encompasses a time of a delivery period (e.g., agent administration period, or treatment period). Alternatively, a regimen is a plurality of predetermined plurality pre-determined vaporized amounts given at pre-determined time intervals.

[0043] The term “carrier” refers to a diluent, adjuvant, excipient, or vehicle with which the compound is administered. Such pharmaceutical carriers can be sterile liquids, such as water and oils, including those of petroleum, animal, vegetable or synthetic origin, such as peanut oil, soybean oil, mineral oil, sesame oil and the like. Water or aqueous solution saline solutions and aqueous dextrose and glycerol solutions are preferably employed as carriers, particularly for injectable solutions. Alternatively, the carrier can be a solid dosage form carrier, including but not limited to one or more of a binder (for compressed pills), a glidant, an encapsulating agent, a flavorant, and a colorant. Suitable pharmaceutical carriers are described in “Remington's Pharmaceutical Sciences” by E. W. Martin.

[0044] The terms “stabilizing,” “stabilize,” “stability,” and “stabilization,” are used herein in reference to maintaining or retaining bioactivity of at least one active agent in a silk fibroin matrix. The addition of “stabilizing agent” or “stabilizer,” to the compositions described herein can further increase the stability of the active agent, i.e., the active agent can retain a higher bioactivity, relative to the bioactivity in the absence of the stabilizing agent. In some embodiments, the stabilizing agent is selected from the

group consisting of a saccharide, a sugar alcohol, an ion, a surfactant, and any combinations thereof. In one embodiment, the saccharide, e.g., sucrose, is added into the compositions described herein. Other stabilizing agents known in the art, e.g., for stabilizing other vaccines, can also be included in the compositions described herein, for example, amino acids, such as sodium glutamate, arginine, lysine, and cysteine; monosaccharides, such as glucose, galactose, fructose, and mannose; disaccharides, such as sucrose, maltose, and lactose; sugar alcohols such as sorbitol and mannitol; polysaccharides, such as oligosaccharide, starch, cellulose, and derivatives thereof; human serum albumin and bovine serum albumin; gelatin, and gelatin derivatives, such as hydrolyzed gelatin; and ascorbic acid as an antioxidant. These materials are described in publications, e.g., “Toketsu-Kanso To Hogo Busshitsu (Lyophilization And Protective Materials)” written by Nei, p. 1-176, published by Tokyo Daigaku Shuppan Kai (Publishing Association of the University of Tokyo), Japan in 1972; and “Shinku Gijutsu Koza (8): Sinku Kanso (Lecture on Vacuum Technology (8): Vacuum Drying)” written by Ota et al., p 176-182, published by Nikkan Kogyo Shimbun Co., Ltd., Japan in 1964.

[0045] As used herein, the term “storage-stable” refers to compositions, wherein the vaccine retains its potency for a period of time under one or more conditions specified herein. In some embodiments, the specified condition can be an environmental condition under which an active agent is stored and/or transported. Non-limiting examples of environmental conditions include temperatures, air pressures, humidity, and light exposure. In some embodiments, the compositions described herein can be immunogenic. In such embodiments, the active agent is an immunogen. In some embodiments, the immunogen is a vaccine. In one embodiment, the vaccine maintains its original immunogenicity at 4°C for more than 5 months with original immunogenicity loss of less than 0.5 Log₁₀.

[0046] The terms “treat” or “treatment” of a state, disorder or condition include: (1) preventing or delaying the appearance of at least one clinical or sub-clinical symptom of the state, disorder or condition developing in a subject that may be afflicted with or predisposed to the state, disorder or condition but does not yet experience or display clinical or subclinical symptoms of the state, disorder or condition; or (2) inhibiting the state, disorder or condition, i.e., arresting, reducing or delaying the development of the disease or a relapse thereof (in case of maintenance treatment) or at least one clinical or sub-clinical symptom thereof; or (3) relieving the disease, i.e., causing regression of the state, disorder or condition or at least one of its clinical or sub-clinical symptoms. The

benefit to a subject to be treated is either statistically significant or at least perceptible to the patient or to the physician.

[0047] As used herein, the term “parainfluenza virus 5” (PIV5) includes, for example and not limitation, strains KNU-11, CC-14, D277, 1168-1, and 08-1990. Non-limiting examples of PIV5 genomes are listed in GenBank Accession Nos. NC_006430.1, AF052755.1, KC852177.1, KP893891.1, KC237065.1, KC237064.1 and KC237063.1, which are hereby incorporated by reference.

[0048] As used herein, a “liquid vaccine” is a vaccine maintained as a liquid (including a liquid multivalent vaccine) that remains efficacious for at least four months when stored at 4° C. In particular embodiments, a liquid vaccine remains efficacious when stored at 4° C. for at least 4 months.

[0049] As used herein, an “efficacious” vaccine comprises a therapeutically effective amount of a given antigen. An “efficacious” vaccine retains sufficient titer for a given antigen to be compliant with the regulatory requirements for that antigen for the jurisdiction where the vaccine is administered, e.g., the administration of a vaccine in the United States is governed by the United States Department of Agriculture (USDA).

[0050] Because the liquid stable vaccines of the present invention ideally range in pH from pH 6.0 to pH 8.0, the liquid stable vaccines of the present invention can comprise a buffer. Buffers for use in the liquid stable vaccines of the present invention include but are not limited to: potassium phosphate, sodium phosphate, Tris, Tris-Histidine, BIS-Tris, BIS-Tris-Propane, sodium or potassium pyrophosphate, imidazole, PIPES, ACES, MOPS, MOPSO, BES, TES, tricine, glycylglycine, and HEPES. The buffers can be brought to the desired pH with the use of any suitable counter ion.

[0051] By “comprising” or “containing” or “including” is meant that at least the named compound, element, particle, or method step is present in the composition or article or method, but does not exclude the presence of other compounds, materials, particles, method steps, even if the other such compounds, material, particles, method steps have the same function as what is named.

[0052] The mention of one or more method steps does not preclude the presence of additional method steps or intervening method steps between those steps expressly identified. Similarly, it is also to be understood that the mention of one or more components in a composition does not preclude the presence of additional components than those expressly identified.

[0053] The materials described as making up the various elements of the disclosure are intended to be illustrative and not restrictive. Many suitable materials that would perform the same or a similar function as the materials described herein are intended to be embraced within the scope of the disclosure. Such other materials not described herein can include, but are not limited to, for example, materials that are developed after the time of the development of the disclosure

II. Compositions

[0054] The disclosure provides storage-stable PIV5-based liquid vaccine formulations, systems and methods for their use in multiple applications including functional genomics, drug discovery, target validation, protein production (e.g., therapeutic proteins, vaccines, monoclonal antibodies), gene therapy, and therapeutic treatments such as vaccination against viruses. In one embodiment, the storage-stable vaccine formulations are in liquid form. In another embodiment, the storage-stable vaccine formulations are against enveloped RNA and DNA viruses, wherein the enveloped virus” refers to any of the viruses with a lipoprotein envelope surrounding the nucleoprotein core of the virus for example to herpesviruses, cytoviruses, poxviruses, arenaviruses, arteriviruses, hepadnaviruses, flaviviruses, togaviruses, coronaviruses, orthomyxoviruses, paramyxoviruses, rhabdoviruses, bunyaviruses, filoviruses, baculoviruses, iridoviruses, and retroviruses.

A. SARS-CoV-2

[0055] As an example, the present disclosure provides a storage-stable liquid vaccine against coronavirus disease 2019 (COVID-19), wherein the vaccine comprises aparainfluenza virus type-5 (PIV5) virus expressing the SARS-CoV-2 envelope spike (S) and nucleocapsid (N) protein, as described in PCT/US2022/076732.

[0056] Coronavirus disease 2019 (COVID-19) is a newly emerging infectious disease currently spreading across the world. It is caused by a novel coronavirus, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) (Zhu et al., 2020, N Engl J Med; 382:727-733). SARS-CoV-2 was first identified in Wuhan, China in December 2019, and has subsequently spread globally to cause the COVID-19 pandemic. According to the World Health Organization (WHO), since 2019, COVID-19 has spread globally, infected more than 519 million people, and caused at least 6 million deaths. SARS-CoV-2 initially infects the upper respiratory tract epithelium (Wölfel, R., et al., Nature, 581(7809):465 (2020)) but can progress to the lower respiratory tract to cause pneumonia and acute respiratory distress syndrome (ARDS) (Huang, C., et al., Lancet,

395(10223):497 (2020)). Since the beginning of the 2019 coronavirus disease (COVID-19) pandemic, numerous SARS-CoV-2 variants have emerged. WHO defines a SARS-CoV-2 variant of concern (VOC) as a variant that affects virus transmissibility and COVID-19 epidemiology, increases virulence and pathogenicity, or decreases the effectiveness of in-place public health measures. Current VOCs include delta and omicron, while previously circulating VOCs include alpha, beta, and gamma.

[0057] SARS-CoV-2 is a single-stranded RNA-enveloped virus belonging to the β coronavirus family (Lu et al., 2020, *Lancet*; 395:565–74). An RNA-based metagenomic next-generation sequencing approach has been applied to characterize its entire genome, which is 29,881 nucleotides (nt) in length (GenBank Sequence Accession MN908947) encoding 9860 amino acids (Chen et al., 2020, *Emerg Microbes Infect*; 9:313–9). Full-genome sequenced genomes available at GenBank include isolate 2019-nCoV WHU01 (GenBank accession number MN988668) and NC_045512 for SARS-CoV-2, both isolates from Wuhan, China, and at least seven additional sequences (MN938384.1, MN975262.1, MN985325.1, MN988713.1, MN994467.1, MN994468.1, and MN997409.1) which are >99.9% identical and are hereby incorporate by reference.

1. SARS-CoV-2 Variants

[0058] Since SARS-CoV-2 was first identified in 2019, multiple genetic variants of SARS-CoV-2 have been emerging and circulating around the world. Viral mutations and variants in the United States are routinely monitored through sequence-based surveillance, laboratory studies, and epidemiological investigations. The US government SARS-CoV-2 Interagency Group (SIG) developed a Variant Classification scheme that defines three classes of SARS-CoV-2 variants: variant of interest, variant of concern and variant of high consequence.

a. Variant of Interest

[0059] A SARS-CoV-2 variant of interest is a variant with specific genetic markers that have been associated with changes to receptor binding, reduced neutralization by antibodies generated against previous infection or vaccination, reduced efficacy of treatments, potential diagnostic impact, or predicted increase in transmissibility or disease severity.

[0060] A variant of interest might require one or more appropriate public health actions, including enhanced sequence surveillance, enhanced laboratory characterization, or epidemiological investigations to assess how easily the virus spreads to others, the severity of disease, the efficacy of therapeutics and whether currently approved or

authorized vaccines offer protection. The growing list variants of interest that are being monitored and characterized include, but are not limited to, Eta, Iota, Kappa, Lambda and Mu.

b. Variant of Concern

[0061] A SARS-CoV-2 variant of concern is a variant for which there is evidence of an increase in transmissibility, more severe disease (e.g., increased hospitalizations or deaths), significant reduction in neutralization by antibodies generated during previous infection or vaccination, reduced effectiveness of treatments or vaccines, or diagnostic detection failures. Possible attributes of a variant of concern include evidence of impact on diagnostics, treatments, or vaccines, widespread interference with diagnostic test targets, evidence of substantially decreased susceptibility to one or more class of therapies, evidence of significant decreased neutralization by antibodies generated during previous infection or vaccination, evidence of reduced vaccine-induced protection from severe disease, evidence of increased transmissibility and evidence of increased disease severity.

[0062] Variants of concern might require one or more appropriate public health actions, such as notification to WHO under the International Health Regulations, reporting to CDC, local or regional efforts to control spread, increased testing, or research to determine the effectiveness of vaccines and treatments against the variant. Based on the characteristics of the variant, additional considerations may include the development of new diagnostics or the modification of vaccines or treatments. The growing list of variants of concern that are being closely monitored and characterized, includes, but is not limited to, Alpha, Beta, Delta, and Gamma.

c. Variant of High Consequence

[0063] A SARS-CoV-2 variant of high consequence has clear evidence that prevention measures or medical countermeasures (MCMs) have significantly reduced effectiveness relative to previously circulating variants. Possible attributes of a variant of high consequence include a demonstrated failure of diagnostic test targets, evidence to suggest a significant reduction in vaccine effectiveness, a disproportionately high number of vaccine breakthrough cases, or very low vaccine-induced protection against severe disease, significantly reduced susceptibility to multiple Emergency Use Authorization (EUA) or approved therapeutics and more severe clinical disease and increased hospitalizations.

[0064] A variant of high consequence would require notification to WHO under the International Health Regulations, reporting to CDC, an announcement of strategies to prevent or contain transmission, and recommendations to update treatments and vaccines. Currently, there are no SARS-CoV-2 variants that rise to the level of high consequence.

B. Parainfluenza Virus 5 (PIV5)

[0065] Parainfluenza virus 5 (PIV5), a negative-stranded RNA virus, is a member of the Rubulavirus genus of the family Paramyxoviridae which includes many important human and animal pathogens such as mumps virus, human parainfluenza virus type 2 and type 4, Newcastle disease virus, Sendai virus, HPIV3, measles virus, canine distemper virus, rinderpest virus and respiratory syncytial virus. PIV5 was previously known as Simian Virus-5 (SV5). Although PIV5 is a virus that infects many animals and humans, no known symptoms or diseases in humans have been associated with PIV5. Unlike most paramyxoviruses, PIV5 infect normal cells with little cytopathic effect. As a negative stranded RNA virus, the genome of PIV5 is very stable. As PIV5 does not have a DNA phase in its life cycle and it replicates solely in cytoplasm, PIV5 is unable to integrate into the host genome. Therefore, using PIV5 as a vector avoids possible unintended consequences from genetic modifications of host cell DNAs. PIV5 can grow to high titers in cells, including Vero cells which have been approved for vaccine production by WHO and FDA. Thus, PIV5 presents many advantages as a vaccine vector.

[0066] A PIV5-based vaccine vector of the present invention may be based on any of a variety of wild type, mutant, or recombinant (rPIV5) strains. Wild type strains include, but are not limited to, the PIV5 strains W3A, WR (ATCC® Number VR-288TM), canine parainfluenza virus strain 78-238 (ATCC number VR-1573) (Evermann et al., 1980, J Am Vet Med Assoc; 177:1132-1134; and Evermann et al., 1981, Arch Virol; 68:165-172), canine parainfluenza virus strain D008 (ATCC number VR-399) (Binn et al., 1967, Proc Soc Exp Biol Med; 126:140-145), MIL, DEN, LN, MEL, cryptovirus, CPI+, CPI-, H221, 78524, T1 and SER. See, for example, Chatziandreou et al., 2004, J Gen Virol; 85(Pt 10):3007-16; Choppin, 1964, Virology; 23:224-233; and Baumgartner et al., 1987, Intervirology; 27:218-223. Additionally, PIV5 strains used in commercial kennel cough vaccines, such as, for example, BI, FD, Merck, and Merial vaccines, may be used.

C. PIV5 CPI Vectored SARS-CoV-2 Constructs

[0067] A PIV5 vaccine vector of the present invention may be constructed using any of a variety of methods, including, but not limited to, the reverse genetics system

described in more detail in He et al. (Virology; 237(2):249-60, 1997). PIV5 encodes eight viral proteins. Nucleocapsid protein (NP), phosphoprotein (P) and large RNA polymerase (L) protein are important for transcription and replication of the viral RNA genome. The V protein plays important roles in viral pathogenesis as well as viral RNA synthesis. The fusion (F) protein, a glycoprotein, mediates both cell-to-cell and virus-to-cell fusion in a pH-independent manner that is essential for virus entry into cells. The structures of the F protein have been determined and critical amino acid residues for efficient fusion have been identified. The hemagglutinin-neuraminidase (HN) glycoprotein is also involved in virus entry and release from the host cells. The matrix (M) protein plays an important role in virus assembly and budding. The hydrophobic (SH) protein is a 44-residue hydrophobic integral membrane protein and is oriented in membranes with its N terminus in the cytoplasm. For reviews of the molecular biology of paramyxoviruses see, for example, Whelan et al., 2004, *Curr Top Microbiol Immunol*; 283:61-119; and Lamb & Parks, (2006). *Paramyxoviridae: the viruses and their replication*. In *Fields Virology*, 5th edn, pp. 1449–1496. Edited by D. M. Knipe & P. M. Howley. Philadelphia, PA: Lippincott Williams & Wilkins.

[0068] Previously, recombinant PIV5 viruses expressing foreign genes from numerous pathogens, including Influenza, Rabies, Respiratory Syncytial Virus, *Tuberculosis*, *Burkholderia*, and MERS-CoV have been generated and tested as vaccine candidates (Li, Z., et al., *J Virol*, 87(1):354 (2013); Chen, Z., et al., *J Virol*, 87(6): 2986 (2013); Wang, D., et al., *J Virol*, 91(11) (2017); Chen, Z., et al., *Vaccine*, 33(51):7217 (2015); Lafontaine, E.R., et al., *Vaccine X*, 1:100002 (2018); Li, K., et al., *mBio*, 11(2) (2020)). Because it actively replicates in the respiratory tract following intranasal immunization, PIV5-vectored vaccines can generate mucosal immunity that includes antigen-specific IgA antibodies and long-lived IgA plasma cells (Wang, D., et al., *J Virol*, 91(11) (2017). Xiao, P., et al., *Front Immunol*, 12:623996 (2021)). Recently a PIV5-vectored vaccine expressing the spike protein from SARS-CoV-2 Wuhan (WA1; CVXGA1) has been shown to be efficacious in mice and ferrets. A single, intranasal dose of CVXGA1 induced WA1-neutralizing antibodies and protected K18-hACE2 mice against lethal infection with SARS-CoV-2 WA1. Furthermore, a single, intranasal dose of CVXGA1 protected ferrets from infection with SARS-CoV-2 WA1 and blocked transmission to cohoused naïve ferrets (An, D., et al., *Sci Adv*, 7(27) (2021)). While these studies determined its efficacy against SARS-CoV-2 WA1, further studies were necessary to establish its efficacy against SARS-CoV-2 variants.

1. PIV5-based Vaccine Vectors Encoding the SARS-CoV-2 Spike (S) Protein

[0069] With the PIV5-based vaccine vectors of the present invention, a heterologous nucleotide sequence encoding the spike (S) protein of a coronavirus, including, but not limited to, the S protein of SARS-CoV-2, is inserted in the PIV5 genome. Coronavirus entry into host cells is mediated by the transmembrane S glycoprotein (Tortorici and Veasley, 2019, *Adv Virus Res*; 105:93-116). As the coronavirus S glycoprotein is surface-exposed and mediates entry into host cells, it is the main target of neutralizing antibodies upon infection and the focus of therapeutic and vaccine design. The spike S protein of SARS-CoV-2 is composed of two subunits, S1 and S2. The S1 subunit contains a receptor-binding domain that recognizes and binds to the host receptor angiotensin-converting enzyme 2, while the S2 subunit mediates viral cell membrane fusion by forming a six-helical bundle via the two-heptad repeat domain. (Huang et al., 2020, *Acta Pharmacol Sinica*; 0:1–9 (available on the worldwide web at doi.org/10.1038/s41401-020-0485-4).

[0070] The total length of SARS-CoV-2 S is 1273 amino acids (aa) and consists of a signal peptide (amino acids 1-13) located at the N-terminus, the S1 subunit (14-685 residues), and the S2 subunit (686-1273 residues); the last two regions are responsible for receptor binding and membrane fusion, respectively. In the S1 subunit, there is an N-terminal domain (14–305 residues) and a receptor-binding domain (RBD, 319-541 residues); the fusion peptide (FP) (788-806 residues), heptapeptide repeat sequence 1 (HR1) (912-984 residues), HR2 (1163-1213 residues), TM domain (1213-1237 residues), and cytoplasm domain (1237-1273 residues) comprise the S2 subunit (Xia et al., 2020, *Cell Mol Immunol*; 17:765–7).

[0071] In some PIV5-based vaccine vectors of the present invention, the heterologous nucleotide sequence encoding the spike (S) protein of a coronavirus, including, but not limited to, the S protein of SARS-CoV-2, has been modified so that the cytoplasmic tail of the coronavirus S protein has been replaced with the cytoplasmic tail of the fusion (F) protein of PIV5. An example of such a PIV5 construct includes the PIV5 construct CVX-GA1, also referred to herein as CVXGA1, CVX-UGA1, pDA27, or DA27. CVXGA1, recombinant PIV5 expressing S from SARS-CoV-2 WA1, has completed phase 1 clinical trial in the US (Clinical Trials. *Phase 1 Study of Intranasal PIV5-vectored COVID-19 Vaccine Expressing SARS-CoV-2 Spike Protein in Healthy Adults (CVXGA1-001)* 2021). A Phase 2 study (NCT05736835) with CVXGA35 vaccine

(PIV5 expressing SARS-CoV-2 XBB.1.5 S protein) is being evaluated in adults and elderly.

[0072] In some PIV5-based vaccine vectors of the present invention, the heterologous nucleotide sequence encoding the coronavirus S protein, including but not limited to, the S protein of SARS-CoV-2, has been modified so that the S protein includes an amino acid substitution at amino acid residue W886 and/or F888. In some aspects, the amino acid substitution at amino acid residue W886 includes a substitution of tryptophan (W) to arginine (R) and/or the amino acid substitution at amino acid residue W888 includes a substitution of phenylalanine (F) to arginine (R).

[0073] In some PIV5-based vaccine vectors of the present invention, the heterologous nucleotide sequence encoding the spike (S) protein of a coronavirus, including, but not limited to, the S protein of SARS-CoV-2, includes both a modification so that the cytoplasmic tail of the coronavirus S protein has been replaced with the cytoplasmic tail of the fusion (F) protein of PIV5 and includes an amino acid substitution at amino acid residue W886 and/or F888. In some aspects, the amino acid substitution at amino acid residue W886 includes a substitution of tryptophan (W) to arginine (R) and/or the amino acid substitution at amino acid residue W888 includes a substitution of phenylalanine (F) to arginine (R). An example of such a PIV5 construct includes the PIV5 construct CVX-GA2, also referred to herein as CVXGA2 or CVX-UGA2.

[0074] The heterologous nucleotide sequence encoding the coronavirus S protein, including but not limited to the S protein of SARS-CoV-2, may be inserted in any of a variety of locations in the PIV5 genome.

[0075] In some embodiments, the heterologous nucleotide sequence encoding the coronavirus S protein, including but not limited to the S protein of SARS-CoV-2, may be inserted between the small hydrophobic protein (SH) gene and the hemagglutinin-neuraminidase (HN) gene of the PIV5 genome.

[0076] The heterologous nucleotide sequence encoding the coronavirus S protein, including but not limited to the S protein of SARS-CoV-2, may be inserted between the hemagglutinin-neuraminidase (HN) and large RNA polymerase protein (L) gene of the PIV5 genome. In some embodiments, the heterologous nucleotide sequence is not inserted at a location between the hemagglutinin-neuraminidase (HN) and large RNA polymerase protein (L) gene of the PIV5 genome. In some embodiments, the heterologous nucleotide sequence is inserted at a location other than between the

hemagglutinin-neuraminidase (HN) and large RNA polymerase protein (L) gene of the PIV5 genome.

[0077] The heterologous nucleotide sequence encoding the coronavirus S protein, including but not limited to the S protein of SARS-CoV-2, may be inserted between the nucleocapsid protein (NP) gene and the V/P gene of the PIV5 genome.

[0078] The heterologous nucleotide sequence encoding the coronavirus S protein, including but not limited to the S protein of SARS-CoV-2, may be inserted between the M gene and the F gene of the PIV5 genome.

[0079] The heterologous nucleotide sequence encoding the coronavirus S protein, including but not limited to the S protein of SARS-CoV-2, may be inserted between the F gene and the SH gene of the PIV5 genome.

[0080] The heterologous nucleotide sequence encoding the coronavirus S protein, including but not limited to the S protein of SARS-CoV-2, may be inserted between the VP gene and the matrix protein (M) gene of the PIV5 genome.

[0081] The heterologous nucleotide sequence encoding the coronavirus S protein, including but not limited to the S protein of SARS-CoV-2, may be inserted between the leader sequence and the nucleocapsid protein (NP) gene of the PIV5 genome.

[0082] The heterologous nucleotide sequence encoding the coronavirus S protein, including but not limited to the S protein of SARS-CoV-2, may be inserted immediately downstream of the leader sequence of the PIV5 genome.

[0083] The heterologous nucleotide sequence encoding the coronavirus S protein, including but not limited to the S protein of SARS-CoV-2, may be inserted to replace all or part of a PIV5 gene within the PIV5 genome. For example, the heterologous nucleotide sequence may replace the F, HN, or SH gene of the PIV5 genome. A heterologous nucleotide sequence may be inserted within a PIV5 gene, resulting in the expression of a chimeric polypeptide. For example, the heterologous nucleotide sequence may be inserted within the SH gene nucleotide sequence, within the NP gene nucleotide sequence, within the V/P gene nucleotide sequence, within the M gene nucleotide sequence, within the F gene nucleotide sequence, within the HN gene nucleotide sequence, and/or within the L gene nucleotide sequence of a PIV5 genome.

[0084] The heterologous nucleotide sequence encoding the coronavirus S protein may be produced by inserting the coronavirus S protein gene from different variants into PIV5 canine parainfluenza virus (CPI) vector: CVXGA1, 3, 4, 5, 6, 13, 14 and 16.2.

PIV5-based vaccine vectors encoding the SARS-CoV-2 spike (S) and nucleocapsid (N) proteins

[0085] PIV5 canine parainfluenza virus (CPI) vaccine vectors encoding SARS-CoV-2 variants of concern or variants of interest are disclosed herein. The PIV5-based vaccine vectors may comprise inserting the Spike (S) protein gene from different variants into PIV5 CPI vector: CVXGA1, 3, 4, 5, 6, 13, 14 and 16.

[0086] To improve vaccine efficacy, the SARS-CoV-2 nucleocapsid (N) may be inserted between the HN and L gene junction in addition to the SARS-CoV-2 spike (S) inserted at the SH and HN junction. In studies disclosed herein, the expression of both the S and N proteins of SARS-CoV-2 have been shown to offer additional protection for the vaccine especially the cellular immune responses offered by the N protein.

[0087] Since the CPI-S+N has reduced titer/yield, the construct may be further modified by moving the SARS-CoV-2 N protein gene to the PIV5 CPI SH gene location as SH deletion has been shown not impacting virus growth. CVXGA7 was constructed to have the N and S genes from SARS-CoV-2 inserted into the CPI Δ SH backbone to produce CPI Δ SH-N+S.

[0088] The mutations in the V/P gene, S157F and S308A, have been shown previously to increase viral polymerase activities and improve viral titer/yield (Timani KA, Sun D, Sun M, et al. A single amino acid residue change in the P protein of parainfluenza virus 5 elevates viral gene expression. J Virol. 2008;82(18):9123-9133. doi:10.1128/JVI.00289-08; Sun D, Luthra P, Li Z, He B. PLK1 down-regulates parainfluenza virus 5 gene expression. PLoS Pathog., 5(7):e1000525 (2009)). Mutations S157F and S308A are herein introduced into the CPI V/P gene to produce CVXGA10 (CPI-S-PLK) and CVXGA12 (CPI Δ SH-S-PLK) viruses.

[0089] A PIV5 viral vaccine of the present invention may also have a mutation, alteration, or deletion in one or more of these eight proteins of the PIV5 genome. For example, a PIV5 viral expression vector may include one or more mutations, including, but not limited to any of those described herein. In some aspects, a combination of two or more (two, three, four, five, six, seven, or more) mutations may be advantageous and may demonstrate enhanced activity.

[0090] A mutation includes, but is not limited to, a mutation of the V/P gene, a mutation of the shared N-terminus of the V and P proteins, a mutation of residues 26, 32, 33, 50, 102, and/or 157 of the shared N-terminus of the V and P proteins, a mutation lacking the C-terminus of the V protein, a mutation lacking the small hydrophobic (SH)

protein, a mutation of the fusion (F) protein, a mutation of the phosphoprotein (P), a mutation of the large RNA polymerase (L) protein, a mutation incorporating residues from canine parainfluenza virus, and/or a mutation that enhances syncytial formation.

[0091] A mutation may include, but is not limited to, rPIV5-V/P-CPI-, rPIV5-CPI-, rPIV5-CPI+, rPIV5V Δ C, rPIV-Rev, rPIV5-RL, rPIV5-P-S157A, rPIV5-P-S308A, rPIV5-L-A1981D and rPIV5-F-S443P, rPIV5-MDA7, rPIV5 Δ SH-CPI-, rPIV5 Δ SH-Rev, and combinations thereof.

[0092] PIV5 can infect cells productively with little cytopathic effect (CPE) in many cell types. In some cell types, PIV5 infection causes formation of syncytia, i.e., fusion of many cells together, leading to cell death. A mutation may include one or more mutations that promote syncytia formation (see, for example Paterson et al., 2000, *Virology*; 270:17-30).

[0093] The V protein of PIV5 plays a critical role in blocking apoptosis induced by virus. Recombinant PIV5 lacking the conserved cysteine-rich C-terminus (rPIV5V Δ C) of the V protein induces apoptosis in a variety of cells through an intrinsic apoptotic pathway, likely initiated through endoplasmic reticulum (ER)-stress (Sun et al., 2004, *J Virol*; 78:5068-5078). Mutant recombinant PIV5 with mutations in the N-terminus of the V/P gene products, such as rPIV5-CPI-, also induce apoptosis (Wansley and Parks, 2002, *J Virol*; 76:10109-10121). A mutation includes, but is not limited to, rPIV5 Δ SH, rPIV5-CPI-, rPIV5V Δ C, and combinations thereof.

[0094] Also included in the present invention are virions and infectious viral particles that include a PIV5 genome including a heterologous nucleotide sequence encoding a coronavirus S protein, including but not limited to the S protein of SARS-CoV-2.

[0095] Also included in the present invention are compositions including one or more of the PIV5 viral constructs or virions, as described herein. Such a composition may include a pharmaceutically acceptable carrier. As used, a pharmaceutically acceptable carrier refers to one or more compatible fillers, diluents or encapsulating substances which are suitable for administration to a human or other vertebrate animal. Such a carrier may be pyrogen free. The present invention also includes methods of making and using the viral vectors and compositions described herein.

[0096] The compositions of the present disclosure may be formulated in pharmaceutical preparations in a variety of forms adapted to the chosen route of

administration. One of skill will understand that the composition will vary depending on mode of administration and dosage unit.

[0097] The agents of this invention can be administered in a variety of ways, including, but not limited to, intravenous, topical, oral, intranasal, subcutaneous, intraperitoneal, intramuscular, and intratumor deliver. In some aspects, the agents of the present invention may be formulated for controlled or sustained release. One advantage of intranasal immunization is the potential to induce a mucosal immune response.

III. Methods of Making

A. Vaccine Formulations

[0098] The global spread of SARS-CoV-2 prompted rapid development of prophylactic vaccines. A parainfluenza virus type 5 (PIV5)-vectored intranasal COVID-19 vaccine was previously introduced. CVXGA1 vaccine, PIV5 expressing SARS-CoV-2 WA1 S protein, has completed a Phase 1 study in adults and teens (NCT04954287) and shown to be well-tolerated and immunogenic. A Phase 2 study (NCT05736835) with CVXGA35 vaccine (PIV5 expressing SARS-CoV-2 XBB.1.5 S protein) is being evaluated in adults and elderly. The current CVXGA vaccine is a frozen formulation formulated in 1xSPG (sucrose phosphate glutamate) buffer with long term stability at temperatures less than -20°C, but not at 4°C. Therefore, a liquid formulation that can be maintained at 4°C is greatly preferred for the commercial product.

1. PIV5 Vaccine Liquid Formulations

[0099] The present disclosure presents compositions and methods of making and using vaccine liquid formulations, including, but not limited to any of those described herein. Presented herein are liquid formulations that can maintain vaccine immunogenicity at 4°C for more than 5 months with immunogenicity loss less than 0.5 Log₁₀ (assay variation limit).

[00100] The intranasal vaccine CVXGA1 (PIV5 expressing SARS-CoV-2 S protein) is a promising next generation COVID-19 vaccine to boost intranasal and cellular immunity. The CVXGA vaccine viruses used in phase 1 clinical (NCT04954287) and Phase 2 studies are frozen products formulated in sucrose phosphate glutamate (SPG, sucrose, KH₂PO₄, K₂HPO₄, and L-glutamic acid) buffer that is stable at temperatures less than -20°C, but not at 4°C for extended time. Ultra-cold chain storage limits vaccine distribution and administration. Developing a new liquid formulation with extended stability at 4°C is critical to the PIV5 vectored intranasal COVID vaccine product development.

[00101] In one embodiment, the pH of the composition is between pH 6.0 and pH 8.0. In another specific embodiment, the pH of the claimed composition is between about pH 6.8 to about pH 7.8. By 'about pH' is meant within 0.2 units of the stated pH value. In particular, the pH of the composition is between pH 6.8 and pH 7.8. For example, the pH of the composition is between about pH 6.0 to about pH 7.0, in particular between pH 6.0 and pH 7.0, typically between pH 6.2 and pH 6.8 or between pH 6.2 and pH 6.6. A pH of about 6.4, in particular of 6.4, is contemplated.

[00102] In another embodiment, the composition according to this disclosure, can be preserved and/or conserved and stored either in liquid form, at about 4° C in the presence of a stabilizer. The pharmaceutically acceptable stabilizers may be sucrose phosphate glutamate (SPG), carbohydrates (e.g., sorbitol, mannitol, lactose, sucrose, glucose, dextran, trehalose), sodium glutamate (Tsvetkov, T. et al., 1983; Israeli, E. et al., 1993), proteins such as peptone, albumin or casein, protein containing agents such as skimmed milk (Mills, C. K. et al., 1988; Wolff, E. et al., 1990), and buffers (e.g., phosphate buffer, alkaline metal phosphate buffer). An adjuvant and/or a vehicle or excipient may be used to make liquid preparations. Many excipients were screened and it was found that sorbitol, histidine, lysine, malic acid, and citrate phosphate improve vaccine stability. The top liquid formulation candidate is citrate phosphate and arginine in 1x SPG.

[00103] In another embodiment, the composition is prepared in a buffer that may be selected from the list comprising of phosphate buffer, citrate buffer, citrate phosphate buffer, borate buffer, tris(hydroxymethyl) aminomethane (Tris) containing buffer, succinate buffer, buffers containing glycine or histidine as one of the buffering agents. In some embodiments, the buffer is citrate phosphate buffer at concentration of 20 mM up to 400 mM of phosphate ions of any pH between 6.0 to pH 8.0. The buffer maintains the pH in a liquid composition above pH 6.5 throughout the bioprocess from viral culture up to preparation of purified inactivated virus bulk. In other embodiments, the composition is formulated in 50-100mM citrate phosphate plus 1% arginine in 1xSPG buffer.

[00104] The liquid stable vaccines of the present invention comprise an amino acid. In certain embodiments the amino acid is arginine. In other embodiments, the amino acid is selected from a group of consisting of histidine, lysine, proline, glycine, methionine, glutamic acid or the combination thereof.

[00105] The Examples presented herein show that the composition as formulated was stable for at least 4 months at 4°C with 50mM citrate phosphate in 1xSPG plus 1% arginine buffer maintained the potency for up to 5 months. In vivo mouse study showed

that CVXGA1 in the top formulation was as immunogenic as the frozen formulation measured by anti-S IgG antibody titers. The identification of liquid stable CVXGA1 will solve the problem of ultra-cold chain storage limits and ease the process of vaccine distribution and administration. The liquid formulation developed in this proposal should be applicable for other PIV5-based vaccines that are in preclinical and clinical development.

IV. Methods of Treating

[00106] In certain embodiments of the disclosure, PIV5 compositions can be utilized to prepare antigenic preparations that be used as vaccines. In other embodiments, the vaccines compositions may be prepared in liquid form. Any suitable antigen(s) can be prepared in accordance with the disclosure, including antigens obtained from prions, viruses, mycobacterium, protozoa (e.g., *Plasmodium falciparum* (malaria)), trypanosomes, bacteria (e.g., *Streptococcus*, *Neisseria*, etc.), etc.

[00107] Vaccines often contain a plurality of antigen components, e.g., derived from different proteins, and/or from different epitopic regions of the same protein. For example, a vaccine against a viral disease can comprise one or more polypeptide sequences obtained from the virus which, when administered to a host, elicit an immunogenic or protective response to viral challenge.

[00108] The disclosure can also be utilized to prepare polypeptide multimers, e.g., where an antigenic preparation is produced which is comprised of more than one polypeptide. For instance, virus capsids can be made up of more than one polypeptide subunit. By transducing a host cell with vectors carrying different viral envelope sequences, the proteins, when expressed in the cell, can self-assemble into three-dimensional structures containing more than one protein subunit (e.g., in their native configuration).

[00109] In further embodiments, the expressible heterologous nucleotide sequence is derived from another virus, other than PIV5. For example, the heterologous nucleotide sequence may encode (from any strain) influenza HA, RSV F, HIV Gag and/or Env, etc. Such embodiments can be useful for developing vaccines and/or methods of vaccination. The examples given here are non-limiting, as it will be understood by those in the art that nucleotide sequences from a variety of pathogenic agents (including also bacteria, parasites, etc.) may be desirable to use for an PIV5 vaccine composition and/or method of vaccination.

[00110] Examples of viruses to which vaccines can be produced in accordance with the disclosure include, e.g., coronaviruses, orthomyxoviruses, influenza virus A (including all strains varying in their HA and NA proteins, such as (non-limiting examples) H1N1, H1N2, H2N2, H3N2, H7N7, and H3N8); influenza B, influenza C, thogoto virus (including Dhori, Batken virus, SiAR 126 virus), and isavirus (e.g., infectious salmon anemia virus) and the like. These include coronaviruses isolated or transmitted from all species types, including isolates from invertebrates, vertebrates, mammals, humans, non-human primates, monkeys, pigs, cows, and other livestock, birds, domestic poultry such as turkeys, chickens, quail, and ducks, wild birds (including aquatic and terrestrial birds), reptiles, etc. These also include existing strains which have changed, e.g., through mutation, antigenic drift, antigenic shift, recombination, etc., especially strains which have increased virulence and/or interspecies transmission (e.g., human-to-human).

V. Methods of Administration

A. Administration by Vaccination

[00111] The present invention includes methods of vaccinating a subject by administering a storage-stable liquid vaccine composition as described herein to the subject.

[00112] As an example, the disclosure provides vaccines against all coronaviruses, including existing subtypes, derivatives thereof, and recombinants thereof, such as subtypes and recombinants which have the ability to spread from human-to-human. Various isolates have been characterized, especially for SARS-CoV-2.

[00113] The disclosure also provides methods for producing PIV5 compositions. Examples of host cells which can be utilized to produce PIV5 compositions, include, any mammalian or human cell line or primary cell. Non-limiting examples include, e.g., 293, HT1080, Jurkat, and SupT1 cells. Other examples are CHO, 293, Hela, Vero, L929, BHK, NIH 3T3, MRC-5, BAE-1, HEP-G2, NSO, U937, Namalwa, HL60, WEHI 231, YAC 1, U 266B1, SH-SY5Y, CHO, e.g., CHO-K1 (CCL-61), 293 (e.g., CRL-1573). Cells are cultured under conditions effective to produce transfection and expression. Such conditions include, e.g., the particular milieu needed to achieve protein production. Such a milieu, includes, e.g., appropriate buffers, oxidizing agents, reducing agents, pH, co-factors, temperature, ion concentrations, suitable age and/or stage of cell (such as, in particular part of the cell cycle, or at a particular stage where particular genes are being expressed) where cells are being used, culture conditions (including cell media,

substrates, oxygen, carbon dioxide, glucose and other sugar substrates, serum, growth factors, etc.).

[00114] The disclosure also provides various treatment methods involving delivering PIV5 vaccine compositions to host cells in vivo. In some embodiments, the PIV5 vaccine composition is delivered into a subject for treating or preventing coronaviruses. In other embodiments, the PIV5 vaccine composition is delivered into a subject for treating or preventing SARS-CoV-2 alpha, delta, omicron strains, or variants thereof or eliciting an immune response to SARS-CoV-2 in a subject.

[00115] It is contemplated that when used to treat various diseases, the compositions and methods of the disclosure can be combined with other therapeutic agents suitable for the same or similar diseases. Also, two or more embodiments of the disclosure may be also co-administered to generate additive or synergistic effects. When co-administered with a second therapeutic agent, the embodiment of the disclosure and the second therapeutic agent may be simultaneously or sequentially (in any order). Suitable therapeutically effective dosages for each agent may be lowered due to the additive action or synergy.

[00116] As a non-limiting example, the disclosure can be combined with other therapies that block inflammation through (e.g., via inhibition, reduction and/or blockage of IL1, $\text{INF}\alpha/\beta$, IL6, TNF, L13, IL23, etc.). In some embodiments, PIV5 compositions and methods disclosed herein are useful to enhance the efficacy of vaccines directed to SARS-CoV-2 infections. The compositions and methods of the disclosure can be administered to a subject either simultaneously with or before (e.g., 1-30 days before) a reagent (including but not limited to small molecules, antibodies, or cellular reagents) that acts to elicit an immune response (e.g., to treat cancer or an infection). The compositions and methods of the disclosure can be also administered in combination with an anti-tumor antibody or an antibody directed at a pathogenic antigen or allergen.

[00117] The pharmaceutical compositions of the invention can be readily employed in a variety of therapeutic or prophylactic applications, e.g., for treating SARS-CoV-2 infection or eliciting an immune response to SARS-CoV-2 in a subject. In various embodiments, the vaccine compositions can be used for treating or preventing infections caused by a pathogen from which the displayed immunogen polypeptide in the PIV5-based vaccine is derived. Thus, the vaccine compositions of the invention can be used in diverse clinical settings for treating or preventing infections caused by various viruses. As exemplification, a SARS-CoV-2 PIV-5-based vaccine composition can be administered to

a subject to induce an immune response to SARS-CoV-2, e.g., to induce production of broadly neutralizing antibodies to the virus. For subjects at risk of developing a SARS-CoV-2 infection, a vaccine composition of the invention can be administered to provide prophylactic protection against viral infection. Therapeutic and prophylactic applications of vaccines derived from the other immunogens described herein can be similarly performed. Depending on the specific subject and conditions, pharmaceutical compositions of the invention can be administered to subjects by a variety of administration modes known to the person of ordinary skill in the art, for example, topical, oral, intranasal, intramuscular, subcutaneous, intravenous, intra-arterial, intra-articular, intraperitoneal, or parenteral routes. In some aspects, administration is to a mucosal surface. A vaccine may be administered by mass administration techniques such as by placing the vaccine in drinking water or by spraying the animals' environment. When administered by injection, the immunogenic composition or vaccine may be administered parenterally. Parenteral administration includes, for example, administration by intravenous, subcutaneous, intramuscular, or intraperitoneal injection.

1. Vaccination by Inhalation

[00118] In one embodiment, the disclosed PIV-5 vaccine compositions are formulated to allow intranasal administration. Intranasal compositions may comprise an inhalable dry powder pharmaceutical formulation comprising a therapeutic agent, wherein the therapeutic agent is present as a freebase or as a mixture of a salt and a freebase. The intranasal compositions disclosed herein may comprise an inhalable liquid pharmaceutical formulation. Pharmaceutical formulations disclosed herein can be formulated as suitable for airway administration, for example, nasal, intranasal, sinusoidal, peroral, and/or pulmonary administration. Typically, formulations are produced such that they have an appropriate particle size for the route, or target, of airway administration. As such, the formulations disclosed herein can be produced so as to be of defined particle size distribution.

[00119] For example, the particle size distribution for a salt form of a therapeutic agent for intranasal administration can be between about 5 μm and about 350 μm . More particularly, the salt form of the therapeutic agent can have a particle size distribution for intranasal administration between about 5 μm to about 250 μm , about 10 μm to about 200 μm , about 15 μm to about 150 μm , about 20 μm to about 100 μm , about 38 μm to about 100 μm , about 53 μm to about 100, about 53 μm to about 150 μm , or about 20 μm to about 53 μm . The salt form of the therapeutic agent in the pharmaceutical compositions

of the invention can a particle size distribution range for intranasal administration that is less than about 200 μm . In other embodiments, the salt form of the therapeutic agent in the pharmaceutical compositions has a particle size distribution that is less than about 150 μm , less than about 100 μm , less than about 53 μm , less than about 38 μm , less than about 20 μm , less than about 10 μm , or less than about 5 μm . The salt form of the therapeutic agent in the pharmaceutical compositions of the invention can have a particle size distribution range for intranasal administration that is greater than about 5 μm , greater than about 10 μm , greater than about 15 μm , greater than about 20 μm , greater than about 38 μm , less than about 53 μm , less than about 70 μm , greater than about 100 μm , or greater than about 150 μm .

[00120] Additionally, the salt form of the therapeutic agent in the pharmaceutical compositions of the invention can have a particle size distribution range for pulmonary administration between about 1 μm and about 10 μm . In other embodiments for pulmonary administration, particle size distribution range is between about 1 μm and about 5 μm , or about 2 μm and about 5 μm . In other embodiments, the salt form of the therapeutic agent has a mean particle size of at least 1 μm , at least 2 μm , at least 3 μm , at least 4 μm , at least 5 μm , at least 10 μm , at least 20 μm , at least 25 μm , at least 30 μm , at least 40 μm , at least 50 μm , at least 60 μm , at least 70 μm , at least 80 μm , at least 90 μm , or at least 100 μm .

[00121] In some embodiments the disclosed cannabinoid compositions include one or more cannabinoids or pharmaceutically acceptable derivatives or salts thereof, a propellant, an alcohol, and a glycol and/or glycol ether. The alcohol may be a monohydric alcohol or a polyhydric alcohol, and is preferably a monohydric alcohol. Monohydric alcohol has a lower viscosity than a glycol or glycol ether. Accordingly, the composition is able to form droplets of a smaller diameter in comparison to compositions in which the monohydric alcohol is not present. The present inventors have surprisingly found that a specific ratio of monohydric alcohol to glycol or glycol ether results in a composition with a desired combination of both long term stability (for example the composition remains as a single phase for at least a week at a temperature of 2-40° C.) and small droplet size.

2. Pulmonary Compositions

[00122] One embodiment provides a formulation and method for treating SARS-CoV-2 in the pulmonary system by inhalation or pulmonary administration. The diffusion characteristics of the particular drug formulation through the pulmonary tissues are

chosen to obtain an efficacious concentration and an efficacious residence time in the tissue to be treated. Doses may be escalated or reduced or given more or less frequently to achieve selected blood levels. Additionally, the timing of administration of administration and amount of the formulation is preferably controlled to optimize the therapeutic effects of the administered formulation on the tissue to be treated and/or titrate to a specific blood level.

[00123] Diffusion through the pulmonary tissues can additionally be modified by various excipients that can be added to the formulation to slow or accelerate the absorption of drugs into the pulmonary tissues. For example, the drug may be combined with surfactants such as the phospholipids, dimyristoylphosphatidyl choline, and of administration dimyristoylphosphatidyl glycerol. The drugs may also be used in conjunction with bronchodilators that can relax the bronchial airways and allow easier entry of the antineoplastic drug to the lung. Albuterol is an example of the latter with many others known in the art. Further, the drug may be complexed with biocompatible polymers, micelle forming structures or cyclodextrins.

[00124] Particle size for the aerosolized drug used in the present examples was measured at about 1.0-5.0 μm with a GSD less than about 2.0 for deposition within the central and peripheral compartments of the lung. As noted elsewhere herein particle sizes are selected depending on the site of desired deposition of the drug particles within the respiratory tract.

[00125] Aerosols useful in the invention include aqueous vehicles such as water or saline with or without ethanol and may contain preservatives or antimicrobial agents such as benzalkonium chloride, paraben, and the like, and/or stabilizing agents such as polyethyleneglycol.

[00126] An agent of the present disclosure may be administered at once or may be divided into a number of multiple doses to be administered at intervals of time. For example, agents of the invention may be administered repeatedly, e.g., at least 2, 3, 4, 5, 6, 7, 8, or more times, or may be administered by continuous infusion. It is understood that the precise dosage and duration of treatment is a function of the disease being treated and may be determined empirically using known testing protocols or by extrapolation from in vivo or in vitro test data. It is to be noted that concentrations and dosage values may also vary with the severity of the condition to be alleviated. It is to be further understood that for any particular subject, specific dosage regimens should be adjusted over time according to the individual need and the professional judgment of the person

administering or supervising the administration of the compositions, and that any concentration ranges set forth herein are exemplary only and are not intended to limit the scope or practice of the claimed compositions and methods.

[00127] In some therapeutic embodiments, an "effective amount" of an agent is an amount that results in a reduction of at least one pathological parameter. Thus, for example, in some aspects of the present disclosure, an effective amount is an amount that is effective to achieve a reduction of at least about 10%, at least about 15%, at least about 20%, or at least about 25%, at least about 30%, at least about 35%, at least about 40%, at least about 45%, at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, or at least about 95%, compared to the expected reduction in the parameter in an individual not treated with the agent.

[00128] In some aspects, any of the PIV5-based constructs and methods described in WO 2013/112690 and WO 2013/112720 (which is hereby incorporated by reference herein in its entirety) may be used in the present invention.

[00129] As used herein, the term "subject" represents an organism, including, for example, a mammal. A mammal includes, but is not limited to, a human, a non-human primate, and other non-human vertebrates. A subject may be an "individual," "patient," or "host." Non-human vertebrates include livestock animals (such as, but not limited to, a cow, a horse, a goat, and a pig), a domestic pet or companion animal, such as, but not limited to, a dog or a cat, and laboratory animals. Non-human subjects also include non-human primates as well as rodents, such as, but not limited to, a rat or a mouse. Non-human subjects also include, without limitation, poultry, horses, cows, pigs, goats, dogs, cats, guinea pigs, hamsters, mink, and rabbits.

[00130] As used herein "in vitro" is in cell culture and "in vivo" is within the body of a subject. As used herein, "isolated" refers to material that has been either removed from its natural environment (e.g., the natural environment if it is naturally occurring), produced using recombinant techniques, or chemically or enzymatically synthesized, and thus is altered "by the hand of man" from its natural state.

B. Booster Vaccines

[00131] The present disclosure provides for the administration of a booster PIV-5 vaccine for use in such a method for inducing in a human subject an immune response, wherein said subject has previously received a primary vaccination against SARS-CoV-2.

[00132] The method of booster vaccination according to the disclosure comprise the step of administering the vaccine composition to the subject.

[00133] The immune response induced by the vaccine composition of the disclosure or by the method of the disclosure is preferably a humoral response, especially a response comprising the production of neutralizing antibodies against the COVID-19 virus, i.e. a neutralizing antibody response.

EXAMPLES

[00134] Materials and Methods

[00135] Materials used in this study are listed in Table 1.

[00136] Table:1 Materials

Item	Source	Catalog No.
Sucrose	J.T. Baker	4074
Potassium phosphate dibasic	J.T. Baker	3252
Potassium phosphate monobasic	J.T. Baker	3246
L-glutamic acid potassium salt monohydrate	Sigma	G1149
Sodium phosphate dibasic	Sigma	71643
Citric acid	Sigma	251275
Arginine	Sigma	A6969
Histidine	Sigma	H5659
Lysine	Sigma	L8662
Proline	Sigma	P5607
Glycine	Sigma	G8790
mannitol	Sigma	M8429
porcine gelatin	Sigma	G0262
Pluronic F68	MP Biomedicals	2750049
malic acid	Sigma	M7397
myo-inositol	Sigma	I7508
sorbitol	Sigma	S3889
dextran sulfate	sigma	51227
HSA	Albumedix	PDP210010
Sodium hydroxide	Spectrum Chemical	S-390

Item	Source	Catalog No.
Hydrochloric acid	Fisher	A144
Filter Units	Thermo Fisher	568-0020
Water For Injection (WFI)	Thermo Fisher	A1287302

[00137] **Potency assay and pH measurement:** Potency assay was performed according to SOP-018: Determination of PIV5-based vaccine virus titer (potency) by plaque assay. The plaque assay was qualified and had acceptable accuracy and precision with %CV <10% or within 0.5 log₁₀PFU/mL assay variation. PH of vaccine in liquid formulations was determined according to SOP-053: pH Measurement and Appearance.

[00138] **Virus Strains:** The virus strains used in the study are shown in Table 2.

[00139] Table 2: Virus strains

Virus name	CVXGA1	CVXGA1	CVXGA1
Lot number	CVXGA1-PP3 MVS	Carbo 1: UEX0013- DSP	220901MN
Titer (Log ₁₀ PFU)	7.41	7.0	7.8
Source	Charles River Laboratories	Exothera	CyanVac
Storage buffer	1 × SPG	1 × SPG +1% Arginine	1 × SPG
Storage conditions	-80°C until use	-80°C until use	-80°C until use
Purpose	Frozen formulation study	Liquid formulation study	Liquid formulation study

[00140] **CVXGA1 MVS stability test (frozen formulation):** CVXGA1-PP3 MVS formulated in 1xSPG was manufactured in Charles River Laboratories. The vaccine virus product was placed at -80, -20 and 4°C for stability study. The titers from two vials from each group of temperature at each time point were determined by the qualified plaque assay (potency assay) per CVL SOP-18. Time points at 0, 1, 3, 6, 9, 12, 18, and 24 months were used for -80 and -20°C stability studies, 0, 1, and 3 months were used for 4°C stability study.

[00141] Preparation of liquid formulation of CVXGA1: CVXGA1-Carbo 1:

UEX0013-DSP was manufactured in Exothera. CVXGA1 harvest was treated by Benzonase to remove host cellular DNA (HCD) and further purified by tangential flow filtration (TFF) followed by formulation in 1xSPG +1% Arginine (SPGR, which is used as part of stabilizer for FluMist (approved vaccine)). The vaccine produced in this process is suitable for phase 3 clinical trial. Thus, most formulation studies were performed with the CVXGA1-Carbo 1: UEX0013-DSP stock. For the study of pH optimization, pH of CVXGA1-Carbo 1: UEX0013-DSP was adjusted to pH 5-9 or 6.6-7.8. The vials were placed at 34°C incubator for accelerated stability study. The titers from two vials from each group at each time point were determined by the plaque assay.

[00142] CVXGA1 lot 220901MN was harvested from infected Vero cells and formulated in 1xSPG. The virus stock was used to determine the stabilizing effects of different excipients. The excipients were listed in Table 3. The pH was adjusted to 7.2±0.2. The vials were placed at 4°C for stability study. The titers from two vials from each group at each time point were determined by the plaque assay.

[00143] Table 3: Concentration of excipients

Excipient	Concentration	Excipient	Concentration
sorbitol	12%	Histidine	150mM
	6%		10mM
	3%	mannitol	12%
sucrose	12%		6%
	10%	porcine gelatin	2.00%
	7.5%		1.0%
	3.75%	Pluronic F68	0.10%
Glycine	150mM		0.01%
	10mM	Lysine	150mM
malic acid	450mM		10mM
	150mM	Proline	150mM
Citrate	200mM		10mM
	20mM	myo-inositol	450mM
rHSA(Prime/Eli te)	1%		150mM

[00144] Once the citrate phosphate was found to be the best excipient compared to others, we determined different concentrations, 20, 30, 40, 50, 100, 150, 200, 300, and 400mM of citrate phosphate plus arginine in liquid formulations. CVXGA1-Carbo 1: UEX0013-DSP was used in the study. The solution pH was adjusted to 7.2 ± 0.2 . The vials were placed at 4°C for stability study. The titers from two-three vials from each group at each time point were determined by the plaque assay.

Example 1: Stability of CVXGA1 in 1xSPG

[00145] The stability of CVXGA1-PP3 MVS virus stock was determined at -80, -20 and 4°C at the indicated time points for two years as of April, 2023. Its stability at -80°C is still ongoing to monitor this clinical lot stability. CVXGA1 formulated in 1xSPG was stable for up to 2 years at -80 and -20°C, however, CVXGA1 in 1xSPG was not stable at 4°C, and lost ~0.58 log titers at month 1, and ~1.80 log titers at month 3 (FIG. 1).

Example 2: Screening optimized pH and buffer

[00146] CVXGA1-Carbo 1: UEX0013-DSP virus was formulated in 1xSPG + 1% arginine buffer. We tested the change of pH values in an accelerated stability study performed at 34°C. The pH was adjusted to 5, 6, 7, 8, 9, then placed at 34°C for potency test. The pH values were stable for up to 18 days, although the virus titers dropped to undetectable level at pH 8 and 9, a bigger than 4 log loss at pH 5, and a relative small 2 log loss at 6 and 7 (FIG. 2A-2B). The study was repeated once and the data is reproducible (data not shown). From these results, we concluded that 1xSPG buffer was able to maintain stable pH and will be used as buffer system for our formulation study. Next, pH range from 6.6 to 7.8 was examined for virus stability. The formulation at pH 6.6 showed about 1.9 log loss in titers, others showed less than 1 log loss with similar trends (FIG. 2C). The study was repeated once and the data is reproducible (data not shown). We concluded that the pH range from 6.8 to 7.8 in 1xSPG +1% arginine buffer is suitable for CVXGA1.

Example 3: Screening excipients for liquid formulations

[00147] Our objective is to identify a formulation that will maintain CVXGA1 potency for at least 4 months at 4°C. We tried many excipients, sucrose, sorbitol, mannitol, arginine, histidine, lysine, Proline, Glycine, recombinant HSA, porcine gelatin, myo-inositol, malic acid, Pluronic F68, and citrate phosphate in SPG buffer to determine whether several excipients could provide better stabilizing effects than others at 4°C. We found that sorbitol, histidine, lysine, malic acid, and citrate phosphate improved vaccine virus potency compared to SPG control, citrate phosphate showed the best effect among

these excipients (Table 4). Citrate acid is used in many biological products, and safe for injected drugs.

[00148] Table 4: Virus titer loss from excipients

Excipient	Concentration	Log10 titer loss		
		W0-W4	W0-W8	W0-W12
sorbitol	12%	0.63	1.05	1.83
	3%	0.72	1.46	2.30
Histidine	150mM	0.56	1.37	1.51
porcine gelatin	2%	0.3	1.65	1.59
Lysine	150mM	0.19	0.98	1.13
malic acid	450mM	0.52	1.16	1.81
	150mM	0.4	0.92	1.77
Citrate phosphate	200mM	-0.04	0.27	0.40
	20mM	0.14	0.50	0.67
Citrate phosphate + arginine	200mM+ 1%	0.079	0.19	0.40
	20mM+ 1%	0.050	0.24	0.46
SPG control		1.15	1.99	2.46

[00149] Therefore, we tested citrate phosphate plus arginine in SPG buffer.

CVXGA1 was formulated in 20 or 200mM citrate phosphate with or without 1% arginine, and placed at 4°C. Potency was tested every month. CVXGA1 virus in 20mM citrate phosphate+1xSPG, or 20mM citrate phosphate+1% Arginine+1xSPG was not stable and lost larger than 0.5 log titers at week 12 or 16, respectively. However, CVXGA1 virus in higher concentration of 200mM citrate phosphate with or without 1% Arginine was stable with potency drops less than 0.5 Log10 PFU/mL (assay limit of variation) up to week 16 (Fig. 3A). Next, we tested the effects of 1% arginine plus different concentrations of citrate phosphate from 50-400mM.

[00150] From this study, 300 and 400mM citrate phosphate lost larger than 0.5 Log10 at week 4, 200mM citrate phosphate did not stabilize the virus after 4 weeks this time, 50 and 100mM citrate phosphate showed stable titers with potency loss less than 0.5 Log10 up to week 16, 50mM citrate phosphate showed potency drops at ~0.23 Log10 at

week 20 (Fig. 3B). We repeated a formulation study with 50, 100, 150 and 200mM citrate phosphate plus 1% arginine. The results also showed 50, 100, and 150mM citrate phosphate plus 1% arginine maintained CVXGA1 potency with potency drops less than 0.5 Log10 up to week 20 (Fig.3C). We concluded that 50-100mM citrate phosphate plus 1% arginine in 1xSPG buffer formulated CVXGA1 is stable at least 4 months at 4°C, the top formulation of 50mM citrate phosphate plus 1% arginine in 1xSPG buffer maintained the best potency for up to 5 months.

[00151] Next, we wanted to know whether the concentrations of citrate phosphate could be reduced below 50mM. We tested CVXGA1 formulated in 20, 30, 40, and 50mM citrate phosphate plus 1% arginine in 1xSPG buffer. We obtained the data from month-3 time point. It was found that 20 and 30mM citrate phosphate did not maintain the stable potency, but 40 and 50mM citrate phosphate showed stable titers with potency drops less than 0.5 Log10 up to week 13 (Fig. 3D). We believe that 40mM citrate phosphate plus 1% arginine in 1xSPG buffer will also maintain the stable potency as 50mM citrate phosphate.

Example 4: Screening excipients for liquid formulations

[00152] To determine if the liquid formulation of 50mM citrate phosphate plus 1% arginine in 1xSPG buffer affected vaccine immunogenicity in comparison to the frozen formulation, we performed a preclinical study using a mouse model (Table 5). Five to 7-week-old female and male BALB/c mice (Envigo) (10/group, 5 females and 5 males) were mock-vaccinated or vaccinated with a single dose at 10⁵ PFU of CVXGA1 in frozen formulation placed at -80°C or liquid formulation of 50mM citrate phosphate plus 1% arginine in 1xSPG buffer placed at 4°C for 4 months. Four weeks post-vaccination, sera were collected. Anti-S IgG antibodies were determined using SARS-CoV-2 WA1 S protein-based ELISA assay.

[00153] Table 5: Mouse immunogenicity study plan

Mouse study 1				
Group	Male/ Female	Test article	Dosing route/volume	Dose level (PFU)
1	5/5	PBS	I.N./100ul	NA
2	5/5	CVXGA1 in frozen formulation	I.N./100ul	10 ⁵
3	4/5	CVXGA1 in liquid formulation	I.N./100ul	10 ⁵
Mouse study 2				
1	3/3	PBS	I.N./50ul	NA
2	3/3	CVXGA1 in frozen formulation	I.N./50ul	10 ⁶
3	3/3	CVXGA1 diluted in liquid formulation	I.N./50ul	10 ⁶

[00154] CVXGA1 in frozen and liquid formulations showed similar anti-S IgG titers in female and male mice (Figs. 4A-4C). No significant difference between frozen and liquid formulation groups was found via one-way analysis of variance (ANOVA). Next, we diluted frozen stock to get virus in a formulation with final concentration at 50mM citrate phosphate plus 1% arginine in 1xSPG, then vaccinated mice at 10⁶ PFU. BALB/c mice (6/group, 3 females and 3 males) were vaccinated. Three weeks post-vaccination, sera were collected. Anti-S IgG antibodies were determined using SARS-CoV-2 WA1 S protein-based ELISA assay. CVXGA1 in frozen and liquid formulations also showed similar anti-S IgG titers in female and male mice (Figs, 4D-4F). No significant difference between frozen and liquid formulation groups was found via one-way analysis of variance (ANOVA). We concluded that CVXGA1 in both frozen and liquid formulations have similar immunogenicity as expected.

[00155] The complete disclosure of all patents, patent applications, and publications, and electronically available material (including, for instance, nucleotide sequence submissions in, e.g., GenBank and RefSeq, and amino acid sequence submissions in, e.g., SwissProt, PIR, PRF, PDB, and translations from annotated coding regions in GenBank and RefSeq) cited herein are incorporated by reference. In the event that any inconsistency exists between the disclosure of the present application and the disclosure(s) of any document incorporated herein by reference, the disclosure of the present application shall govern. The foregoing detailed description and examples have

been given for clarity of understanding only. No unnecessary limitations are to be understood therefrom. The invention is not limited to the exact details shown and described, for variations obvious to one skilled in the art will be included within the invention defined by the claims.

What is claimed is:

1. A liquid vaccine formulation for administering to a recipient comprising:
 - a) an immunogen;
 - b) at least 1X of a stabilizing agent, and
 - c) at least 20-400 mM of a buffer;wherein the immunogen is capable of eliciting a protective immune response in the recipient;
wherein the liquid formulation has a pH of about pH 6.0 and pH 8.0; and
wherein the liquid formulation maintains storage-stable immunogenicity at 4°C for at least 4 months with a vaccine potency loss of less than 0.5 Log₁₀.
2. The liquid formulation of claim 1, wherein the vaccine is against an enveloped virus selected from a group consisting of herpesviruses, cytoviruses, poxviruses, arenaviruses, arteriviruses, hepadnaviruses, flaviviruses, togaviruses, coronaviruses, orthomyxoviruses, paramyxoviruses, rhabdoviruses, bunyaviruses, filoviruses, baculoviruses, iridoviruses, and retroviruses.
3. The liquid formulation of claim 1, wherein the vaccine is against a coronavirus.
4. The liquid formulation of claim 2, wherein the vaccine comprises a viral expression vector expressing a heterologous polypeptide comprising a spike (S) and/or nucleocapsid (N) proteins of a coronavirus.
5. The liquid formulation of claim 2, wherein the coronavirus is a severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), a variant of interest or a variant of concern of SARS-CoV-2.
6. The liquid formulation of claim 5, wherein the variant of interest or a variant of concern of SARS-CoV-2 is a SARS-CoV-2 beta variant, a SARS-CoV-2 gamma variant, a SARS-CoV-2 delta variant, a SARS-CoV-2 omicron variant, or other variants SARS-CoV-2.

7. The liquid formulation of claim 1, wherein the stabilizing agent is selected from a group consisting of sucrose phosphate glutamate (SPG), carbohydrates, sodium glutamate, proteins such as peptone, albumin or casein, protein containing agents such as skimmed milk, an amino acid, buffers and combinations thereof.
8. The liquid formulation of claim 7, wherein the carbohydrate is selected from a group consisting of sorbitol, mannitol, lactose, sucrose, glucose, dextran, and trehalose.
9. The liquid formulation of claim 7, wherein the amino acid is selected from a group of consisting of arginine, histidine, lysine, proline, glycine, methionine, and glutamic acid and combinations thereof.
10. The liquid formulation of claim 1, wherein the stabilizing agent is the combination of sucrose phosphate glutamate and arginine.
11. The liquid formulation of claim 1, wherein the buffer is selected from a group consisting of a phosphate buffer, citrate buffer, citrate phosphate buffer, borate buffer, tris(hydroxymethyl) aminomethane (Tris) containing buffer, succinate buffer, buffers containing glycine or histidine as one of the buffering agents.
12. The liquid formulation of claim 11, wherein the buffer is at a concentration of about 20, 30, 40, 50 mM, 60 mM, 70 mM, 80 mM, 90 mM, 100 mM, 110 mM, 120 mM, 130 mM, 140 mM, 150 mM, 160 mM, 170 mM, 180 mM, 190 mM, 200 mM, 210 mM, 220 mM, 230 mM, 240 mM, 250 mM, 260 mM, 270 mM, 280 mM, 290 mM, 300 mM, 310 mM, 320 mM, 330 mM, 340 mM, 350 mM, 360 mM, 370 mM, 380 mM, 390 mM, or 400 mM.
13. The liquid formulation of claim 1, wherein the pH of the liquid formulation is about pH 6.0, pH 6.2, pH 6.4, pH 6.8, pH 7.0, pH 7.2, pH 7.4, pH 7.6, pH 7.8, pH 8.0, pH 8.2, pH 8.4, pH 8.6, pH 8.8, or pH 9.0.
14. The liquid formulation of claim 1, wherein the liquid formulation maintains storage-stable immunogenicity for at least 1 month, 2 months, 3 months, 4 months, 5 months, 6 months or longer.

15. The liquid formulation of claim 1, wherein the liquid formulation maintains storage-stable immunogenicity with a 0 Log₁₀, 0.1 Log₁₀, 0.2 Log₁₀, 0.3 Log₁₀, 0.4 Log₁₀, or 0.5 Log₁₀ loss in vaccine potency.