PERSONALIZED CANCER VACCINE EPITOPE SELECTION

RELATED APPLICATIONS

This application claims the benefit of priority under 35 U.S.C. § 119(e) of U.S. provisional application number 62/690,441, filed June 27, 2018, U.S. provisional application number 62/757,045, filed November 7, 2018, U.S. provisional application number 62/814,200, filed March 5, 2019, and U.S. provisional application number 62/855,311, filed May 31, 2019, each of which is incorporated by reference herein in its entirety.

BACKGROUND OF INVENTION

Recent theories in cancer evolution have focused on three steps including stress-induced genome instability, population diversity or heterogeneity, and genome-mediated macroevolution. The theory explains why most of the known molecular mechanisms can contribute to cancer yet there is no single dominant mechanism for the majority of clinical cases. However, the common mechanisms suggest that cancer vaccines may provide a universal solution in the treatment of cancer.

Cancer vaccines include preventive or prophylactic vaccines, which are intended to prevent cancer from developing in healthy people; and therapeutic vaccines, which are intended to treat an existing cancer by strengthening the body's natural defenses against the cancer. Cancer preventive vaccines may, for instance, target infectious agents that cause or contribute to the development of cancer in order to prevent infectious diseases from causing cancer. Gardasil® and Cervarix®, are two examples of commercially available prophylactic vaccines that protect against HPV infection and resultant cancers. Other preventive cancer vaccines may target host proteins or fragments that are predicted to increase the likelihood of an individual developing cancer in the future.

Many commercial or developing vaccines are based on whole microorganisms, protein antigens, peptides, or polysaccharides and their combinations. Certain developing vaccines are also based on nucleic acid vaccines (*e.g.*, deoxyribonucleic acid (DNA) vaccines or ribonucleic acid (RNA) vaccines). Such nucleic acid vaccines are generally not optimized to have the greatest efficacy for their size or length.

SUMMARY OF INVENTION

Provided herein is a nucleic acid (*e.g.*, ribonucleic acid (RNA)) cancer vaccine having a maximized anti-cancer efficacy for a given length and comprising one or more nucleic acids

that can direct the body's cellular machinery to produce nearly any cancer protein or fragment thereof of interest. In some embodiments, the disclosure also provides methods of making a nucleic acid cancer vaccine having a maximized anti-cancer efficacy for a given length. In some embodiments, the disclosure also provides methods of treating a patient having cancer with a cancer vaccine having a maximized anti-cancer efficacy for a given length. Additionally, in certain embodiments, the disclosure provides a computerized system for creating a nucleic acid cancer vaccine that has a maximized cancer efficacy for a given length.

In one aspect, the instant disclosure provides a nucleic acid cancer vaccine, comprising: one or more nucleic acids each having one or more open reading frames encoding 5-130 peptide epitopes, wherein each of the peptide epitopes are portions of personalized cancer antigens, and wherein at least two peptide epitopes have different lengths. In another aspect, the instant disclosure provides a nucleic acid cancer vaccine, comprising: one or more nucleic acids each having one or more open reading frames encoding 5-130, 20-40, 30-35, or 34 peptide epitopes, wherein each of the peptide epitopes are portions of personalized cancer antigens, and wherein each of the peptide epitopes have different lengths. In another aspect, the instant disclosure provides a nucleic acid cancer vaccine, comprising: one or more nucleic acids each having one or more open reading frames encoding 5-130, 20-40, 30-35, or 34 peptide epitopes, wherein each of the peptide epitopes are portions of personalized cancer antigens, and wherein each of the peptide epitopes have equal lengths. In some embodiments the cancer vaccine composition comprises one or more mRNAs each having one or more open reading frames encoding 34 peptide epitopes and wherein 29 epitopes are MHC class I epitopes and 5 epitopes are MHC class II or MHC class I and II epitopes.

In some embodiments, the length of each peptide epitope is determined such that the anti-cancer efficacy of the nucleic acid cancer vaccine is maximized for a given total length of the one or more nucleic acids. In some embodiments, the minimum length of any peptide epitope is 8 amino acids. In some embodiments, the maximum length of any peptide epitope is 31 amino acids. In some embodiments, the minimum length of any or all of the peptide epitopes is 13 amino acids. In some embodiments, the maximum length of any or all of the peptide epitopes is 35 amino acids. In some embodiments, the length of any or all of the peptide epitopes is 25 amino acids.

In some embodiments, the cancer vaccine is a DNA cancer vaccine. In some embodiments, the cancer vaccine is an RNA cancer vaccine. In some embodiments, the

cancer vaccine is an mRNA cancer vaccine, and the one or more nucleic acids are mRNA. In some embodiments, the one or more mRNA each comprise a 5′ UTR and/or a 3′ UTR. In some embodiments, the one or more mRNA each comprise a poly-A tail. In some embodiments, the poly-A tail comprises about 100 nucleotides. In some embodiments, the one or more mRNA each comprise a cap structure or a modified cap structure. In some embodiments, the cap structure or the modified cap structure is a 5′ cap structure, a 5′ cap-0 structure, a 5′ cap-1 structure, or a 5′ cap-2 structure.

In some embodiments, the one or more mRNA comprise at least one chemical modification. In certain embodiments, the chemical modification is selected from the group consisting of pseudouridine, N1-methylpseudouridine, N1-ethylpseudouridine, 2-thiouridine, 4′-thiouridine, 5-methylcytosine, 2-thio-1-methyl-1-deaza-pseudouridine, 2-thio-1-methyl-pseudouridine, 2-thio-5-aza-uridine, 2-thio-dihydropseudouridine, 2-thio-dihydrouridine, 2-thio-pseudouridine, 4-methoxy-2-thio-pseudouridine, 4-methoxy-pseudouridine, 4-thio-1-methyl-pseudouridine, 4-thio-pseudouridine, 5-aza-uridine, dihydropseudouridine, 5-methyluridine, 5-methyluridine, 5-methoxyuridine, and 2′-O-methyl uridine. In some embodiments, the one or more mRNA is fully modified.

In some embodiments, the one or more nucleic acids encode 3-10 peptide epitopes, 5-10 peptide epitopes, 10-20 peptide epitopes, 20-30 peptide epitopes, 30-40 peptide epitopes, 40-50 peptide epitopes, 50-60 peptide epitopes, 60-70 peptide epitopes, 70-80 peptide epitopes, 80-90 peptide epitopes, 90-100 peptide epitopes, 100-110 peptide epitopes, 110-120 peptide epitopes, or 120-130 peptide epitopes. In some embodiments, each of the peptide epitopes is encoded by a separate open reading frame. In some embodiments, the peptide epitopes are in the form of a concatemeric cancer antigen comprised of 3-130 peptide epitopes. In some embodiments, the cancer vaccine composition comprises one mRNA having one open reading frame encoding 15 peptide epitopes.

In some embodiments, one or more of the following conditions are met: a) the 3-130 peptide epitopes are interspersed by cleavage sensitive sites (e.g., a linker such as a peptide linker comprising a cleavage sensitive site or a cleavage sensitive site as part of adjacent epitopes); and/or b) each peptide epitope is linked directly to one another without a linker; and/or c) each peptide epitope is linked to one another with a single amino acid linker; and/or d) each peptide epitope is linked to one another with a short linker; and/or e) each peptide epitope comprises 8-31 amino acids and includes one or more SNP mutations; and/or f) each peptide epitope comprises 8-31 amino acids and includes a mutation causing a unique expressed peptide sequence; and/or g) at least 30% of the peptide epitopes have a highest

affinity for class I MHC molecules from a subject; and/or h) at least 30% of the peptide epitopes have a highest affinity for class II MHC molecules from a subject; and/or i) none of the peptide epitopes have a highest affinity for class II MHC molecules from a subject; and/or j) at least 50% of the peptide epitopes have a predicted binding affinity of IC_{50} <500nM for HLA-A, HLA-B and/or DRB1; and/or k) the nucleic acid encoding the peptide epitopes is arranged such that the peptide epitopes are ordered to minimize pseudo-epitopes; and/or l) the ratio of class I MHC molecule peptide epitopes to class II MHC molecule peptide epitopes is at least 1:1, 2:1, 3:1, 4:1, or 5:1; and/or m) no class II MHC molecule peptide epitopes are present. In other embodiments at least 30% of the peptide epitopes have a highest affinity for class I MHC molecules and/or class II MHC class molecules from a subject. In other embodiments at least 50% of the peptide epitopes have a probability percent rank greater than 0.5% for HLA-A, HLA-B, and/or DRB1. The probability percentile rank provides a threshold for determining strong binders and is a calculation of a percentage of scores in a frequency distribution that are equal to or lower than it.

In some embodiments, at least one of the peptide epitopes is a predicted T cell reactive epitope. In certain embodiments, at least one of the peptide epitopes is a predicted B cell reactive epitope. In some embodiments, the peptide epitopes comprise a combination of predicted T cell reactive epitopes and predicted B cell reactive epitopes. In some embodiments, the peptide epitopes are predicted T cell reactive epitopes and/or predicted B cell reactive epitopes. In some embodiments, at least one of the peptide epitopes is a predicted neoepitope. In certain embodiments, at least one nucleic acid has an open reading frame encoding at least a fragment of one or more traditional cancer antigens or one or more cancer/testis antigens.

In some embodiments, each nucleic acid is formulated in a lipid nanoparticle. In some embodiments, each nucleic acid is formulated in a different lipid nanoparticle. In some embodiments, each nucleic acid is formulated in the same lipid nanoparticle.

In some embodiments, the total length of the one or more nucleic acids encodes a total protein length of 50-100 amino acids, 100-200 amino acids, 200-300 amino acids, 300-400 amino acids, 400-500 amino acids, 500-600 amino acids, 600-700 amino acids, 700-800 amino acids, 800-900 amino acids, 900-1000 amino acids, 1000-1100 amino acids, or 1100-1200 amino acids.

In some embodiments, the anti-cancer efficacy is calculated at least in part based on one or more factors selected from the group consisting of gene expression, RNA Seq, transcript abundance, DNA allele frequency, amino acid conservation, physiochemical

similarity, oncogene, predicted binding affinity to a specific HLA allele, clonality, binding efficiency and presence in an indel. In some embodiments, the one or more factors are inputted into a statistical model (*e.g.*, a regression model (such as a linear regression model, a logistic regression model, a generalized linear model, *etc.*), a generalized linear model (such as a logistic regression model, a probit regression model, *etc.*), a random forest regression model, a neural network, a support vector machine, a Gaussian mixture model, a hierarchical Bayesian model, and/or any other suitable statistical model).

In another aspect, the disclosure provides a method of making a cancer vaccine comprising: a) identifying between 3-130 personalized cancer antigens for a patient; b) determining the anti-tumor efficacy of at least two peptide epitopes for each of the 3-130 personalized cancer antigens; and c) preparing a cancer vaccine in which the total anti-cancer efficacy of the cancer vaccine is maximized for a given total length of the cancer vaccine.

In another aspect, the disclosure provides a method for treating a patient having cancer, comprising: a) analyzing a sample derived from the patient is in order to identify one or more personalized cancer antigens; b) determining the anti-tumor efficacy of at least two peptide epitopes for each of the identified personalized cancer antigens; c) preparing a cancer vaccine in which the total anti-cancer efficacy of the cancer vaccine is maximized for a given total length of the cancer vaccine; and d) administering the cancer vaccine to the patient. Optionally, any of the methods described herein may comprise manufacture of the cancer vaccine.

In some embodiments, the cancer vaccine is a nucleic acid cancer vaccine comprising one or more nucleic acids each having one or more open reading frames. In some embodiments, the cancer vaccine is a DNA cancer vaccine. In some embodiments, the cancer vaccine is an RNA cancer vaccine. In some embodiments, the cancer vaccine is an mRNA cancer vaccine. In some embodiments, the cancer vaccine is a peptide cancer vaccine.

In some embodiments, the cancer vaccine is administered at a dosage level sufficient to deliver between 0.02-1.0 mg of the cancer vaccine to the subject. In some embodiments, the cancer vaccine is administered to the subject twice, three times, four times, or more. In some embodiments, the cancer vaccine is administered by intradermal, intramuscular, intravascular, intratumoral, and/or subcutaneous administration. In some embodiments, the cancer vaccine is administered by intramuscular administration.

In certain embodiments, the methods and compositions described herein may be used with or for any type of cancer. In some embodiments, the cancer is selected from the group

consisting of non-small cell lung cancer (NSCLC), small cell lung cancer, melanoma, bladder urothelial carcinoma, HPV-negative head and neck squamous cell carcinoma (HNSCC), a solid malignancy that is microsatellite high (MSI H) / mismatch repair (MMR) deficient, renal cancer, gastric cancer, and tumor mutational burden high tumors. In some embodiments, the NSCLC lacks an EGFR sensitizing mutation and/or an ALK translocation. In some embodiments, the solid malignancy that is microsatellite high (MSI H) / mismatch repair (MMR) deficient is selected from the group consisting of colorectal cancer, stomach adenocarcinoma, esophageal adenocarcinoma, and endometrial cancer. In some embodiments that cancer is any one of melanoma, bladder carcinoma, HPV negative HNSCC, NSCLC, SCLC, MSI-High tumors, or TMB (tumor mutational burden) High cancers.

In certain embodiments, the one or more mRNA each comprise a 5′ UTR and/or a 3′ UTR. In some embodiments, the one or more mRNA each comprise a poly-A tail. In some embodiments, the poly-A tail comprises about 100 nucleotides. In some embodiments, the one or more mRNA each comprise a cap structure or a modified cap structure. In some embodiments, the cap structure or the modified cap structure is a 5′ cap structure, a 5′ cap-0 structure, a 5′ cap-1 structure, or a 5′ cap-2 structure. In certain embodiments, the one or more mRNA comprise at least one chemical modification. In some embodiments, the chemical modification is selected from the group consisting of pseudouridine, N1-methylpseudouridine, N1-ethylpseudouridine, 2-thiouridine, 4′-thiouridine, 5-methylcytosine, 2-thio-1-methyl-1-deaza-pseudouridine, 2-thio-1-methyl-pseudouridine, 2-thio-5-aza-uridine, 2-thio-dihydropseudouridine, 2-thio-dihydrouridine, 2-thio-pseudouridine, 4-methoxy-2-thio-pseudouridine, 4-methoxy-pseudouridine, 4-thio-1-methyl-pseudouridine, 4-thio-pseudouridine, 5-aza-uridine, dihydropseudouridine, 5-methyluridine, 5-methyluridine, 5-methoxyuridine, and 2′-O-methyl uridine. In some embodiments, the one or more mRNA is fully modified.

In certain embodiments, the one or more nucleic acids encode 3-10 peptide epitopes, 5-10 peptide epitopes, 10-20 peptide epitopes, 20-30 peptide epitopes, 30-40 peptide epitopes, 40-50 peptide epitopes, 50-60 peptide epitopes, 60-70 peptide epitopes, 70-80 peptide epitopes, 80-90 peptide epitopes, 90-100 peptide epitopes, 100-110 peptide epitopes, 110-120 peptide epitopes, or 120-130 peptide epitopes. In some embodiments, each of the peptide epitopes is encoded by a separate open reading frame. In some embodiments, the peptide epitopes are in the form of a concatemeric cancer antigen comprised of 5-130 peptide epitopes.

In some embodiments, one or more of the following conditions are met: a) the 3-130 peptide epitopes are interspersed by cleavage sensitive sites; and/or b) each peptide epitope is linked directly to one another without a linker; and/or c) each peptide epitope is linked to one or another with a single amino acid linker; and/or d) each peptide epitope is linked to one another with a short linker; and/or e) each peptide epitope comprises 8-31 amino acids and includes one or more SNP mutations; and/or f) each peptide epitope comprises 8-31 amino acids and includes a mutation causing a unique expressed peptide sequence; and/or g) at least 30% of the peptide epitopes have a highest affinity for class I MHC molecules from a subject; and/or h) at least 30% of the peptide epitopes have a highest affinity for class II MHC molecules from a subject; and/or i) none of the peptide epitopes have a highest affinity for class II MHC molecules from a subject; and/or j) at least 50% of the peptide epitopes have a predicted binding affinity of IC_{50} <500nM for HLA-A, HLA-B and/or DRB1; and/or k) the nucleic acid encoding the peptide epitopes is arranged such that the peptide epitopes are ordered to minimize pseudo-epitopes; and/or l) the ratio of class I MHC molecule peptide epitopes to class II MHC molecule peptide epitopes is at least 1:1, 2:1, 3:1, 4:1, or 5:1; and/or m) no class II MHC molecule peptide epitopes are present.

In some embodiments, at least one of the peptide epitopes is a predicted T cell reactive epitope. In certain embodiments, at least one of the peptide epitopes is a predicted B cell reactive epitope. In some embodiments, the peptide epitopes comprise a combination of predicted T cell reactive epitopes and predicted B cell reactive epitopes. In certain embodiments, the peptide epitopes are predicted T cell reactive epitopes and/or predicted B cell reactive epitopes. In some embodiments, at least one of the peptide epitopes is a predicted neoepitope. In some embodiments, at least one nucleic acid has an open reading frame encoding at least a fragment of one or more traditional cancer antigens or one or more cancer/testis antigens.

In some embodiments, each nucleic acid is formulated in a lipid nanoparticle. In some embodiments, each nucleic acid is formulated in a different lipid nanoparticle. In certain embodiments, each nucleic acid is formulated in the same lipid nanoparticle.

In some embodiments, the total length of the one or more nucleic acids encodes a total protein length of 50-100 amino acids, 100-200 amino acids, 200-300 amino acids, 300-400 amino acids, 400-500 amino acids, 500-600 amino acids, 600-700 amino acids, 700-800 amino acids, 800-900 amino acids, 900-1000 amino acids, 1000-1100 amino acids, or 1100-1200 amino acids. In some embodiments, the anti-cancer efficacy is calculated at least in part based on one or more factors selected from the group consisting of gene expression,

RNA Seq, transcript abundance, DNA allele frequency, amino acid conservation, physiochemical similarity, oncogene, predicted binding affinity to a specific HLA allele, clonality, binding efficiency and presence in an indel. In certain embodiments, the one or more factors are inputted into a statistical model (*e.g.*, a regression model (such as a linear regression model, a logistic regression model, a generalized linear model, *etc.*), a generalized linear model (such as a logistic regression model, a probit regression model, *etc.*), a random forest regression model, a neural network, a support vector machine, a Gaussian mixture model, a hierarchical Bayesian model, and/or any other suitable statistical model).

In another aspect, the present disclosure provides a computerized system for selecting nucleic acids to include in a nucleic acid cancer vaccine having a maximum length, the system comprising: a communication interface configured to receive a plurality of sequences of nucleic acids encoding a plurality of peptide epitopes, wherein each of the peptide epitopes are portions of personalized cancer antigens; and at least one computer processor programmed to: for each of the plurality of peptide epitopes, calculate a score for each of a plurality of nucleic acids in the peptide, each of which includes at least one of the one or more peptide epitopes, wherein at least two of the nucleic acid sequences have different lengths; and ranking based on the calculated scores, the plurality of nucleic acid sequences in the plurality of peptides; and selecting based on the ranking and the maximum length of the vaccine, nucleic acid sequences for inclusion in the vaccine.

In some embodiments, the minimum length of any peptide epitope is 8 amino acids. In some embodiments, the maximum length of any peptide epitope is 31 amino acids. In certain embodiments, the plurality of nucleic acids encode 3-10 peptide epitopes, 5-10 peptide epitopes, 10-20 peptide epitopes, 20-30 peptide epitopes, 30-40 peptide epitopes, 40-50 peptide epitopes, 50-60 peptide epitopes, 60-70 peptide epitopes, 70-80 peptide epitopes, 80-90 peptide epitopes, 90-100 peptide epitopes, 100-110 peptide epitopes, 110-120 peptide epitopes, or 120-130 peptide epitopes.

In some embodiments, one or more of the following conditions are met: a) each peptide epitope comprises 8-31 amino acids and includes one or more SNP mutations; and/or b) each peptide epitope comprises 8-31 amino acids and includes a mutation causing a unique expressed peptide sequence; and/or c) at least 30% of the peptide epitopes have a highest affinity for class I MHC molecules from a subject; and/or d) at least 30% of the peptide epitopes have a highest affinity for class II MHC molecules from a subject; and/or e) none of the peptide epitopes have a highest affinity for class II MHC molecules from a subject; and/or f) at least 50% of the peptide epitopes have a predicted binding affinity of IC_{50} <500nM for

HLA-A, HLA-B and/or DRB1; and/or g) the ratio of class I MHC molecule peptide epitopes to class II MHC molecule peptide epitopes is at least 1:1, 2:1, 3:1, 4:1, or 5:1; and/or h) no class II MHC molecule peptide epitopes are present.

In some embodiments, at least one of the peptide epitopes is a predicted T cell reactive epitope. In some embodiments, at least one of the peptide epitopes is a predicted B cell reactive epitope. In some embodiments, the peptide epitopes comprise a combination of predicted T cell reactive epitopes and predicted B cell reactive epitopes. In certain embodiments, the peptide epitopes are predicted T cell reactive epitopes and/or predicted B cell reactive epitopes. In some embodiments, at least one of the peptide epitopes is a predicted neoepitope. In some embodiments, at least one nucleic acid has an open reading frame encoding at least a fragment of one or more traditional cancer antigens or one or more cancer/testis antigens.

In some embodiments, the total length of the vaccine encodes a total protein length of 50-100 amino acids, 100-200 amino acids, 200-300 amino acids, 300-400 amino acids, 400-500 amino acids, 500-600 amino acids, 600-700 amino acids, 700-800 amino acids, 800-900 amino acids, 900-1000 amino acids, 1000-1100 amino acids, or 1100-1200 amino acids. In some embodiments, the score is calculated at least in part based on one or more factors selected from the group consisting of gene expression, RNA Seq, transcript abundance, DNA allele frequency, amino acid conservation, physiochemical similarity, oncogene, predicted binding affinity to a specific HLA allele, clonality, binding efficiency and presence in an indel. In certain embodiments, the one or more factors are inputted into a statistical model (*e.g.*, a regression model (such as a linear regression model, a logistic regression model, a generalized linear model, *etc.*), a generalized linear model (such as a logistic regression model, a probit regression model, *etc.*), a random forest regression model, a neural network, a support vector machine, a Gaussian mixture model, a hierarchical Bayesian model, and/or any other suitable statistical model).

In some embodiments an anti-tumor T-cell responses is evaluated for each neoantigen. In some embodiments the evaluation is based on confidence in the variant call from WES and RNA-Seq data; mRNA transcript abundance from RNA-Seq data; variant allele frequency from WES and RNA-Seq data; and predicted HLA binding affinity from NetMHCpan and NetMHCIIpan.

In some embodiments an HLA allotype of the patient is identified and antigens which are predicted to bind to the patient's HLA are incorporated. More weight may be assigned in some embodiments to predicted binders of HLA-A, -B and DR (core targets), and lower

(although non-zero) weight to other HLA allotypes of the patient (supplementary targets). Nearly all individuals have at least one HLA-A, -B and DR functional allotype (i.e. core MHC alleles) and these are the restricting elements for ~90% of all known human epitopes (FIG. 5). HLA-C-restricted or alloreactive T-cells are rarely observed and HLA-C's cell surface expression is 10% of that seen for HLA-A and B. The remaining supplementary targets encode for class II molecules and individuals can be null for genes encoding them. Moreover, 4-digit precision typing of these supplementary Class II targets is often ambiguous even when using state of the art NGS- and other sequence-based typing methods. In some embodiments if the NGS-based allele typing for either core or supplemental HLA targets is ambiguous, the allele(s) may not be considered when ranking neoantigens.

In some embodiments a selfness check of each neoantigen may be performed. A patient-specific set of transcripts are created using protein-coding transcript amino acid sequences from a reference human genome annotation, by tailoring the sequences to the patient's own set of germline protein-coding variants in some embodiments. This patient-specific exome (excluding the gene containing the neoantigen) may be used to check each HLA class I binding neoantigen epitope (8- to 11-mer) for 100% exact self-matches in some embodiments. Any neoantigen identified as 100% self-matches elsewhere in the genome and/or transcriptome using this tool may be excluded from the mRNA construct in some embodiments.

All variants that are not excluded by the selfness check may be evaluated for inclusion in the patient-specific mRNA construct design. In some embodiments pre-defined weights may be used rather than hard filters based on the knowledge that MHC binding predictions are imperfect and RNA-Seq sensitivity may be limited by tumor content of the biopsy and depth of sequencing.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a table depicting hotspot mutations by indication.

FIG. 2 shows a comparison of the predicted %rank by netMHCpan v3.0 vs. netMHCpan 4.0 EL for HLA-A*02:01. A large number of peptides move in and out of the 0.5% rank, which is generally considered to be the cutoff for "strong binders".

FIGs. 3A-3B show different methods of binding prediction. FIG. 3A is a graph showing the evenness of predicted binders to major HLA alleles. Switching to the percent rank (% rank) leads to a more balanced distribution of predicted binders across different HLA alleles. Likewise, FIG. 3B is a graph showing the area under the curve (AUC) of different

samples using different methods for predicting MHC binding. The percent rank method was shown to improve prediction performance over other alternatives (*e.g.*, IC_{50}).

FIGs. 4A-4C show the results from an *in vivo* immunogenicity study. Comparable immune responses to class I epitopes were detected by the 20mer/31 flank and 34mer/25 flank vaccines, but not the 40mer/21 flank at both the 3 and 10 μg doses. For several of the restimulations, only the 34mer constructs demonstrated a detectable response under the testing conditions.

FIGs. 5A-5B show core and supplementary HLA targets for neoantigens. FIG. 5A: An analysis of all known human T cell epitopes (positive in human T cell stimulation assays) using the Immune Epitope Database (IEDB; www.iedb.org/) revealed a clear hierarchy of HLA-restricting elements with HLA-A, -B and DR accounting for ~90% of all described human epitopes in the data base (n=8101). FIG. 5B: Limiting the IEDB search tool to viral epitopes only (n=4472) strengthened the apparent preference of T-cells for these core class I and class II loci. This analysis suggests that neoantigen selection can be prioritized on mutations predicted to bind the HLA-A, -B and –DRB1 allotypes of a patient.

FIG. 6 shows population analysis of somatic mutation load. Distribution of non-synonymous mutations in cancer histology cohorts from cBioPortal. Red, blue, and green lines represent 20, 34, and 100 mutations, respectively.

FIGs. 7A-7D show reproducibility of next generation sequencing (NGS) and bioinformatics system outputs. Independent processing of 4 related tumor samples from a single patient is used. A primary tumor sample and 3 tumor cell lines derived from it, were run through NGS, variant calling and the Bioinformatics System (FIG. 7A). Minimal differences in the variants called between the 4 samples was observed (FIG. 7B). Correlations between raw neoantigen scores for the 369 mutations identified (Spearman's Rank Correlation Coefficients: Tumor vs. Line 1: ρ=0.86; p=1.92E-101; Tumor vs. Line 2: ρ=0.84; p=3.01E-89 and Tumor vs. Line 3: ρ=0.84; p=5.77E-91) (FIG. 7C). Venn diagram of common and unique neoantigens selected for inclusion in a representative mRNA sequence (FIG. 7D).

DETAILED DESCRIPTION

Embodiments of the present disclosure provide nucleic acid (*e.g.*, DNA or RNA such as mRNA) vaccines that include one or more nucleic acids having one or more open reading frames encoding peptide epitopes. As provided herein, nucleic acid cancer vaccines encoding peptide epitopes of non-uniform length may be used to induce a balanced immune response,

comprising cellular and/or humoral immunity. Methods of making a nucleic acid cancer vaccine having a maximized anti-cancer efficacy for a given length are also provided herein, as are methods of treating a patient having cancer with a cancer vaccine having a maximized anti-cancer efficacy for a given length. Additionally, provided herein is a computerized system for creating a nucleic acid cancer vaccine that has a maximized cancer efficacy for a given length. A maximized anti-cancer efficacy may be determined by identifying a T-cell activation value or survival value, such as a maximal T-cell activation value or survival value, based on the length of the epitopes or nucleic acid encoding the epitope. T-cell activation values or survival values can be determined using any method known in the art, for example, using commercially available assays (Thermo Fisher Scientific, Promega Corporation, etc.). Typically T-cell activation values are determined based on changes in expression levels of cytokines, such as interferon gamma associated with T-cell activation or upregulation of cell surface activation markers such as 41BB and/or OX40. Survival values can be assessed relative to survival in controls or population based data on survival.

Although attempts have been made to produce nucleic acid cancer vaccines, such as RNA (*e.g.*, mRNA) cancer vaccines, the efficacy of such vaccines remains variable. Quite surprisingly, the inventors have discovered that immune responses to such cancer vaccines may be optimized through the evaluation and selection of peptide epitopes of varying sizes for inclusion in the cancer vaccine (as opposed to the selection of peptide epitopes of uniform length/size).

The generation of cancer antigens that elicit a desired immune response (*e.g.,* T-cell responses) against targeted polypeptide sequences in vaccine development remains a challenging task. The invention involves technology that overcome hurdles associated with vaccine development. In some embodiments the nucleic acid vaccines of the invention are superior to conventional vaccines (*e.g.*, those encoding peptide epitopes of uniform length) by a factor of at least 10 fold, 20 fold, 40 fold, 50 fold, 100 fold, 500 fold or 1,000 fold.

As a non-limiting example, when an RNA (*e.g.*, mRNA) nucleic acid cancer vaccine as described herein is delivered to a cell, the RNA (*e.g.*, mRNA) will be processed into a polypeptide by the intracellular machinery which can then process the polypeptide into immunosensitive fragments capable of stimulating an immune response against a tumor or population of cancerous cells.

Peptide Epitopes

The nucleic acid cancer vaccines of the disclosure may encode one or more peptide epitopes (which are portions of personalized cancer antigens). Portions of personalized cancer antigens are segments of personalized cancer antigens that are less than the full-length personalized cancer antigen. A personalized cancer antigen is a tumor-specific antigen, also referred to as a neoantigen that is present in a tumor of an individual that is not expressed or is expressed at low levels in normal non-cancerous tissue of the individual. The antigen may or may not be present in tumors of other individuals.

In one embodiment, the nucleic acid cancer vaccine is composed of open reading frames that may contain any number of peptide epitopes. In some embodiments the nucleic acid cancer vaccine is composed of open reading frames encoding 2 or more, 3 or more, 4 or more, 5 or more, 6 or more, 7 or more, 8 or more, 9 or more, 10 or more, 11 or more, 12 or more, 13 or more, 14 or more, 15 or more, 16 or more, 17 or more, 18 or more, 19 or more, 20 or more, 21 or more, 22 or more, 23 or more, 24 or more, 25 or more, 26 or more, 27 or more, 28 or more, 29 or more, 30 or more, 31 or more, 32 or more, 33 or more, 34 or more, 35 or more, 36 or more, 37 or more, 38 or more, 39 or more, 40 or more, 45 or more, 50 or more, 55 or more, 60 or more, 65 or more, 70 or more, 75 or more, 80 or more, 85 or more, 90 or more, 95 or more, 100 or more, 105 or more, 110 or more, 115 or more, 120 or more, 125 or more, 130 or more, 135 or more, 140 or more, 145 or more, 150 or more, 155 or more, 160 or more, 165 or more, 170 or more, 175 or more, 180 or more, 185 or more, 190 or more, 195 or more, or 200 or more peptide epitopes. In other embodiments the nucleic acid cancer vaccine is composed of open reading frames encoding 200 or less, 195 or less, 190 or less, 185 or less, 180 or less, 175 or less, 170 or less, 165 or less, 160 or less, 155 or less, 150 or less, 145 or less, 140 or less, 135 or less, 130 or less, 125 or less, 120 or less, 115 or less, 110 or less, 100 or less, 95 or less, 90 or less, 85 or less, 80 or less, 75 or less, 70 or less, 65 or less, 60 or less, 55 or less, 50 or less, 45 or less, 40 or less, 35 or less, 30 or less, 25 or less, 20 or less, 15 or less, 10 or less, or 5 or less peptide epitopes. In other embodiments the nucleic acid cancer vaccine is composed of open reading frames encoding up to 200, up to 195, up to 190, up to 185, up to 180, up to 175, up to 170, up to 165, up to 160, up to 155, up to 150, up to 145, up to 140, up to 135, up to 130, up to 125, up to 120, up to 115, up to 110, up to 100, up to 95, up to 90, up to 85, up to 80, up to 75, up to 70, up to 65, up to 60, up to 55, up to 50, up to 45, up to 40, up to 35, up to 30, up to 25, up to 20, up to 15, up to 10 peptide epitopes, up to 5 peptide epitopes, or up to 3 peptide epitopes.

In certain embodiments, the nucleic acid cancer vaccine encodes 3-10 peptide epitopes, 5-10 peptide epitopes, 10-20 peptide epitopes, 20-30 peptide epitopes, 30-40 peptide epitopes, 40-50 peptide epitopes, 50-60 peptide epitopes, 60-70 peptide epitopes, 70-80 peptide epitopes, 80-90 peptide epitopes, 90-100 peptide epitopes, 100-110 peptide epitopes, 110-120 peptide epitopes, 120-130 peptide epitopes, 130-140 peptide epitopes, 140-150 peptide epitopes, 150-160 peptide epitopes, 160-170 peptide epitopes, 170-180 peptide epitopes, 180-190 peptide epitopes, or 190-200 peptide epitopes.

In certain embodiments, the nucleic acid cancer vaccine encodes 2-200, 5-200, 8-200, 10-200, 2-190, 5-190, 8-190, 10-190, 2-180, 5-180, 8-180, 10-180, 2-170, 5-170, 8-170, 10-170, 2-160, 5-160, 8-160, 10-160, 2-150, 5-150, 8-150, 10-150, 2-145, 5-145, 8-145, 10-145, 2-140, 5-140, 8-140, 10-140, 2-139, 5-139, 8-139, 10-139, 2-138, 5-138, 8-138, 10-138, 2-137, 5-137, 8-137, 10-137, 2-136, 5-136, 8-136, 10-136, 2-135, 5-135, 8-135, 10-135, 2-134, 5-134, 8-134, 10-134, 2-133, 5-133, 8-133, 10-133, 2-132, 5-132, 8-132, 10-132, 2-131, 5-131, 8-131, 10-131, 2-130, 5-130, 8-130, 10-130, 2-129, 5-129, 8-129, 10-129, 2-128, 5-128, 8-128, 10-128, 2-127, 5-127, 8-127, 10-127, 2-126, 5-126, 8-126, 10-126, 2-125, 5-125, 8-125, 10-125, 2-124, 5-124, 8-124, 10-124, 2-123, 5-123, 8-123, 10-123, 2-122, 5-122, 8-122, 10-122, 2-121, 5-121, 8-121, 10-121, 2-120, 5-120, 8-120, 10-120, 2-119, 5-119, 8-119, 10-119, 2-118, 5-118, 8-118, 10-118, 2-117, 5-117, 8-117, 10-117, 2-116, 5-116, 8-116, 10-116, 2-115, 5-115, 8-115, 10-115, 2-114, 5-114, 8-114, 10-114, 2-113, 5-113, 8-113, 10-113, 2-112, 5-112, 8-112, 10-112, 2-111, 5-111, 8-111, 10-111, 2-110, 5-110, 8-110, 10-110, 2-100, 5-100, 8-100, or 10-100 peptide epitopes.

In other embodiments, the nucleic acid cancer vaccine encodes 2-95, 5-95, 8-95, 10-95, 2-90, 5-90, 8-90, 10-85, 2-85, 5-85, 8-85, 10-85, 2-80, 5-80, 8-80, 10-80, 2-85, 5-85, 8-85, 10-85, 2-80, 5-80, 8-80, 10-80, 2-75, 5-75, 8-75, 10-75, 2-70, 5-70, 8-70, 10-70, 2-65, 5-65, 8-65, 10-65, 2-60, 5-60, 8-60, 10-60, 2-55, 5-55, 8-55, 10-55, 2-50, 5-50, 8-50, 10-50, 2-45, 5-45, 8-45, 10-45, 2-40, 5-40, 8-40, 10-40, 2-39, 5-39, 8-39, 10-39, 2-38, 5-38, 8-38, 10-38, 2-37, 5-37, 8-37, 10-37, 2-36, 5-36, 8-36, 10-36, 2-35, 5-35, 8-35, 10-35, 2-34, 5-34, 8-34, 10-34, 2-33, 5-33, 8-33, 10-33, 2-32, 5-32, 8-32, 10-32, 2-31, 5-31, 8-31, 10-31, 2-30, 5-30, 8-30, 10-30, 2-29, 5-29, 8-29, 10-29, 2-28, 5-28, 8-28, 10-28, 2-27, 5-27, 8-27, 10-27, 2-26, 5-26, 8-26, 10-26, 2-25, 5-25, 8-25, 10-25, 2-24, 5-24, 8-24, 10-24, 2-23, 5-23, 8-23, 10-23, 2-22, 5-22, 8-22, 10-22, 2-21, 5-21, 8-21, 10-21, 2-20, 5-20, 8-20, 10-20, 2-19, 5-19, 8-19, 10-19, 2-18, 5-18, 8-18, 10-18, 2-17, 5-17, 8-17, 10-17, 2-16, 5-16, 8-16, 10-16, 2-15, 5-15, 8-15, 10-15, 2-14, 5-14, 8-14, 10-14, 2-13, 5-13, 8-13, 10-13, 2-12, 5-12, 8-12, 10-12, 2-11, 5-11, 8-11, 10-11, 2-10, 5-10, or 8-10 peptide epitopes.

In yet other embodiments the nucleic acid cancer vaccine encodes 20-200, 30-200, 40-200, 50-200, 20-180, 30-180, 40-180, 50-180, 20-170, 30-170, 40-170, 50-170, 20-160, 30-160, 40-160, 20-150, 30-150, 40-150, 50-150, 20-140, 30-140, 40-140, 50-140, 20-130, 20-130, 40-130, 50-130, 20-120, 30-120, 40-120, 50-120, 20-110, 30-110, 40-110, 50-110, 20-100, 30-100, 40-100, or 50-100 peptide epitopes. In one embodiment, the nucleic acid vaccine encodes 34 peptide epitopes.

In some embodiments the nucleic acid cancer vaccines and vaccination methods described herein include open reading frames that encode epitopes or antigens based on specific mutations (neoepitopes) and/or those expressed by cancer-germline genes (antigens common to tumors found in multiple patients).

An epitope, also known as an antigenic determinant, as used herein is a portion of an antigen that is recognized by the immune system in the appropriate context, specifically by antibodies, B cells, or T cells. Epitopes may include B cell epitopes (*e.g.*, predicted B cell reactive epitopes) and T cell epitopes (*e.g.*, predicted T cell reactive epitopes). B-cell epitopes (*e.g.*, predicted B cell reactive epitopes) are peptide sequences which are required for recognition by specific antibody producing B-cells. B cell epitopes (*e.g.*, predicted B cell reactive epitopes) refer to a specific region of the antigen that is recognized by an antibody. T-cell epitopes (*e.g.*, predicted T cell reactive epitopes) are peptide sequences which, in association with proteins on APC, are required for recognition by specific T-cells. T cell epitopes (*e.g.*, predicted T cell reactive epitopes) are processed intracellularly and presented on the surface of APCs, where they are bound to MHC molecules including MHC class II and MHC class I molecules. The portion of an antibody that binds to the epitope is called a paratope. An epitope may be a conformational epitope or a linear epitope, based on the structure and interaction with the paratope. A linear, or continuous, epitope is defined by the primary amino acid sequence of a particular region of a protein. The sequences that interact with the antibody are situated next to each other sequentially on the protein, and the epitope can usually be mimicked by a single peptide. Conformational epitopes are epitopes that are defined by the conformational structure of the native protein. These epitopes may be continuous or discontinuous (*i.e.,* may be components of the epitope can be situated on disparate parts of the protein, which are brought close to each other in the folded native protein structure).

Each peptide epitope may be any length that is reasonable for an epitope. In some embodiments, the length of each peptide epitope is not necessarily equal. In some embodiments, each peptide epitope in a nucleic acid cancer vaccine is a different length. In

certain embodiments, at least two (*e.g.*, at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, at least 10, at least 11, at least 12, at least 13, at least 14, at least 15, and up to and including all) of the peptide epitopes in a nucleic acid cancer vaccine are different lengths.

In some embodiments, the length of at least one of the peptide epitopes is at least 2, at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, at least 10, at least 11, at least 12, at least 13, at least 14, at least 15, at least 16, at least 17, at least 18, at least 19, at least 20, at least 21, at least 22, at least 23, at least 24, at least 25, at least 26, at least 27, at least 28, at least 29, at least 30, at least 31, at least 32, at least 33, at least 34, at least 35, at least 36, at least 37, at least 38, at least 39, at least 40, at least 45, at least 50, at least 55, at least 60, at least 65, at least 70, at least 75, at least 80, at least 85, at least 90, at least 95, or at least 100 amino acids. In other embodiments, the length of at least one of the peptide epitopes is 100 or less, 95 or less, 90 or less, 85 or less, 80 or less, 75 or less, 70 or less, 65 or less, 60 or less, 55 or less, 50 or less, 45 or less, 40 or less, 35 or less, 30 or less, 25 or less, 20 or less, 15 or less, 14 or less, 13 or less, 12 or less, 11 or less, 10 or less, 9 or less, 8 or less, 7 or less, 6 or less, 5 or less, 4 or less, 3 or less, or 2 or less amino acids. In other embodiments, the length of at least one of the peptide epitopes is up to 100, up to 95, up to 90, up to 85, up to 80, up to 75, up to 70, up to 65, up to 60, up to 55, up to 50, up to 45, up to 40, up to 35, up to 30, up to 25, up to 20, up to 15, or up to 10 amino acids.

In some embodiments each peptide epitope may be from 5-100 amino acids long (inclusive). In some embodiments the length of at least one of the peptide epitopes is 5-100, 5-95, 5-90, 5-85, 5-80, 5-75, 5-70, 5-65, 5-60, 5-55, 5-50, 5-45, 5-40, 5-39, 5-38, 5-37, 5-36, 5-35, 5-34, 5-33, 5-32, 5-31, 5-30, 5-29, 5-28, 5-27, 5-26, 5-25, 5-24, 5-23, 5-22, 5-21, 5-20, 8-100, 8-95, 8-90, 8-85, 8-80, 8-75, 8-70, 8-65, 8-60, 8-55, 8-50, 8-45, 8-40, 8-39, 8-38, 8-37, 8-36, 8-35, 8-34, 8-33, 8-32, 8-31, 8-30, 8-29, 8-28, 8-27, 8-26, 8-25, 8-24, 8-23, 8-22, 8-21, 8-20, 10-100, 10-95, 10-90, 10-85, 10-80, 10-75, 10-70, 10-65, 10-60, 10-55, 10-50, 10-45, 10-40, 10-39, 10-38, 10-37, 10-36, 10-35, 10-34, 10-33, 10-32, 10-31, 10-30, 10-29, 10-28, 10-27, 10-26, 10-25, 10-24, 10-23, 10-22, 10-21, or 10-20 amino acids.

In some embodiments, each of the peptide epitopes encoded by the nucleic acid cancer vaccine may have a different length. In certain embodiments, at least one of the peptide epitopes has a different length than another peptide epitope encoded by the nucleic acid cancer vaccine. Each peptide epitope may be any length that is reasonable for an epitope.

In some embodiments, different percentages of peptide epitope lengths are encoded by the nucleic acids. All of the percentages described in the following listings may be approximate (*i.e.*, within 5% of the stated amount). The use of the terms "approximate" and "about" is equivalent.

In some embodiments, the percentages of peptide epitope lengths encoded by the nucleic acids may be as follows: about 100% < 15 amino acids, about 0% ≥ 15 amino acids; about 95% < 15 amino acids, about 5% ≥ 15 amino acids; about 90% < 15 amino acids, about 10% ≥ 15 amino acids; about 85% < 15 amino acids, about 15% ≥ 15 amino acids; about 80% < 15 amino acids, about 20% ≥ 15 amino acids; about 75% < 15 amino acids, about 25% ≥ 15 amino acids; about 70% < 15 amino acids, about 30% ≥ 15 amino acids; about 65% < 15 amino acids, about 35% ≥ 15 amino acids; about 60% < 15 amino acids, about 40% ≥ 15 amino acids; about 55% < 15 amino acids, about 45% ≥ 15 amino acids; about 50% < 15 amino acids, about 50% ≥ 15 amino acids; about 45% < 15 amino acids, about 55% ≥ 15 amino acids; about 40% < 15 amino acids, about 60% ≥ 15 amino acids; about 35% < 15 amino acids, about 65% ≥ 15 amino acids; about 30% < 15 amino acids, about 70% ≥ 15 amino acids; about 25% < 15 amino acids, about 75% ≥ 15 amino acids; about 20% < 15 amino acids, about 80% ≥ 15 amino acids; about 15% < 15 amino acids, about 85% ≥ 15 amino acids; about 10% < 15 amino acids, about 90% ≥ 15 amino acids; about 5% < 15 amino acids, about 95% ≥ 15 amino acids; or about 0% < 15 amino acids, about 100% ≥ 15 amino acids.

In some embodiments, the percentages of peptide epitope lengths encoded by the nucleic acids may be as follows: about 100% < 17 amino acids, about 0% ≥ 17 amino acids; about 95% < 17 amino acids, about 5% ≥ 17 amino acids; about 90% < 17 amino acids, about 10% ≥ 17 amino acids; about 85% < 17 amino acids, about 17% ≥ 17 amino acids; about 80% < 17 amino acids, about 20% ≥ 17 amino acids; about 75% < 17 amino acids, about 25% ≥ 17 amino acids; about 70% < 17 amino acids, about 30% ≥ 17 amino acids; about 65% < 17 amino acids, about 35% ≥ 17 amino acids; about 60% < 17 amino acids, about 40% ≥ 17 amino acids; about 55% < 17 amino acids, about 45% ≥ 17 amino acids; about 50% < 17 amino acids, about 50% ≥ 17 amino acids; about 45% < 17 amino acids, about 55% ≥ 17 amino acids; about 40% < 17 amino acids, about 60% ≥ 17 amino acids; about 35% < 17 amino acids, about 65% ≥ 17 amino acids; about 30% < 17 amino acids, about 70% ≥ 17 amino acids; about 25% < 17 amino acids, about 75% ≥ 17 amino acids; about 20% < 17 amino acids, about 80% ≥ 17 amino acids; about 17% < 17 amino acids, about 85% ≥ 17 amino acids; about 10% < 17 amino acids, about 90% ≥ 17 amino acids; about 5% < 17

amino acids, about 95% ≥ 17 amino acids; or about 0% < 17 amino acids, about 100% ≥ 17 amino acids.

In some embodiments, the percentages of peptide epitope lengths encoded by the nucleic acids may be as follows: about 100% < 19 amino acids, about 0% ≥ 19 amino acids; about 95% < 19 amino acids, about 5% ≥ 19 amino acids; about 90% < 19 amino acids, about 10% ≥ 19 amino acids; about 85% < 19 amino acids, about 19% ≥ 19 amino acids; about 80% < 19 amino acids, about 20% ≥ 19 amino acids; about 75% < 19 amino acids, about 25% ≥ 19 amino acids; about 70% < 19 amino acids, about 30% ≥ 19 amino acids; about 65% < 19 amino acids, about 35% ≥ 19 amino acids; about 60% < 19 amino acids, about 40% ≥ 19 amino acids; about 55% < 19 amino acids, about 45% ≥ 19 amino acids; about 50% < 19 amino acids, about 50% ≥ 19 amino acids; about 45% < 19 amino acids, about 55% ≥ 19 amino acids; about 40% < 19 amino acids, about 60% ≥ 19 amino acids; about 35% < 19 amino acids, about 65% ≥ 19 amino acids; about 30% < 19 amino acids, about 70% ≥ 19 amino acids; about 25% < 19 amino acids, about 75% ≥ 19 amino acids; about 20% < 19 amino acids, about 80% ≥ 19 amino acids; about 19% < 19 amino acids, about 85% ≥ 19 amino acids; about 10% < 19 amino acids, about 90% ≥ 19 amino acids; about 5% < 19 amino acids, about 95% ≥ 19 amino acids; or about 0% < 19 amino acids, about 100% ≥ 19 amino acids.

In some embodiments, the peptide epitope lengths may be categorized in one of the following groups (for a total of 100%): 8-12 amino acids, 13-17 amino acids, 18-21 amino acids, 22-26 amino acids, or 27-31 amino acids. About 0%, 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 100% of the peptide epitopes encoded by the open reading frames of the nucleic acids may be 8-12 amino acids in length. About 0%, 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 100% of the peptide epitopes encoded by the open reading frames of the nucleic acids may be 13-17 amino acids in length. About 0%, 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 100% of the peptide epitopes encoded by the open reading frames of the nucleic acids may be 18-21 amino acids in length. About 0%, 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 100% of the peptide epitopes encoded by the open reading frames of the nucleic acids may be 22-26 amino acids in length. About 0%, 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 100% of the peptide epitopes encoded by the open reading frames of the nucleic acids may be 27-31 amino acids in length. Several

non-limiting examples of the percentages of peptide epitope lengths encoded by the open reading frames of the nucleic acids follow.

In some embodiments, the percentages of peptide epitope lengths encoded by the nucleic acids may be as follows: 50% 8-12 amino acids, 50% 13-17 amino acids, 0% 18-21 amino acids, 0% 22-26 amino acids, and 0% 27-31 amino acids; 0% 8-12 amino acids, 50% 13-17 amino acids, 50% 18-21 amino acids, 0% 22-26 amino acids, and 0% 27-31 amino acids; 0% 8-12 amino acids, 0% 13-17 amino acids, 50% 18-21 amino acids, 50% 22-26 amino acids, and 0% 27-31 amino acids; 0% 8-12 amino acids, 0% 13-17 amino acids, 0% 18-21 amino acids, 50% 22-26 amino acids, and 50% 27-31 amino acids; 50% 8-12 amino acids, 0% 13-17 amino acids, 50% 18-21 amino acids, 0% 22-26 amino acids, and 0% 27-31 amino acids; 50% 8-12 amino acids, 0% 13-17 amino acids, 0% 18-21 amino acids, 50% 22-26 amino acids, and 0% 27-31 amino acids; 50% 8-12 amino acids, 0% 13-17 amino acids, 0% 18-21 amino acids, 0% 22-26 amino acids, and 50% 27-31 amino acids; 0% 8-12 amino acids, 50% 13-17 amino acids, 50% 18-21 amino acids, 0% 22-26 amino acids, and 0% 27-31 amino acids; 0% 8-12 amino acids, 50% 13-17 amino acids, 0% 18-21 amino acids, 50% 22-26 amino acids, and 0% 27-31 amino acids; 0% 8-12 amino acids, 50% 13-17 amino acids, 0% 18-21 amino acids, 0% 22-26 amino acids, and 50% 27-31 amino acids; or 0% 8-12 amino acids, 0% 13-17 amino acids, 50% 18-21 amino acids, 0% 22-26 amino acids, and 50% 27-31 amino acids.

In some embodiments, the percentages of peptide epitope lengths encoded by the nucleic acids may be as follows: 10% 8-12 amino acids, 40% 13-17 amino acids, 40% 18-21 amino acids, 10% 22-26 amino acids, and 0% 27-31 amino acids; 10% 8-12 amino acids, 10% 13-17 amino acids, 40% 18-21 amino acids, 40% 22-26 amino acids, and 0% 27-31 amino acids; 40% 8-12 amino acids, 40% 13-17 amino acids, 10% 18-21 amino acids, 10% 22-26 amino acids, and 0% 27-31 amino acids; 10% 8-12 amino acids, 40% 13-17 amino acids, 10% 18-21 amino acids, 40% 22-26 amino acids, and 0% 27-31 amino acids; 40% 8-12 amino acids, 10% 13-17 amino acids, 40% 18-21 amino acids, 10% 22-26 amino acids, and 0% 27-31 amino acids; 0% 8-12 amino acids, 10% 13-17 amino acids, 40% 18-21 amino acids, 40% 22-26 amino acids, and 10% 27-31 amino acids; 0% 8-12 amino acids, 10% 13-17 amino acids, 10% 18-21 amino acids, 40% 22-26 amino acids, and 40% 27-31 amino acids; 0% 8-12 amino acids, 40% 13-17 amino acids, 40% 18-21 amino acids, 10% 22-26 amino acids, and 10% 27-31 amino acids; 0% 8-12 amino acids, 10% 13-17 amino acids, 40% 18-21 amino acids, 10% 22-26 amino acids, and 40% 27-31 amino acids; 0% 8-12 amino acids,

40% 13-17 amino acids, 10% 18-21 amino acids, 40% 22-26 amino acids, and 10% 27-31 amino acids.

In some embodiments, the percentages of peptide epitope lengths encoded by the nucleic acids may be as follows: 25% 8-12 amino acids, 25% 13-17 amino acids, 25% 18-21 amino acids, 25% 22-26 amino acids, and 0% 27-31 amino acids; 25% 8-12 amino acids, 25% 13-17 amino acids, 25% 18-21 amino acids, 0% 22-26 amino acids, and 25% 27-31 amino acids; 25% 8-12 amino acids, 25% 13-17 amino acids, 0% 18-21 amino acids, 25% 22-26 amino acids, and 25% 27-31 amino acids; 25% 8-12 amino acids, 0% 13-17 amino acids, 25% 18-21 amino acids, 25% 22-26 amino acids, and 25% 27-31 amino acids; 0% 8-12 amino acids, 25% 13-17 amino acids, 25% 18-21 amino acids, 25% 22-26 amino acids, and 25% 27-31 amino acids.

In some embodiments, the percentages of peptide epitope lengths encoded by the nucleic acids may be as follows: 15% 8-12 amino acids, 15% 13-17 amino acids, 15% 18-21 amino acids, 15% 22-26 amino acids, and 40% 27-31 amino acids; 15% 8-12 amino acids, 15% 13-17 amino acids, 15% 18-21 amino acids, 15% 22-26 amino acids, and 40% 27-31 amino acids; 15% 8-12 amino acids, 15% 13-17 amino acids, 15% 18-21 amino acids, 15% 22-26 amino acids, and 40% 27-31 amino acids; 15% 8-12 amino acids, 15% 13-17 amino acids, 15% 18-21 amino acids, 15% 22-26 amino acids, and 40% 27-31 amino acids; 15% 8-12 amino acids, 15% 13-17 amino acids, 15% 18-21 amino acids, 15% 22-26 amino acids, and 40% 27-31 amino acids; 40% 8-12 amino acids, 15% 13-17 amino acids, 15% 18-21 amino acids, 15% 22-26 amino acids, and 15% 27-31 amino acids; 40% 8-12 amino acids, 15% 13-17 amino acids, 15% 18-21 amino acids, 15% 22-26 amino acids, and 15% 27-31 amino acids; 40% 8-12 amino acids, 15% 13-17 amino acids, 15% 18-21 amino acids, 15% 22-26 amino acids, and 15% 27-31 amino acids; 40% 8-12 amino acids, 15% 13-17 amino acids, 15% 18-21 amino acids, 15% 22-26 amino acids, and 15% 27-31 amino acids.

In some embodiments, the percentages of peptide epitope lengths encoded by the nucleic acids may be as follows: 10% 8-12 amino acids, 10% 13-17 amino acids, 10% 18-21 amino acids, 10% 22-26 amino acids, and 60% 27-31 amino acids; 10% 8-12 amino acids, 10% 13-17 amino acids, 10% 18-21 amino acids, 10% 22-26 amino acids, and 60% 27-31 amino acids; 10% 8-12 amino acids, 10% 13-17 amino acids, 10% 18-21 amino acids, 10% 22-26 amino acids, and 60% 27-31 amino acids; 10% 8-12 amino acids, 10% 13-17 amino acids, 10% 18-21 amino acids, 10% 22-26 amino acids, and 60% 27-31 amino acids; 10% 8-

12 amino acids, 10% 13-17 amino acids, 10% 18-21 amino acids, 10% 22-26 amino acids, and 60% 27-31 amino acids; 60% 8-12 amino acids, 10% 13-17 amino acids, 10% 18-21 amino acids, 10% 22-26 amino acids, and 10% 27-31 amino acids; 60% 8-12 amino acids, 10% 13-17 amino acids, 10% 18-21 amino acids, 10% 22-26 amino acids, and 10% 27-31 amino acids; 60% 8-12 amino acids, 10% 13-17 amino acids, 10% 18-21 amino acids, 10% 22-26 amino acids, and 10% 27-31 amino acids; 60% 8-12 amino acids, 10% 13-17 amino acids, 10% 18-21 amino acids, 10% 22-26 amino acids, and 10% 27-31 amino acids; 60% 8-12 amino acids, 10% 13-17 amino acids, 10% 18-21 amino acids, 10% 22-26 amino acids, and 10% 27-31 amino acids.

In some embodiments, the percentages of peptide epitope lengths encoded by the nucleic acids may be as follows: 15% 8-12 amino acids, 20% 13-17 amino acids, 20% 18-21 amino acids, 15% 22-26 amino acids, and 30% 27-31 amino acids; 15% 8-12 amino acids, 15% 13-17 amino acids, 20% 18-21 amino acids, 20% 22-26 amino acids, and 30% 27-31 amino acids; 20% 8-12 amino acids, 20% 13-17 amino acids, 15% 18-21 amino acids, 15% 22-26 amino acids, and 30% 27-31 amino acids; 15% 8-12 amino acids, 20% 13-17 amino acids, 15% 18-21 amino acids, 20% 22-26 amino acids, and 30% 27-31 amino acids; 20% 8-12 amino acids, 15% 13-17 amino acids, 20% 18-21 amino acids, 15% 22-26 amino acids, and 30% 27-31 amino acids; 30% 8-12 amino acids, 15% 13-17 amino acids, 20% 18-21 amino acids, 20% 22-26 amino acids, and 15% 27-31 amino acids; 30% 8-12 amino acids, 15% 13-17 amino acids, 15% 18-21 amino acids, 20% 22-26 amino acids, and 20% 27-31 amino acids; 30% 8-12 amino acids, 20% 13-17 amino acids, 20% 18-21 amino acids, 15% 22-26 amino acids, and 15% 27-31 amino acids; 30% 8-12 amino acids, 15% 13-17 amino acids, 20% 18-21 amino acids, 15% 22-26 amino acids, and 20% 27-31 amino acids; 30% 8-12 amino acids, 20% 13-17 amino acids, 15% 18-21 amino acids, 20% 22-26 amino acids, and 15% 27-31 amino acids.

In some embodiments, the percentages of peptide epitope lengths encoded by the nucleic acids may be as follows: 35% 8-12 amino acids, 35% 13-17 amino acids, 10% 18-21 amino acids, 10% 22-26 amino acids, and 10% 27-31 amino acids; 10% 8-12 amino acids, 35% 13-17 amino acids, 35% 18-21 amino acids, 10% 22-26 amino acids, and 10% 27-31 amino acids; 10% 8-12 amino acids, 10% 13-17 amino acids, 35% 18-21 amino acids, 35% 22-26 amino acids, and 10% 27-31 amino acids; 10% 8-12 amino acids, 10% 13-17 amino acids, 10% 18-21 amino acids, 35% 22-26 amino acids, and 35% 27-31 amino acids; 35% 8-12 amino acids, 10% 13-17 amino acids, 35% 18-21 amino acids, 10% 22-26 amino acids, and 10% 27-31 amino acids; 35% 8-12 amino acids, 10% 13-17 amino acids, 10% 18-21

amino acids, 35% 22-26 amino acids, and 10% 27-31 amino acids; 35% 8-12 amino acids, 10% 13-17 amino acids, 10% 18-21 amino acids, 10% 22-26 amino acids, and 35% 27-31 amino acids; 10% 8-12 amino acids, 35% 13-17 amino acids, 10% 18-21 amino acids, 35% 22-26 amino acids, and 10% 27-31 amino acids; 10% 8-12 amino acids, 35% 13-17 amino acids, 10% 18-21 amino acids, 10% 22-26 amino acids, and 35% 27-31 amino acids.

In some embodiments, the percentages of peptide epitope lengths encoded by the nucleic acids may be as follows: 30% 8-12 amino acids, 30% 13-17 amino acids, 30% 18-21 amino acids, 5% 22-26 amino acids, and 5% 27-31 amino acids; 5% 8-12 amino acids, 30% 13-17 amino acids, 30% 18-21 amino acids, 30% 22-26 amino acids, and 5% 27-31 amino acids; 5% 8-12 amino acids, 5% 13-17 amino acids, 30% 18-21 amino acids, 30% 22-26 amino acids, and 30% 27-31 amino acids; 30% 8-12 amino acids, 5% 13-17 amino acids, 5% 18-21 amino acids, 30% 22-26 amino acids, and 30% 27-31 amino acids; 30% 8-12 amino acids, 30% 13-17 amino acids, 5% 18-21 amino acids, 5% 22-26 amino acids, and 30% 27-31 amino acids; 5% 8-12 amino acids, 30% 13-17 amino acids, 5% 18-21 amino acids, 30% 22-26 amino acids, and 30% 27-31 amino acids; 5% 8-12 amino acids, 30% 13-17 amino acids, 30% 18-21 amino acids, 5% 22-26 amino acids, and 30% 27-31 amino acids; 30% 8-12 amino acids, 30% 13-17 amino acids, 5% 18-21 amino acids, 30% 22-26 amino acids, and 5% 27-31 amino acids; 30% 8-12 amino acids, 5% 13-17 amino acids, 30% 18-21 amino acids, 5% 22-26 amino acids, and 30% 27-31 amino acids.

In some embodiments, the percentages of peptide epitope lengths encoded by the nucleic acids may be as follows: 20% 8-12 amino acids, 20% 13-17 amino acids, 20% 18-21 amino acids, 20% 22-26 amino acids, and 20% 27-31 amino acids.

In some embodiments, the optimal length of a peptide epitope may be obtained through the following procedure: synthesizing a V5 tag concatemer-test protease site, introducing it into DC cells (for example, using an RNA Squeeze procedure), lysing the cells, and then running an anti-V5 Western blot to assess the cleavage at protease sites.

The RNA Squeeze technique is an intracellular delivery method by which a variety of materials can be delivered to a broad range of live cells. Cells are subjected to microfluidic construction, which causes rapid mechanical deformation. The deformation results in temporary membrane disruption and the newly-formed transient pores. Material is then passively diffused into the cell cytosol via the transient pores. The technique can be used in a variety of cell types, including primary fibroblasts, embryonic stem cells, and a host of immune cells, and has been shown to have relatively high viability in most applications and

does not damage sensitive materials, such as quantum dots or proteins, through its actions. Sharei et al., PNAS (2013); 110(6):2082-7.

The peptide epitopes described herein may be encoded in any order in the nucleic acid. For example, each of the peptide epitopes may have a length that may be categorized in one of the following groups (for a total of 100%): 8-12 amino acids (represented by "A"), 13-17 amino acids (represented by "B"), 18-21 amino acids (represented by "C"), 22-26 amino acids (represented by "D"), or 27-31 amino acids (represented by "E"). One or more peptide epitopes of any group (*e.g.*, 8-12 aa) may be encoded consecutively by the nucleic acid (*e.g.*, the nucleic acid may encode two or more peptide epitopes of length "A" in a row and these epitopes may be directly linked or indirectly linked as described elsewhere herein). Additionally, the peptide epitopes of different groups may be interspersed and the nucleic acid may encode epitopes of different groups consecutively (*e.g.*, the nucleic acid may encode a peptide epitope of length A next to a peptide epitope of length B, C, D, or E and these epitopes may be directly linked or indirectly linked as described elsewhere herein).

As a non-limiting example, the peptide epitopes may be encoded as follows in a nucleic acid or the nucleic acid may encode (at least in part) one of the following combinations of peptide epitopes:

$(A)_{1-50}(B)_{1-50}(C)_{1-50}(D)_{1-50}(E)_{1-50}$, $(A)_{1-50}(B)_{1-50}(C)_{1-50}(E)_{1-50}(D)_{1-50}$, $(A)_{1-50}(B)_{1-50}(D)_{1-50}(C)_{1-50}(E)_{1-50}$, $(A)_{1-50}(B)_{1-50}(D)_{1-50}(E)_{1-50}(C)_{1-50}$, $(A)_{1-50}(B)_{1-50}(E)_{1-50}(C)_{1-50}(D)_{1-50}$, $(A)_{1-50}(B)_{1-50}(E)_{1-50}(D)_{1-50}(C)_{1-50}$, $(A)_{1-50}(C)_{1-50}(D)_{1-50}(E)_{1-50}(B)_{1-50}$, $(A)_{1-50}(C)_{1-50}(D)_{1-50}(B)_{1-50}(E)_{1-50}$, $(A)_{1-50}(C)_{1-50}(E)_{1-50}(D)_{1-50}(B)_{1-50}$, $(A)_{1-50}(C)_{1-50}(E)_{1-50}(B)_{1-50}(D)_{1-50}$, $(A)_{1-50}(C)_{1-50}(B)_{1-50}(E)_{1-50}(D)_{1-50}$, $(A)_{1-50}(C)_{1-50}(B)_{1-50}(D)_{1-50}(E)_{1-50}$, $(A)_{1-50}(D)_{1-50}(C)_{1-50}(B)_{1-50}(E)_{1-50}$, $(A)_{1-50}(D)_{1-50}(C)_{1-50}(E)_{1-50}(B)_{1-50}$, $(A)_{1-50}(D)_{1-50}(B)_{1-50}(C)_{1-50}(E)_{1-50}$, $(A)_{1-50}(D)_{1-50}(B)_{1-50}(E)_{1-50}(C)_{1-50}$, $(A)_{1-50}(D)_{1-50}(E)_{1-50}(B)_{1-50}(C)_{1-50}$, $(A)_{1-50}(D)_{1-50}(E)_{1-50}(C)_{1-50}(B)_{1-50}$, $(A)_{1-50}(E)_{1-50}(C)_{1-50}(B)_{1-50}(D)_{1-50}$, $(A)_{1-50}(E)_{1-50}(C)_{1-50}(D)_{1-50}(B)_{1-50}$, $(A)_{1-50}(E)_{1-50}(B)_{1-50}(C)_{1-50}(D)_{1-50}$, $(A)_{1-50}(E)_{1-50}(B)_{1-50}(D)_{1-50}(C)_{1-50}$, $(A)_{1-50}(E)_{1-50}(D)_{1-50}(B)_{1-50}(C)_{1-50}$, $(A)_{1-50}(E)_{1-50}(D)_{1-50}(C)_{1-50}(B)_{1-50}$, $(B)_{1-50}(A)_{1-50}(C)_{1-50}(D)_{1-50}(E)_{1-50}$, $(B)_{1-50}(A)_{1-50}(C)_{1-50}(E)_{1-50}(D)_{1-50}$, $(B)_{1-50}(A)_{1-50}(D)_{1-50}(C)_{1-50}(E)_{1-50}$, $(B)_{1-50}(A)_{1-50}(D)_{1-50}(E)_{1-50}(C)_{1-50}$, $(B)_{1-50}(A)_{1-50}(E)_{1-50}(C)_{1-50}(D)_{1-50}$, $(B)_{1-50}(A)_{1-50}(E)_{1-50}(D)_{1-50}(C)_{1-50}$, $(B)_{1-50}(C)_{1-50}(D)_{1-50}(E)_{1-50}(A)_{1-50}$, $(B)_{1-50}(C)_{1-50}(D)_{1-50}(A)_{1-50}(E)_{1-50}$, $(B)_{1-50}(C)_{1-50}(E)_{1-50}(D)_{1-50}(A)_{1-50}$, $(B)_{1-50}(C)_{1-50}(E)_{1-50}(A)_{1-50}(D)_{1-50}$, $(B)_{1-50}(C)_{1-50}(A)_{1-50}(E)_{1-50}(D)_{1-50}$, $(B)_{1-50}(C)_{1-50}(A)_{1-50}(D)_{1-50}(E)_{1-50}$, $(B)_{1-50}(D)_{1-50}(C)_{1-50}(A)_{1-50}(E)_{1-50}$, $(B)_{1-50}(D)_{1-50}(C)_{1-50}(E)_{1-50}(A)_{1-50}$, $(B)_{1-50}(D)_{1-50}(A)_{1-50}(C)_{1-50}(E)_{1-50}$, $(B)_{1-50}(D)_{1-50}(A)_{1-50}(E)_{1-50}(C)_{1-50}$, $(B)_{1-50}(D)_{1-50}(E)_{1-50}(A)_{1-50}(C)_{1-50}$, $(B)_{1-50}(D)_{1-50}(E)_{1-50}(C)_{1-50}(A)_{1-50}$, $(B)_{1-50}(E)_{1-50}(C)_{1-50}(A)_{1-50}(D)_{1-50}$, $(B)_{1-50}(E)_{1-50}(C)_{1-50}(D)_{1-50}(A)_{1-50}$, $(B)_{1-50}(E)_{1-50}(A)_{1-50}(C)_{1-50}(D)_{1-50}$, $(B)_{1-50}(E)_{1-50}$

$_{50}$(A)$_{1-50}$(D)$_{1-50}$(C)$_{1-50}$, (B)$_{1-50}$ (E)$_{1-50}$(D)$_{1-50}$(A)$_{1-50}$(C)$_{1-50}$, (B)$_{1-50}$ (E)$_{1-50}$(D)$_{1-50}$(C)$_{1-50}$(A)$_{1-50}$, (C)$_{1-50}$ (B)$_{1-50}$(A)$_{1-50}$(D)$_{1-50}$(E)$_{1-50}$, (C)$_{1-50}$ (B)$_{1-50}$(A)$_{1-50}$(E)$_{1-50}$(D)$_{1-50}$, (C)$_{1-50}$ (B)$_{1-50}$(D)$_{1-50}$(A)$_{1-50}$(E)$_{1-50}$, (C)$_{1-50}$ (B)$_{1-50}$(D)$_{1-50}$(E)$_{1-50}$(A)$_{1-50}$, (C)$_{1-50}$ (B)$_{1-50}$(E)$_{1-50}$(A)$_{1-50}$(D)$_{1-50}$, (C)$_{1-50}$ (B)$_{1-50}$(E)$_{1-50}$(D)$_{1-50}$(A)$_{1-50}$, (C)$_{1-50}$ (A)$_{1-50}$(D)$_{1-50}$(E)$_{1-50}$(B)$_{1-50}$, (C)$_{1-50}$ (A)$_{1-50}$(D)$_{1-50}$(B)$_{1-50}$(E)$_{1-50}$, (C)$_{1-50}$ (A)$_{1-50}$(E)$_{1-50}$(D)$_{1-50}$(B)$_{1-50}$, (C)$_{1-50}$ (A)$_{1-50}$(E)$_{1-50}$(B)$_{1-50}$(D)$_{1-50}$, (C)$_{1-50}$ (A)$_{1-50}$(B)$_{1-50}$(E)$_{1-50}$(D)$_{1-50}$, (C)$_{1-50}$ (A)$_{1-50}$(B)$_{1-50}$(D)$_{1-50}$(E)$_{1-50}$, (C)$_{1-50}$ (D)$_{1-50}$(A)$_{1-50}$(B)$_{1-50}$(E)$_{1-50}$, (C)$_{1-50}$ (D)$_{1-50}$(A)$_{1-50}$(E)$_{1-50}$(B)$_{1-50}$, (C)$_{1-50}$ (D)$_{1-50}$(B)$_{1-50}$(A)$_{1-50}$(E)$_{1-50}$, (C)$_{1-50}$ (D)$_{1-50}$(B)$_{1-50}$(E)$_{1-50}$(A)$_{1-50}$, (C)$_{1-50}$ (D)$_{1-50}$(E)$_{1-50}$(B)$_{1-50}$(A)$_{1-50}$, (C)$_{1-50}$ (D)$_{1-50}$(E)$_{1-50}$(A)$_{1-50}$(B)$_{1-50}$, (C)$_{1-50}$ (E)$_{1-50}$(A)$_{1-50}$(B)$_{1-50}$(D)$_{1-50}$, (C)$_{1-50}$ (E)$_{1-50}$(A)$_{1-50}$(D)$_{1-50}$(B)$_{1-50}$, (C)$_{1-50}$ (E)$_{1-50}$(B)$_{1-50}$(A)$_{1-50}$(D)$_{1-50}$, (C)$_{1-50}$ (E)$_{1-50}$(B)$_{1-50}$(D)$_{1-50}$(A)$_{1-50}$, (C)$_{1-50}$ (E)$_{1-50}$(D)$_{1-50}$(B)$_{1-50}$(A)$_{1-50}$, (C)$_{1-50}$ (E)$_{1-50}$(D)$_{1-50}$(A)$_{1-50}$(B)$_{1-50}$, (D)$_{1-50}$ (B)$_{1-50}$(C)$_{1-50}$(A)$_{1-50}$(E)$_{1-50}$, (D)$_{1-50}$ (B)$_{1-50}$(C)$_{1-50}$(E)$_{1-50}$(A)$_{1-50}$, (D)$_{1-50}$ (B)$_{1-50}$(A)$_{1-50}$(C)$_{1-50}$(E)$_{1-50}$, (D)$_{1-50}$ (B)$_{1-50}$(A)$_{1-50}$(E)$_{1-50}$(C)$_{1-50}$, (D)$_{1-50}$ (B)$_{1-50}$(E)$_{1-50}$(C)$_{1-50}$(A)$_{1-50}$, (D)$_{1-50}$ (B)$_{1-50}$(E)$_{1-50}$(A)$_{1-50}$(C)$_{1-50}$, (D)$_{1-50}$ (C)$_{1-50}$(A)$_{1-50}$(E)$_{1-50}$(B)$_{1-50}$, (D)$_{1-50}$ (C)$_{1-50}$(A)$_{1-50}$(B)$_{1-50}$(E)$_{1-50}$, (D)$_{1-50}$ (C)$_{1-50}$(E)$_{1-50}$(A)$_{1-50}$(B)$_{1-50}$, (D)$_{1-50}$ (C)$_{1-50}$(E)$_{1-50}$(B)$_{1-50}$(A)$_{1-50}$, (D)$_{1-50}$ (C)$_{1-50}$(B)$_{1-50}$(E)$_{1-50}$(A)$_{1-50}$, (D)$_{1-50}$ (C)$_{1-50}$(B)$_{1-50}$(A)$_{1-50}$(E)$_{1-50}$, (D)$_{1-50}$ (A)$_{1-50}$(C)$_{1-50}$(B)$_{1-50}$(E)$_{1-50}$, (D)$_{1-50}$ (A)$_{1-50}$(C)$_{1-50}$(E)$_{1-50}$(B)$_{1-50}$, (D)$_{1-50}$ (A)$_{1-50}$(B)$_{1-50}$(C)$_{1-50}$(E)$_{1-50}$, (D)$_{1-50}$ (A)$_{1-50}$(B)$_{1-50}$(E)$_{1-50}$(C)$_{1-50}$, (D)$_{1-50}$ (A)$_{1-50}$(E)$_{1-50}$(B)$_{1-50}$(C)$_{1-50}$, (D)$_{1-50}$ (A)$_{1-50}$(E)$_{1-50}$(C)$_{1-50}$(B)$_{1-50}$, (D)$_{1-50}$ (E)$_{1-50}$(C)$_{1-50}$(B)$_{1-50}$(A)$_{1-50}$, (D)$_{1-50}$ (E)$_{1-50}$(C)$_{1-50}$(A)$_{1-50}$(B)$_{1-50}$, (D)$_{1-50}$ (E)$_{1-50}$(B)$_{1-50}$(C)$_{1-50}$(A)$_{1-50}$, (D)$_{1-50}$ (E)$_{1-50}$(B)$_{1-50}$(A)$_{1-50}$(C)$_{1-50}$, (D)$_{1-50}$ (E)$_{1-50}$(A)$_{1-50}$(B)$_{1-50}$(C)$_{1-50}$, (D)$_{1-50}$ (E)$_{1-50}$(A)$_{1-50}$(C)$_{1-50}$(B)$_{1-50}$, (E)$_{1-50}$ (B)$_{1-50}$(C)$_{1-50}$(D)$_{1-50}$(A)$_{1-50}$, (E)$_{1-50}$ (B)$_{1-50}$(C)$_{1-50}$(A)$_{1-50}$(D)$_{1-50}$, (E)$_{1-50}$ (B)$_{1-50}$(D)$_{1-50}$(C)$_{1-50}$(A)$_{1-50}$, (E)$_{1-50}$ (B)$_{1-50}$(D)$_{1-50}$(A)$_{1-50}$(C)$_{1-50}$, (E)$_{1-50}$ (B)$_{1-50}$(A)$_{1-50}$(C)$_{1-50}$(D)$_{1-50}$, (E)$_{1-50}$ (B)$_{1-50}$(A)$_{1-50}$(D)$_{1-50}$(C)$_{1-50}$, (E)$_{1-50}$ (C)$_{1-50}$(D)$_{1-50}$(A)$_{1-50}$(B)$_{1-50}$, (E)$_{1-50}$ (C)$_{1-50}$(D)$_{1-50}$(B)$_{1-50}$(A)$_{1-50}$, (E)$_{1-50}$ (C)$_{1-50}$(A)$_{1-50}$(D)$_{1-50}$(B)$_{1-50}$, (E)$_{1-50}$ (C)$_{1-50}$(A)$_{1-50}$(B)$_{1-50}$(D)$_{1-50}$, (E)$_{1-50}$ (C)$_{1-50}$(B)$_{1-50}$(A)$_{1-50}$(D)$_{1-50}$, (E)$_{1-50}$ (C)$_{1-50}$(B)$_{1-50}$(D)$_{1-50}$(A)$_{1-50}$, (E)$_{1-50}$ (D)$_{1-50}$(C)$_{1-50}$(B)$_{1-50}$(A)$_{1-50}$, (E)$_{1-50}$ (D)$_{1-50}$(C)$_{1-50}$(A)$_{1-50}$(B)$_{1-50}$, (E)$_{1-50}$ (D)$_{1-50}$(B)$_{1-50}$(C)$_{1-50}$(A)$_{1-50}$, (E)$_{1-50}$ (D)$_{1-50}$(B)$_{1-50}$(A)$_{1-50}$(C)$_{1-50}$, (E)$_{1-50}$ (D)$_{1-50}$(A)$_{1-50}$(B)$_{1-50}$(C)$_{1-50}$, (E)$_{1-50}$ (D)$_{1-50}$(A)$_{1-50}$(C)$_{1-50}$(B)$_{1-50}$, (E)$_{1-50}$ (A)$_{1-50}$(C)$_{1-50}$(B)$_{1-50}$(D)$_{1-50}$, (E)$_{1-50}$ (A)$_{1-50}$(B)$_{1-50}$(C)$_{1-50}$(D)$_{1-50}$, (E)$_{1-50}$ (A)$_{1-50}$(B)$_{1-50}$(D)$_{1-50}$(C)$_{1-50}$, (E)$_{1-50}$ (A)$_{1-50}$(D)$_{1-50}$(B)$_{1-50}$(C)$_{1-50}$, or (E)$_{1-50}$ (A)$_{1-50}$(D)$_{1-50}$(C)$_{1-50}$(B)$_{1-50}$

wherein a peptide epitopes of 8-12 amino acids are represented by "A", peptide epitopes of 13-17 amino acids are represented by "B", peptide epitopes of 18-21 amino acids are represented by "C", peptide epitopes of 22-26 amino acids are represented by "D", and peptide epitopes of 27-31 amino acids are represented by "E".

Any of the foregoing combinations of peptide epitopes may be combined. For example, any of the nucleic acid cancer vaccines described herein may encode more than one of the listed groups of peptide epitopes.

In some embodiments, the peptide epitopes comprise at least one MHC class I epitope and at least one MHC class II epitope. In some embodiments, at least 10% of the peptide epitopes are MHC class I epitopes. In some embodiments, at least 20% of the peptide epitopes are MHC class I epitopes. In some embodiments, at least 30% of the peptide epitopes are MHC class I epitopes. In some embodiments, at least 40% of the peptide epitopes are MHC class I epitopes. In some embodiments, at least 0%, 60%, 70%, 80%, 90%, or 100% of the peptide epitopes are MHC class I epitopes. In some embodiments, none (0%) of the peptide epitopes are MHC class II epitopes. In some embodiments, at least 10% of the peptide epitopes are MHC class II epitopes. In some embodiments, at least 20% of the peptide epitopes are MHC class II epitopes. In some embodiments, at least 30% of the peptide epitopes are MHC class II epitopes. In some embodiments, at least 40% of the peptide epitopes are MHC class II epitopes. In some embodiments, at least 50%, 60%, 70%, 80%, 90% or 100% of the peptide epitopes are MHC class II epitopes. In some embodiments, the ratio of MHC class I epitopes to MHC class II epitopes is a ratio selected from about 10%:about 90%; about 20%:about 80%; about 30%:about 70%; about 40%:about 60%; about 50%:about 50%; about 60%:about 40%; about 70%:about 30%; about 80%: about 20%; about 90%: about 10% MHC class 1: MHC class II epitopes. In one embodiment, the ratio of MHC class I : MHC class II epitopes is 1:1. In one embodiment, the ratio of MHC class I : MHC class II epitopes is 2:1. In one embodiment, the ratio of MHC class I : MHC class II epitopes is 3:1. In one embodiment, the ratio of MHC class I : MHC class II epitopes is 4:1. In one embodiment, the ratio of MHC class I : MHC class II epitopes is 5:1. In some embodiments, the ratio of MHC class II epitopes to MHC class I epitopes is a ratio selected from about 10%:about 90%; about 20%:about 80%; about 30%:about 70%; about 40%:about 60%; about 50%:about 50%; about 60%:about 40%; about 70%:about 30%; about 80%: about 20%; about 90%: about 10% MHC class II: MHC class I epitopes. In one embodiment, the ratio of MHC class II : MHC class I epitopes is 1:1. In one embodiment, the ratio of MHC class II : MHC class I epitopes is 1:2. In one embodiment, the ratio of MHC class II : MHC class I epitopes is 1:3. In one embodiment, the ratio of MHC class II : MHC class I epitopes is 1:4. In one embodiment, the ratio of MHC class II : MHC class I epitopes is 1:5. In some embodiments, at least one of the peptide epitopes of the cancer vaccine is a B cell epitope. In some embodiments, one or more predicted T cell

reactive epitope of the cancer vaccine comprises between 8-11 amino acids. In some embodiments, one or more predicted B cell reactive epitope of the cancer vaccine comprises between 13-17 amino acids.

The cancer vaccine of the disclosure, in some aspects comprises an mRNA vaccine encoding multiple peptide epitope antigens arranged with a single amino acid spacer between the peptide epitopes, a short linker between the peptide epitopes, or directly to one another without a spacer between the peptide epitopes. The multiple epitope antigens may include a mixture of MHC class I epitopes and MHC class II epitopes. As a non-limiting example, the multiple peptide epitope antigens may be a polypeptide having the structure:

$(X\text{-}G\text{-}X)_{1\text{-}10}(G\text{-}Y\text{-}G\text{-}Y)_{1\text{-}10}(G\text{-}X\text{-}G\text{-}X)_{0\text{-}10}(G\text{-}Y\text{-}G\text{-}Y)_{0\text{-}10}$, $(X\text{-}G)_{1\text{-}10}(G\text{-}Y)_{1\text{-}10}(G\text{-}X)_{0\text{-}10}(G\text{-}Y)_{0\text{-}10}$, $(X\text{-}G\text{-}X\text{-}G\text{-}X)_{1\text{-}10}(G\text{-}Y\text{-}G\text{-}Y)_{1\text{-}10}(X\text{-}G\text{-}X)_{0\text{-}10}(G\text{-}Y\text{-}G\text{-}Y)_{0\text{-}10}$, $(X\text{-}G\text{-}X)_{1\text{-}10}(G\text{-}Y\text{-}G\text{-}Y\text{-}G\text{-}Y)_{1\text{-}10}(X\text{-}G\text{-}X)_{0\text{-}10}(G\text{-}Y\text{-}G\text{-}Y)_{0\text{-}10}$, $(X\text{-}G\text{-}X\text{-}G\text{-}X\text{-}G\text{-}X)_{1\text{-}10}(G\text{-}Y\text{-}G\text{-}Y)_{1\text{-}10}(X\text{-}G\text{-}X)_{0\text{-}10}(G\text{-}Y\text{-}G\text{-}Y)_{0\text{-}10}$, $(X\text{-}G\text{-}X)_{1\text{-}10}(G\text{-}Y\text{-}G\text{-}Y\text{-}G\text{-}Y\text{-}G\text{-}Y)_{1\text{-}10}(X\text{-}G\text{-}X)_{0\text{-}10}(G\text{-}Y\text{-}G\text{-}Y)_{0\text{-}10}$, $(X)_{1\text{-}10}(Y)_{1\text{-}10}(X)_{0\text{-}10}(Y)_{0\text{-}10}$, $(Y)_{1\text{-}10}(X)_{1\text{-}10}(Y)_{0\text{-}10}(X)_{0\text{-}10}$, $(XX)_{1\text{-}10}(Y)_{1\text{-}10}(X)_{0\text{-}10}(Y)_{0\text{-}10}$, $(YY)_{1\text{-}10}(XX)_{1\text{-}10}(Y)_{0\text{-}10}(X)_{0\text{-}10}$, $(X)_{1\text{-}10}(YY)_{1\text{-}10}(X)_{0\text{-}10}(Y)_{0\text{-}10}$, $(XXX)_{1\text{-}10}(YYY)_{1\text{-}10}(XX)_{0\text{-}10}(YY)_{0\text{-}10}$, $(YYY)_{1\text{-}10}(XXX)_{1\text{-}10}(YY)_{0\text{-}10}(XX)_{0\text{-}10}$, $(XY)_{1\text{-}10}(Y)_{1\text{-}10}(X)1_{\text{-}10}(Y)1_{\text{-}10}$, $(YX)_{1\text{-}10}(Y)_{1\text{-}10}(X)1_{\text{-}10}(Y)1_{\text{-}10}$, $(YX)_{1\text{-}10}(X)_{1\text{-}10}(Y)1_{\text{-}10}(Y)1_{\text{-}10}$, $(Y\text{-}G\text{-}Y)_{1\text{-}10}(G\text{-}X\text{-}G\text{-}X)_{1\text{-}10}(G\text{-}Y\text{-}G\text{-}Y)_{0\text{-}10}(G\text{-}X\text{-}G\text{-}X)_{0\text{-}10}$, $(Y\text{-}G)_{1\text{-}10}(G\text{-}X)_{1\text{-}10}(G\text{-}Y)_{0\text{-}10}(G\text{-}X)_{0\text{-}10}$, $(Y\text{-}G\text{-}Y\text{-}G\text{-}Y)_{1\text{-}10}(G\text{-}X\text{-}G\text{-}X)_{1\text{-}10}(Y\text{-}G\text{-}Y)_{0\text{-}10}(G\text{-}X\text{-}G\text{-}X)_{0\text{-}10}$, $(Y\text{-}G\text{-}Y)_{1\text{-}10}(G\text{-}X\text{-}G\text{-}X\text{-}G\text{-}X)_{1\text{-}10}(Y\text{-}G\text{-}Y)_{0\text{-}10}(G\text{-}X\text{-}G\text{-}X)_{0\text{-}10}$, $(Y\text{-}G\text{-}Y\text{-}G\text{-}Y\text{-}G\text{-}Y)_{1\text{-}10}(G\text{-}X\text{-}G\text{-}X)_{1\text{-}10}(Y\text{-}G\text{-}Y)_{0\text{-}10}(G\text{-}X\text{-}G\text{-}X)_{0\text{-}10}$, $(Y\text{-}G\text{-}Y)_{1\text{-}10}(G\text{-}X\text{-}G\text{-}X\text{-}G\text{-}X\text{-}G\text{-}X)_{1\text{-}10}(Y\text{-}G\text{-}Y)_{0\text{-}10}(G\text{-}X\text{-}G\text{-}X)_{0\text{-}10}$, $(XY)_{1\text{-}10}(YX)_{1\text{-}10}(XY)_{0\text{-}10}(YX)_{0\text{-}10}$, $(YX)_{1\text{-}10}(XY)_{1\text{-}10}(Y)_{0\text{-}10}(X)_{0\text{-}10}$, $(YY)_{1\text{-}10}(X)_{1\text{-}10}(Y)_{0\text{-}10}(X)_{0\text{-}10}$, $(XY)_{1\text{-}10}(XY)_{1\text{-}10}(X)_{0\text{-}10}(X)_{0\text{-}10}$, $(Y)_{1\text{-}10}(YX)_{1\text{-}10}(X)_{0\text{-}10}(Y)_{0\text{-}10}$, $(XYX)_{1\text{-}10}(YXX)_{1\text{-}10}(YX)_{0\text{-}10}(YY)_{0\text{-}10}$, or $(YYX)_{1\text{-}10}(XXY)_{1\text{-}10}(YX)_{0\text{-}10}(XY)_{0\text{-}10}$,

where X is an MHC class I epitope of 5-100 amino acids (*e.g.*, any of the lengths described herein including 8-31 amino acids) in length, Y is an MHC class II epitope of 5-100 amino acids (*e.g.*, any of the lengths described herein including 8-31 amino acids) in length, and G is glycine.

The nucleic acid cancer vaccine of the disclosure, in some aspects, comprises a nucleic acid encoding one or more peptide epitopes that include a mutation causing a unique expressed peptide sequence. In some embodiments, a mutation causing a unique expressed peptide sequence may be, but is not limited to, an insertion, deletion, frameshift mutation, and/or splicing variant. In some embodiments, the nucleic acid cancer vaccine encodes multiple peptide epitope antigens including one or more single nucleotide polymorphism (SNP) mutations with flanking amino acids on each side of the SNP mutation. In some

embodiments, the number of flanking amino acids on each side of the SNP mutation may be 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 22, 24, 26, 28, or 30. In some embodiments, the SNP mutation is centrally located and the number of flanking amino acids on each side of the SNP mutation is approximately the same. In other embodiments, the SNP mutation does not have an equivalent number of flanking amino acids on each side. In an embodiment, an epitope of the cancer vaccine comprises an SNP flanked by two Class I sequences, each sequence comprising seven amino acids. In another embodiment, an epitope of the cancer vaccine comprises a SNP flanked by two Class II sequences, each sequence comprising 10 amino acids. In some embodiments, an epitope may comprise a centrally located SNP and flanks which are both Class I sequences, both Class II sequences, or one Class I and one Class II sequence.

In another embodiment, the peptide epitopes are in the form of a concatemeric cancer antigen comprised of peptide epitopes. Any number of peptide epitopes may be used. In certain embodiments, the peptide epitopes are in the form of a concatemeric cancer antigen comprised of 5-200 peptide epitopes. In certain embodiments, the peptide epitopes are in the form of a concatemeric cancer antigen comprised of 3-130 peptide epitopes. In some embodiments, the concatemeric cancer antigen comprises one or more of: a) the peptide epitopes (*e.g.*, the 3-200 or 3-130 peptide epitopes) are interspersed by cleavage sensitive sites; and/or b) each peptide epitope is linked directly to one another without a linker; and/or c) each peptide epitope is linked to one or another with a single amino acid linker; and/or d) each peptide epitope is linked to one or another with a short linker; and/or e) each peptide epitope comprises 8-31 amino acids and includes one or more SNP mutations (*e.g.*, a centrally located SNP mutation); and/or f) each peptide epitope comprises 8-31 amino acids and includes a mutation causing a unique expressed peptide sequence; and/or g) at least 30% of the peptide epitopes have a highest affinity for class I MHC molecules from a subject; and/or h) at least 30% of the peptide epitopes have a highest affinity for class II MHC molecules from a subject; and/or i) none of the peptide epitopes have a highest affinity for class II MHC molecules from a subject; and/or j) at least 50% of the peptide epitopes have a predicted binding affinity of IC50 <500nM for HLA-A, HLA-B and/or DRB1; and/or k) the nucleic acids encoding the peptide epitopes are arranged such that the peptide epitopes are ordered to minimize pseudo-epitopes, l) the ratio of class I MHC molecule peptide epitopes to class II MHC molecule peptide epitopes is at least 1:1, 2:1, 3:1, 4:1, or 5:1; and/or m) no class II MHC molecules peptide epitopes are present. In some embodiments, peptide epitopes having a "highest affinity" for a class I MHC molecule specifically bind (*i.e.*, bind

with greatest affinity) to that class I MHC molecule. In some embodiments, peptide epitopes having a "highest affinity" for a class I MHC molecule have greater binding affinity for that class I MHC molecule than a class II MHC molecule. In some embodiments, peptide epitopes having a "highest affinity" for a class II MHC molecule specifically bind (*i.e.*, bind with greatest affinity) to that class II MHC molecule. In some embodiments, peptide epitopes having a "highest affinity" for a class II MHC molecule have greater binding affinity for that class II MHC molecule than a class I MHC molecule.

It will be appreciated that a concatemer of 2 or more peptides, *e.g.,* 2 or more neoantigens, may create unintended new epitopes (pseudoepitopes) at peptide boundaries. To prevent or eliminate such pseudoepitopes, class I alleles may be scanned for hits across peptide boundaries in a concatemer. In some embodiments, the peptide order within the concatemer is shuffled to reduce or eliminate pseudoepitope formation. In some embodiments, a linker is used between peptides, *e.g.,* a single amino acid linker such as glycine, to reduce or eliminate pseudoepitope formation. In some embodiments, anchor amino acids can be replaced with other amino acids which will reduce or eliminate pseudoepitope formation. In some embodiments, peptides are trimmed at the peptide boundary within the concatemer to reduce or eliminate pseudoepitope formation.

In some embodiments the multiple peptide epitope antigens are arranged and ordered to minimize pseudoepitopes. In other embodiments the multiple peptide epitope antigens are a polypeptide that is free of pseudoepitopes. When the cancer antigen epitopes are arranged in a concatemeric structure in a head to tail formation a junction is formed between each of the cancer antigen epitopes. That includes several, *i.e.,* 1-10, amino acids from an epitope on a N-terminus of the peptide and several, *i.e.,* 1-10, amino acids on a C-terminus of an adjacent directly linked epitope. It is important that the junction not be an immunogenic peptide that may produce an immune response. In some embodiments the junction forms a peptide sequence that binds to an HLA protein of a subject for which the personalized cancer vaccine is designed with an IC_{50} greater than about 50 nM. In other embodiments the junction peptide sequence binds to an HLA protein of a subject with an IC_{50} greater than about 10 nM, 150 nM, 200 nM, 250 nM, 300 nM, 350 nM, 400 nM, 450 nm, or 500 nM.

Personalized Cancer Vaccines

In some aspects, the present disclosure provides a nucleic acid cancer vaccine comprising one or more nucleic acids, wherein each of the nucleic acids encodes at least one suitable cancer antigen such as a personalized antigen specific for a cancer subject.

For instance, the nucleic acid cancer vaccine may include nucleic acids encoding one or more cancer antigens specific for each subject, referred to as neoepitopes. Antigens that are expressed in or by tumor cells are referred to as "tumor associated antigens." A particular tumor associated antigen may or may not also be expressed in non-cancerous cells. Many tumor mutations are well known in the art. Tumor associated antigens that are not expressed or rarely expressed in non-cancerous cells, or whose expression in non-cancerous cells is sufficiently reduced in comparison to that in cancerous cells and that induce an immune response induced upon vaccination, are referred to as neoepitopes. Neoepitopes are completely foreign to the body and thus would not produce an immune response against healthy tissue or be masked by the protective components of the immune system. In some embodiments personalized vaccines based on neoepitopes are desirable because such vaccine formulations will maximize specificity against a patient's specific tumor. Mutation-derived neoepitopes can arise from point mutations, non-synonymous mutations leading to different amino acids in the protein; read-through mutations in which a stop codon is modified or deleted, leading to translation of a longer protein with a novel tumor-specific sequence at the C-terminus; splice site mutations that lead to the inclusion of an intron in the mature mRNA and thus a unique tumor-specific protein sequence; chromosomal rearrangements that give rise to a chimeric protein with tumor-specific sequences at the junction of 2 proteins (*i.e.,* gene fusion); frameshift mutations or deletions that lead to a new open reading frame with a novel tumor-specific protein sequence; and/or translocations.

Methods for generating personalized cancer vaccines generally involve identification of mutations, *e.g.,* using deep nucleic acid or protein sequencing techniques, identification of neoepitopes, *e.g.,* using application of validated peptide-MHC binding prediction algorithms or other analytical techniques to generate a set of candidate T cell epitopes that may bind to patient HLA alleles and are based on mutations present in tumors, optional demonstration of antigen-specific T cells against selected neoepitopes or demonstration that a candidate neoepitope is bound to HLA proteins on the tumor surface and development of the vaccine. Examples of techniques for identifying mutations include but are not limited to dynamic allele-specific hybridization (DASH), microplate array diagonal gel electrophoresis (MADGE), pyrosequencing, oligonucleotide-specific ligation, the TaqMan system as well as various DNA "chip" technologies (*e.g..,* Affymetrix SNP chips), and methods based on the generation of small signal molecules by invasive cleavage followed by mass spectrometry or immobilized padlock probes and rolling-circle amplification.

Several deep nucleic acid and protein sequencing techniques are known in the art. Any type of sequence analysis method can be used. For instance nucleic acid sequencing may be performed on whole tumor genomes, tumor exomes (protein-encoding DNA), and/or tumor transcriptomes. Real-time single molecule sequencing-by-synthesis technologies rely on the detection of fluorescent nucleotides as they are incorporated into a nascent strand of DNA that is complementary to the template being sequenced. Other rapid high throughput sequencing methods also exist. Protein sequencing may be performed on tumor proteomes. Additionally, protein mass spectrometry may be used to identify or validate the presence of mutated peptides bound to MHC proteins on tumor cells. Peptides can be acid-eluted from tumor cells or from HLA molecules that are immunoprecipitated from tumors, and then identified using mass spectrometry. The results of the sequencing may be compared with known control sets or with sequencing analysis performed on normal tissue of the patient. In some embodiments, these neoepitopes bind to class I HLA proteins with a greater affinity than the wild-type peptide and/or are capable of activating anti-tumor CD8 T-cells. Identical mutations in any particular gene are rarely found across tumors.

Proteins of MHC class I are present on the surface of almost all cells of the body, including most tumor cells. The proteins of MHC class I are loaded with antigens that usually originate from endogenous proteins or from pathogens present inside cells, and are then presented to cytotoxic T-lymphocytes (CTLs). T-Cell receptors are capable of recognizing and binding peptides complexed with the molecules of MHC class I. Each cytotoxic T-lymphocyte expresses a unique T-cell receptor which is capable of binding specific MHC/peptide complexes.

Using computer algorithms, it is possible to predict potential neoepitopes such as putative T-cell reactive epitopes, *i.e.,* peptide sequences, which are bound by the MHC molecules of class I or class II in the form of a peptide-presenting complex and then, in this form, recognized by the T-cell receptors of T-lymphocytes. Examples of programs useful for identifying peptides which will bind to MHC include, for instance: Lonza Epibase, SYFPEITHI (Rammensee et al., Immunogenetics, 50 (1999), 213-219) and HLA_BIND (Parker et al., J. Immunol., 152 (1994), 163-175).

Once putative neoepitopes are selected, they can be further tested using *in vitro* and/or *in vivo* assays. Conventional *in vitro* lab assays, such as Elispot assays, may be used with an isolate from each patient to refine the list of neoepitopes selected based on the algorithm's predictions.

In some embodiments the nucleic acid cancer vaccines and vaccination methods described herein may include peptide epitopes or antigens based on specific mutations (neoepitopes) and those expressed by cancer-germline genes (antigens common to tumors found in multiple patients, referred to herein as "traditional cancer antigens" or "shared cancer antigens"). In some embodiments, a traditional antigen is one that is known to be found in cancers or tumors generally or in a specific type of cancer or tumor. In some embodiments, a traditional cancer antigen is a non-mutated tumor antigen. In some embodiments, a traditional cancer antigen is a mutated tumor antigen.

In some embodiments, the nucleic acid cancer vaccines and methods described herein may include peptide epitopes based on cancer/testis (CT) antigens. Cancer/testis antigen expression is limited to male germ cells in healthy adults, but ectopic expression has been observed in tumor cells of multiple types of human cancer. Since male germ cells are devoid of HLA-class I molecules and cannot present antigens to T cells, cancer/testis antigens are generally considered neoantigens when expressed in cancer cells and have the capacity to elicit immune responses that are strictly cancer-specific. Cancer/testis antigens for use with the compositions and methods described herein may be any such cancer/testis antigen known in the field including, but not limited to, MAGEA1, MAGEA2, MAGEA3, MAGEA4, MAGEA5, MAGEA6, MAGEA8, MAGEA9, MAGEA10, MAGEA11, MAGEA12, BAGE, BAGE2, BAGE3, BAGE4, BAGE5, MAGEB1, MAGEB2, MAGEB5, MAGEB6, MAGEB3, MAGEB4, GAGE1, GAGE2A, GAGE3, GAGE4, GAGE5, GAGE6, GAGE7, GAGE8, SSX1, SSX2, SSX2b, SSX3, SSX4, CTAG1B, LAGE-1b, CTAG2, MAGEC1, MAGEC3, SYCP1, BRDT, MAGEC2, SPANXA1, SPANXB1, SPANXC, SPANXD, SPANXN1, SPANXN2, SPANXN3, SPANXN4, SPANXN5, XAGE1D, XAGE1C, XAGE1B, XAGE1, XAGE2, XAGE3, XAGE-3b, XAGE-4/RP11-167P23.2, XAGE5, DDX43, SAGE1, ADAM2, PAGE5, CT16.2, PAGE1, PAGE2, PAGE2B, PAGE3, PAGE4, LIPI, VENTXP1, IL13RA2, TSP50, CTAGE1, CTAGE-2, CTAGE5, SPA17, ACRBP, CSAG1, CSAG2, DSCR8, MMA1b, DDX53, CTCFL, LUZP4, CASC5, TFDP3, JARID1B, LDHC, MORC1, DKKL1, SPO11, CRISP2, FMR1NB, FTHL17, NXF2, TAF7L, TDRD1, TDRD6, TDRD4, TEX15, FATE1, TPTE, CT45A1, CT45A2, CT45A3, CT45A4, CT45A5, CT45A6, HORMAD1, HORMAD2, CT47A1, CT47A2, CT47A3, CT47A4, CT47A5, CT47A6, CT47A7, CT47A8, CT47A9, CT47A10, CT47A11, CT47B1, SLCO6A1, TAG, LEMD1, HSPB9, CCDC110, ZNF165, SPACA3, CXorf48, THEG, ACTL8, NLRP4, COX6B2, LOC348120, CCDC33, LOC196993, PASD1, LOC647107, TULP2, CT66/AA884595, PRSS54, RBM46, CT69/BC040308, CT70/BI818097, SPINLW1, TSSK6,

ADAM29, CCDC36, LOC440934, SYCE1, CPXCR1, TSPY3, TSGA10, HIWI, MIWI, PIWI, PIWIL2, ARMC3, AKAP3, Cxorf61, PBK, C21orf99, OIP5, CEP290, CABYR, SPAG9, MPHOSPH1, ROPN1, PLAC1, CALR3, PRM1, PRM2, CAGE1, TTK, LY6K, IMP-3, AKAP4, DPPA2, KIAA0100, DCAF12, SEMG1, POTED, POTEE, POTEA, POTEG, POTEB, POTEC, POTEH, GOLGAGL2 FA, CDCA1, PEPP2, OTOA, CCDC62, GPATCH2, CEP55, FAM46D, TEX14, CTNNA2, FAM133A, LOC130576, ANKRD45, ELOVL4, IGSF11, TMEFF1, TMEFF2, ARX, SPEF2, GPAT2, TMEM108, NOL4, PTPN20A, SPAG4, MAEL, RQCD1, PRAME, TEX101, SPATA19, ODF1, ODF2, ODF3, ODF4, ATAD2, ZNF645, MCAK, SPAG1, SPAG6, SPAG8, SPAG17, FBXO39, RGS22, cyclin A1, C15orf60, CCDC83, TEKT5, NR6A1, TMPRSS12, TPPP2, PRSS55, DMRT1, EDAG, NDR, DNAJB8, CSAG3B, CTAG1A, GAGE12B, GAGE12C, GAGE12D, GAGE12E, GAGE12F, GAGE12G, GAGE12H, GAGE12I, GAGE12J, GAGE13, LOC728137, MAGEA2B, MAGEA9B/LOC728269, NXF2B, SPANXA2, SPANXB2, SPANXE, SSX4B, SSX5, SSX6, SSX7, SSX9, TSPY1D, TSPY1E, TSPY1F, TSPY1G, TSPY1H, TSPY1I, TSPY2, XAGE1E, XAGE2B/CTD-2267G17.3, and/or variants thereof.

In some embodiments, the nucleic acid cancer vaccines may further include one or more nucleic acids encoding for one or more non-mutated tumor antigens. In some embodiments, the nucleic acid cancer vaccines may further include one or more nucleic acids encoding for one or more mutated tumor antigens.

Many tumor antigens are known in the art. Cancer or tumor antigens (*e.g.*, traditional cancer antigens) for use with the compositions and methods described herein may be any such cancer or tumor antigens known in the field. In some embodiments, the cancer or tumor antigen (*e.g.*, the traditional cancer antigen) is one of the following antigens: CD2, CD19, CD20, CD22, CD27, CD33, CD37, CD38, CD40, CD44, CD47, CD52, CD56, CD70, CD79, CD137, 4- IBB, 5T4, AGS-5, AGS-16, Angiopoietin 2, B2M, B7.1, B7.2, B7DC, B7H1, B7H2, B7H3, BT-062, BTLA, CAIX, Carcinoembryonic antigen, CTLA4, Cripto, ED-B, ErbBl, ErbB2, ErbB3, ErbB4, EGFL7, EpCAM, EphA2, EphA3, EphB2, FAP, Fibronectin, Folate Receptor, Ganglioside GM3, GD2, glucocorticoid-induced tumor necrosis factor receptor (GITR), gpl00, gpA33, GPNMB, ICOS, IGF1R, Integrin av, Integrin $\alpha v\beta$, LAG-3, Lewis Y, Mesothelin, c-MET, MN Carbonic anhydrase IX, MUC1, MUC16, Nectin-4, NKGD2, NOTCH, OX40, OX40L, PD-1, PDL1, PSCA, PSMA, RANKL, ROR1, ROR2, SLC44A4, Syndecan-1, TACI, TAG-72, Tenascin, TIM3, TRAILR1 , TRAILR2,VEGFR- 1 , VEGFR-2, VEGFR-3, and/or variants thereof.

Epitopes can be identified using a free or commercial database (Lonza Epibase, antitope for example). Such tools are useful for predicting the most immunogenic epitopes within a target antigen protein. The selected peptides may then be synthesized and screened in human HLA panels, and the most immunogenic sequences are used to construct the nucleic acids encoding the peptide epitope(s). One strategy for mapping epitopes of Cytotoxic T-Cells based on generating equimolar mixtures of the four C-terminal peptides for each nominal 11-mer across a protein. This strategy would produce a library antigen containing all the possible active CTL epitopes.

The neoepitopes may be designed to optimally bind to MHC in order to promote a robust immune response. In some embodiments each peptide epitope comprises an antigenic region and a MHC stabilizing region. An MHC stabilizing region is a sequence which stabilizes the peptide in the MHC.

All of the MHC stabilizing regions within the epitopes may be the same or they may be different. The MHC stabilizing regions may be at the N terminal portion of the peptide or the C terminal portion of the peptide. Alternatively the MHC stabilizing regions may be in the central region of the peptide.

The MHC stabilizing region may be 5-10, 5-15, 8-10, 1-5, 3-7, or 3-8 amino acids in length. In yet other embodiments the antigenic region is 5-100 amino acids in length. The peptides interact with the molecules of MHC class I by competitive affinity binding within the endoplasmic reticulum, before they are presented on the cell surface. The affinity of an individual peptide is directly linked to its amino acid sequence and the presence of specific binding motifs in defined positions within the amino acid sequence. The peptide being presented in the MHC is held by the floor of the peptide-binding groove, in the central region of the $\alpha 1/\alpha 2$ heterodimer (a molecule composed of two non-identical subunits). The sequence of residues of the peptide-binding groove's floor determines which particular peptide residues it binds.

Optimal binding regions may be identified by a computer assisted comparison of the affinity of a binding site (MHC pocket) for a particular amino acid at each amino acid in the binding site for each of the target epitopes to identify an ideal binder for all of the examined antigens. The MHC stabilization regions of the epitopes may be identified using amino acid prediction matrices of data points for a binding site. An amino acid prediction matrix is a table having a first and a second axis defining data points. Prediction matrices can be generated as shown in Singh, H. and Raghava, G.P.S. (2001), "ProPred: prediction of HLA-DR binding sites." Bioinformatics, 17(12), 1236-37). In some embodiments, the prediction

matrix is based on evolutionary conservation, in another embodiment, the prediction matrix uses physiochemical similarity to examine how similar a somatic amino acid is to the germline amino acid (*e.g.,* Kim et al., J Immunol. 2017: 3360-3368). The similarity of the somatic amino acid to the germline amino acid approximates how a mutation affects binding (e.g., T cell receptor recognition). In some embodiments, less similarity is indicative of improved binding (e.g., T cell receptor recognition).

In some embodiments the MHC stabilizing region is designed based on the subject's particular MHC. In that way the MHC stabilizing region can be optimized for each patient.

The neoepitopes selected for inclusion in the cancer vaccine (*e.g.*, nucleic acid cancer vaccine) will typically be high affinity binding peptides. In some aspect the neoepitope binds an HLA protein with greater affinity than a wild-type peptide. The neoepitope has an IC_{50} of at least less than 5000 nM, at least less than 500 nM, at least less than 250 nM, at least less than 200 nM, at least less than 150 nM, at least less than 100 nM, at least less than 50 nM or less in some embodiments. Typically, peptides with predicted IC_{50} <50 nM, are generally considered medium to high affinity binding peptides and will be selected for testing their affinity empirically using biochemical assays of HLA-binding. Finally, it will be determined whether the human immune system can mount effective immune responses against these mutated tumor antigens and thus effectively kill tumor but not normal cells.

In some embodiments, the neoepitopes are 13 residues or less in length and may consist of between about 8 and about 11 residues, particularly 9 or 10 residues. In other embodiments the neoepitopes may be designed to be longer. For instance, the neoepitopes may have extensions of 2-5 amino acids toward the N- and C-terminus of each corresponding gene product. The use of a longer peptide may allow endogenous processing by patient cells and may lead to more effective antigen presentation and induction of T cell responses.

Neoepitopes having the desired activity may be modified as necessary to provide certain desired attributes, *e.g.,* improved pharmacological characteristics, while increasing or at least retaining substantially all of the biological activity of the unmodified peptide to bind the desired MHC molecule and activate the appropriate T cell or B cell. For instance, the neoepitopes may be subject to various changes, such as substitutions, either conservative or non-conservative, where such changes might provide for certain advantages in their use, such as improved MHC binding. By conservative substitutions is meant replacing an amino acid residue with another which is biologically and/or chemically similar, *e.g.,* one hydrophobic residue for another, or one polar residue for another. The substitutions include combinations such as Gly, Ala; Val, Ile, Leu, Met; Asp, Glu; Asn, Gln; Ser, Thr; Lys, Arg; and Phe, Tyr.

The effect of single amino acid substitutions may also be probed using D-amino acids. Such modifications may be made using well known peptide synthesis procedures, as described in *e.g.,* Merrifield, Science 232:341-347 (1986), Barany & Merrifield, The Peptides, Gross & Meienhofer, eds. (N.Y., Academic Press), pp. 1-284 (1979); and Stewart & Young, Solid Phase Peptide Synthesis, (Rockford, Ill., Pierce), 2d Ed. (1984).

The neoepitopes can also be modified by extending or decreasing the compound's amino acid sequence, *e.g.,* by the addition or deletion of amino acids. The peptides, polypeptides or analogs can also be modified by altering the order or composition of certain residues, it being readily appreciated that certain amino acid residues essential for biological activity, *e.g.,* those at critical contact sites or conserved residues, may generally not be altered without an adverse effect on biological activity.

Typically, a series of peptides with single amino acid substitutions are employed to determine the effect of electrostatic charge, hydrophobicity, etc. on binding. For instance, a series of positively charged (*e.g.,* Lys or Arg) or negatively charged (*e.g.,* Glu) amino acid substitutions are made along the length of the peptide revealing different patterns of sensitivity towards various MHC molecules and T cell or B cell receptors. In addition, multiple substitutions using small, relatively neutral moieties such as Ala, Gly, Pro, or similar residues may be employed. The substitutions may be homo-oligomers or hetero-oligomers. The number and types of residues which are substituted or added depend on the spacing necessary between essential contact points and certain functional attributes which are sought (*e.g.,* hydrophobicity versus hydrophilicity). Increased binding affinity for an MHC molecule or T cell receptor may also be achieved by such substitutions, compared to the affinity of the parent peptide. In any event, such substitutions should employ amino acid residues or other molecular fragments chosen to avoid, for example, steric and charge interference which might disrupt binding.

The neoepitopes may also comprise isosteres of two or more residues in the neoepitopes. An isostere as defined here is a sequence of two or more residues that can be substituted for a second sequence because the steric conformation of the first sequence fits a binding site specific for the second sequence. The term specifically includes peptide backbone modifications well known to those skilled in the art. Such modifications include modifications of the amide nitrogen, the alpha-carbon, amide carbonyl, complete replacement of the amide bond, extensions, deletions or backbone crosslinks. See, generally, Spatola, Chemistry and Biochemistry of Amino Acids, Peptides and Proteins, Vol. VII (Weinstein ed., 1983).

The consideration of immunogenicity is an important component in the selection of optimal neoepitopes for inclusion in a vaccine. As a set of non-limiting examples, immunogenicity may be assessed by analyzing the MHC binding capacity of a neoepitope, HLA promiscuity, mutation position, predicted T cell reactivity, actual T cell reactivity, structure leading to particular conformations and resultant solvent exposure, and representation of specific amino acids. Known algorithms such as the NetMHC prediction algorithm can be used to predict capacity of a peptide to bind to common HLA-A and -B alleles. In some embodiments, the NetMHC prediction algorithm uses the IC_{50} to determine binding capacity. In other embodiments, the NetMHC prediction algorithm uses percent rank and eluted ligand data to determine binding capacity (Jurtz et al., J Immunol. 2017 Nov 1;199(9):3360-3368). As shown in FIGs. 2-3B, the percent rank method results in a more balanced distribution of predicted binders across different HLA alleles. Structural assessment of a MHC bound peptide may also be conducted by in silico 3-dimensional analysis and/or protein docking programs. Use of a predicted epitope structure when bound to a MHC molecule, such as acquired from a Rosetta algorithm, may be used to evaluate the degree of solvent exposure of an amino acid residues of an epitope when the epitope is bound to a MHC molecule. T cell reactivity may be assessed experimentally with epitopes and T cells *in vitro*. Alternatively T cell reactivity may be assessed using T cell response/sequence datasets.

One important aspect of a neoepitope included in a vaccine is a lack of self-reactivity. The putative neoepitopes may be screened to confirm that the epitope is restricted to tumor tissue, for instance, arising as a result of genetic change within malignant cells. Ideally, the epitope should not be present in normal tissue of the patient and thus, self-similar epitopes are filtered out of the dataset. A personalized coding genome may be used as a reference for comparison of neoantigen candidates to determine lack of self-reactivity. In some embodiments, a personalized coding genome is generated from an individualized transcriptome and/or exome.

The nature of peptide composition may also be considered in the epitope design. For instance a score can be provided for each putative epitope on the value of conserved versus non-conserved amino acids found in the epitope.

In some embodiments, the analysis performed by the tools described herein may include comparing different sets of properties acquired at different times from a patient, *i.e.,* prior to and following a therapeutic intervention, from different tissue samples, from different patients having similar tumors, etc. In some embodiments, an average of peak values from

one set of properties may be compared with an average of peak values from another set of properties. For example, an average value for HLA binding may be compared between two different sets of distributions. The two sets of distributions may be determined for time durations separated by days, months, or years, for instance.

A neoepitope characterization system in accordance with the techniques described herein may take any suitable form, as embodiments are not limited in this respect. One or more computer systems may be used to implement any of the functionality described above. The computer system may include one or more processors and one or more computer-readable storage media (*i.e.*, tangible, non-transitory computer-readable media), *e.g.,* volatile storage and one or more non-volatile storage media, which may be formed of any suitable data storage media. The processor may control writing data to and reading data from the volatile storage and the non-volatile storage device in any suitable manner, as embodiments are not limited in this respect. To perform any of the functionality described herein, the processor may execute one or more instructions stored in one or more computer-readable storage media (*e.g.,* volatile storage and/or non-volatile storage), which may serve as tangible, non-transitory computer-readable media storing instructions for execution by the processor.

Methods for Preparation

In other aspects the disclosure provides a method for preparing a cancer vaccine, comprising: a) identifying between personalized cancer antigens for a patient; b) determining the anti-tumor efficacy of at least two peptide epitopes for each of the 3-130 personalized cancer antigens; and c) preparing a cancer vaccine in which the total anti-cancer efficacy of the cancer vaccine is maximized (*e.g.*, the predicted total anti-cancer efficacy of the cancer vaccine is maximized) for a given total length of the cancer vaccine.

Methods for generating cancer vaccines according to the disclosure may involve identification of mutations using techniques such as deep nucleic acid or protein sequencing methods as described herein of tissue samples. In some embodiments an initial identification of mutations in a subject's (*e.g.*, a patient's) transcriptome is performed. The data from the subject's (*e.g.*, the patient's) transcriptome is compared with sequence information from the subject's (*e.g.*, the patient's) exome in order to identify patient specific and tumor specific mutations that are expressed. The comparison produces a dataset of putative neoepitopes, referred to as a mutanome. The mutanome may include approximately 100-10,000 candidate mutations per patient. The mutanome is subject to a data probing analysis using a set of

inquiries or algorithms to identify an optimal mutation set for generation of a neoantigen vaccine. In some embodiments an mRNA neoantigen vaccine is designed and manufactured. The patient is then treated with the vaccine. In certain embodiments, such a neoantigen-containing vaccine may be a polycistronic vaccine including multiple neoepitopes or one or more single RNA vaccines or a combination thereof.

In some embodiments the entire method from the initiation of the mutation identification process to the start of patient treatment is achieved in less than 2 months. In other embodiments the whole process is achieved in 7 weeks or less, 6 weeks or less, 5 weeks or less, 4 weeks or less, 3 weeks or less, 2 weeks or less or less than 1 week. In some embodiments the whole method is performed in less than 30 days.

In a personalized cancer vaccine, the subject specific cancer antigens may be identified in a sample of a patient. The term "biological sample" refers to a sample that contains biological materials such as a DNA, a RNA and a protein. In some embodiments, the biological sample may suitably comprise a bodily fluid from a subject. The bodily fluids can be fluids isolated from anywhere in the body of the subject, preferably a peripheral location, including but not limited to, for example, blood, plasma, serum, urine, sputum, spinal fluid, cerebrospinal fluid, pleural fluid, nipple aspirates, lymph fluid, fluid of the respiratory, intestinal, and genitourinary tracts, tear fluid, saliva, breast milk, fluid from the lymphatic system, semen, cerebrospinal fluid, intra-organ system fluid, ascitic fluid, tumor cyst fluid, amniotic fluid and combinations thereof. In some embodiments, the sample may be a tissue sample or a tumor sample. For instance, a sample of one or more tumor cells may be examined for the presence of subject specific cancer antigens.

The identification process for specific cancer antigens may involve both transcriptome and exome analysis or only transcriptome or exome analysis. In some embodiments transcriptome analysis is performed first and exome analysis is performed second. The analysis is performed on a biological or tissue sample. In some embodiments a biological or tissue sample is a blood or serum sample. In other embodiments the sample is a tissue bank sample or EBV transformation of B-cells.

Alternatively the subject specific cancer antigens may be identified in an exosome of the subject. When the antigens for a vaccine are identified in an exosome of the subject, such antigens are said to be representative of exosome antigens of the subject.

Exosomes are small microvesicles shed by cells, typically having a diameter of approximately 30-100 nm. Exosomes are classically formed from the inward invagination and pinching off of the late endosomal membrane, resulting in the formation of a

multivesicular body (MVB) laden with small lipid bilayer vesicles, each of which contains a sample of the parent cell's cytoplasm. Fusion of the MVB with the cell membrane results in the release of these exosomes from the cell, and their delivery into the blood, urine, cerebrospinal fluid, or other bodily fluids. Exosomes can be recovered from any of these biological fluids for further analysis.

Nucleic acids within exosomes have a role as biomarkers for tumor antigens. An advantage of analyzing exosomes in order to identify subject specific cancer antigens, is that the method circumvents the need for biopsies. This can be particularly advantageous when the patient needs to have several rounds of therapy including identification of cancer antigens, and vaccination.

A number of methods of isolating exosomes from a biological sample have been described in the art. For example, the following methods can be used: differential centrifugation, low speed centrifugation, anion exchange and/or gel permeation chromatography, sucrose density gradients or organelle electrophoresis, magnetic activated cell sorting (MACS), nanomembrane ultrafiltration concentration, Percoll gradient isolation and using microfluidic devices. Exemplary methods are described in US Patent Publication No. 2014/0212871, for instance.

Once an mRNA vaccine is synthesized, it is administered to the patient. In some embodiments the vaccine is administered on a schedule for up to two months, up to three months, up to four month, up to five months, up to six months, up to seven months, up to eight months, up to nine months, up to ten months, up to eleven months, up to 1 year, up to 1 and ½ years, up to two years, up to three years, or up to four years. The schedule may be the same or varied. In some embodiments the schedule is weekly for the first 3 weeks and then monthly thereafter.

At any point in the treatment the patient may be examined to determine whether the mutations in the vaccine are still appropriate. Based on that analysis the vaccine may be adjusted or reconfigured to include one or more different mutations or to remove one or more mutations.

It has been recognized and appreciated that, by analyzing certain properties of cancer associated mutations, optimal neoepitopes may be assessed and/or selected for inclusion in a cancer vaccine. A property of a neoepitope or set of neoepitopes may include, for instance, an assessment of gene or transcript-level expression in patient RNA-seq or other nucleic acid analysis, tissue-specific expression in available databases, known oncogenes/tumor suppressors, variant call confidence score, RNA-seq allele-specific expression, conservative

vs. non-conservative AA substitution, position of point mutation (Centering Score for increased TCR engagement), position of point mutation (Anchoring Score for differential HLA binding), Selfness: <100% core epitope homology with patient WES data, HLA-A and –B IC_{50} for 8mers-11mers, HLA-DRB1 IC_{50} for 15mers-20mers, promiscuity Score (*i.e.,* number of patient HLAs predicted to bind), HLA-C IC_{50} for 8mers-11mers, HLA-DRB3-5 IC_{50} for 15mers-20mers, HLA-DQB1/A1 IC_{50} for 15mers-20mers, HLA-DPB1/A1 IC_{50} for 15mers-20mers, Class I vs Class II proportion, Diversity of patient HLA-A, -B and DRB1 allotypes covered, proportion of point mutation vs complex epitopes (*e.g.,* frameshifts), and /or pseudo-epitope HLA binding scores.

In some embodiments, the properties of cancer associated mutations used to identify optimal neoepitopes are properties related to the type of mutation, abundance of mutation in patient sample, immunogenicity, lack of self-reactivity, and nature of peptide composition. The type of mutation should be determined and considered as a factor in determining whether a putative epitope should be included in a vaccine. The type of mutation may vary. In some instances it may be desirable to include multiple different types of mutations in a single vaccine. In other instances a single type of mutation may be more desirable. A value for each particular mutation can be weighted and calculated. In some embodiments, a particular mutation is a single nucleotide polymorphism (SNP). In some embodiments, a particular mutation is a complex variant, for example, a peptide sequence resulting from intron retention, complex splicing events, or insertion / deletion mutations changing the reading frame of a sequence.

The abundance of the mutation in a patient sample may also be scored and factored into the decision of whether a putative epitope should be included in a vaccine. Highly abundant mutations may promote a more robust immune response.

In some embodiments, the personalized mRNA cancer vaccines described herein may be used for treatment of cancer. As one non-limiting example, the disclosure provides methods for treating a patient having cancer, comprising: a) analyzing a sample derived from the patient is in order to identify one or more personalized cancer antigens; b) determining the anti-tumor efficacy of at least two peptide epitopes for each of the identified personalized cancer antigens; c) preparing a cancer vaccine in which the total anti-cancer efficacy of the cancer vaccine is maximized (*e.g.*, the predicted total anti-cancer efficacy of the cancer vaccine is maximized) for a given total length of the cancer vaccine; and d) administering the cancer vaccine to the patient.

Cancer vaccines (*e.g.*, nucleic acid cancer vaccines) may be administered prophylactically or therapeutically as part of an active immunization scheme to healthy individuals or early in cancer or late stage and/or metastatic cancer. In one embodiment, the effective amount of the cancer vaccine (*e.g.*, nucleic acid cancer vaccines) provided to a cell, a tissue or a subject may be enough for immune activation, and in particular antigen specific immune activation.

In some embodiments, the cancer vaccine (*e.g.*, nucleic acid cancer vaccine) may be administered with an anti-cancer therapeutic agent. The cancer vaccine (*e.g.*, nucleic acid cancer vaccine) and anti-cancer therapeutic can be combined to enhance immune therapeutic responses even further. The cancer vaccine (*e.g.*, nucleic acid cancer vaccines) and other therapeutic agent may be administered simultaneously or sequentially. When the other therapeutic agents are administered simultaneously they can be administered in the same or separate formulations, but are administered at the same time. The other therapeutic agents are administered sequentially with one another and with the cancer vaccine (*e.g.*, nucleic acid cancer vaccine), when the administration of the other therapeutic agents and the cancer vaccine (*e.g.*, nucleic acid cancer vaccine) is temporally separated. The separation in time between administrations of these compounds may be a matter of minutes or it may be longer, *e.g.,* hours, days, weeks, months. Other therapeutic agents include but are not limited to anti-cancer therapeutic, adjuvants, cytokines, antibodies, antigens, etc.

In some embodiments, the progression of the cancer can be monitored to identify changes in the expressed antigens. Thus, in some embodiments the method also involves at least one month after the administration of a cancer mRNA vaccine, identifying at least 2 cancer antigens from a sample of the subject to produce a second set of cancer antigens, and administering to the subject a mRNA vaccine having an open reading frame encoding the second set of cancer antigens to the subject. The mRNA vaccine having an open reading frame encoding second set of antigens, in some embodiments, is administered to the subject 2 months, 3 months, 4 months, 5 months, 6 months, 8 months, 10 months, or 1 year after the mRNA vaccine having an open reading frame encoding the first set of cancer antigens. In other embodiments the mRNA vaccine having an open reading frame encoding second set of antigens is administered to the subject 1 ½, 2, 2 ½ , 3, 3 ½, 4, 4 ½, or 5 years after the mRNA vaccine having an open reading frame encoding the first set of cancer antigens.

Hotspot mutations as neoantigens

In population analyses of cancer, certain mutations occur in a higher percentage of patients than would be expected by chance. These "recurrent" or "hotspot" mutations have often been shown to have a "driver" role in the tumor, producing some change in the cancer cell function that is important to tumor initiation, maintenance, or metastasis, and is therefore selected for in the evolution of the tumor. In addition to their importance in tumor biology and therapy, recurrent mutations provide the opportunity for precision medicine, in which the patient population is stratified into groups more likely to respond to a particular therapy, including but not limited to targeting the mutated protein itself.

Therefore, in some embodiments, the cancer vaccine further comprises one or more cancer hotspot neoepitopes in addition the personalized cancer epitopes. In some embodiments, cancer hotspot mutations that occur over a threshold prevalence in an indication of interest are included in the vaccine. The threshold prevalence, in some embodiments, is greater than 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, or 10%. Indications of interest include, but are not limited to bladder urothelial carcinoma (BLCA), colon adenocarcinoma (COAD), esophageal carcinoma (ESCA), hepatocellular carcinoma (HCC), head and neck squamous cell carcinoma (HNSC), lung adenocarcinoma (LUAD), pancreatic adenocarcinoma (PAAD), prostate adenocarcinoma (PRAD), rectum adenocarcinoma (READ), small cell lung cancer (SCLC), skin cutaneous melanoma (SKCM), serous ovarian cancer (SOC), stomach adenocarcinoma (STAD), and uterine endometrial cancer (UEC). Exemplary mutations are provided in the table below, and an exemplary graph of hotspot mutations by indication is provided as FIG. 1.

Gene	Mutated position
KRAS	G12, G13
NRAS	Q61
BRAF	V600
PIK3CA	R88, E545, H1047
TP53	R175, R282
EGFR	L858
FGFR3	S249
ERBB2	S310
PTEN	R130
BCOR	N1459

Much effort and research on recurrent mutations has focused on non-synonymous (or "missense") single nucleotide variants (SNVs), but population analyses have revealed that a variety of more complex (non-SNV) variant classifications, such as synonymous (or "silent"),

splice site, multi-nucleotide variants, insertions, and deletions, can also occur at high frequencies.

The p53 gene (official symbol TP53) is mutated more frequently than any other gene in human cancers. Large cohort studies have shown that, for most p53 mutations, the genomic position is unique to one or only a few patients and the mutation cannot be used as recurrent neoantigens for therapeutic vaccines designed for a specific population of patients. Surprisingly, a small subset of p53 loci do, however, exhibit a "hotspot" pattern, in which several positions in the gene are mutated with relatively high frequency. Strikingly, a large portion of these recurrently mutated regions occur near exon-intron boundaries, disrupting the canonical nucleotide sequence motifs recognized by the mRNA splicing machinery. Mutation of a splicing motif can alter the final mRNA sequence even if no change to the local amino acid sequence is predicted (*i.e.,* , for synonymous or intronic mutations). Therefore, these mutations are often annotated as "noncoding" by common annotation tools and neglected for further analysis, even though they may alter mRNA splicing in unpredictable ways and exert severe functional impact on the translated protein. If an alternatively spliced isoform produces an in-frame sequence change (*i.e.,* no PTC is produced), it can escape depletion by NMD and be readily expressed, processed, and presented on the cell surface by the HLA system. Further, mutation-derived alternative splicing is usually "cryptic", *i.e.,* not expressed in normal tissues, and therefore may be recognized by T-cells as non-self neoantigens.

Mutations are typically obtained from a patient's DNA sequencing data to derive neo-epitopes for prior art peptide vaccines. mRNA expression, however, is a more direct measurement of the global space of possible neo-epitopes. For example, some tumor-specific neo-epitopes may arise from splicing changes, insertions/deletions (InDels) resulting in frameshifts, alternative promoters, or epigenetic modifications that are not easily identified using only the exome sequencing data. In some aspects, the neoantigens from InDels are enriched for predicted high-affinity binders versus nsSNVs. Such neoantigens may be immunogenic. For example, frameshift InDels have been found to be significantly associated with checkpoint inhibitor responses across three melanoma cohorts. All neoepitopes may be scored in the same manner as those neoepitopes arising from SNVs, although, at most, one neoantigen candidate per InDels is included, in order to avoid a bias toward InDels. There is untapped value in identifying these types of complex mutations for neoantigen vaccines because they will increase the number of epitopes capable of binding a patient's unique HLA allotypes. Moreover, the complex variants will be more immunogenic and likely lead to

more effective immune responses against tumors due to their difference from self-proteins compared to variants resulting from a single amino acid change.

In some aspects, the invention involves a method for identifying patient specific complex mutations and formulating these mutations into effective personalized cancer vaccines (*e.g.*, nucleic acid cancer vaccines). The methods involve the use of short read RNA-Seq. A major challenge inherent to using short reads for RNA-seq is the fact that multiple mRNA transcript isoforms can be obtained from the same genomic locus, due to alternative splicing and other mechanisms. Due to the sequencing reads being much shorter than the full-length mRNA transcript, it becomes difficult to map a set of reads back to the correct corresponding isoform within a known gene annotation model. As a result, complex variants that diverge from the known gene annotations (as are common in cancer) can be difficult to discover by standard approaches. However, short peptides may be identified rather than the exact exon composition of the full-length transcript. The methods for identifying short peptides that will be representative of these complex mutations involves a short k-mer counting approach to neo-epitope prediction of complex variants.

Nucleic Acids/Polynucleotides

Cancer vaccines (*e.g.*, nucleic acid cancer vaccines), as provided herein, comprise at least one (one or more) nucleic acid having an open reading frame encoding at least one peptide epitope. The term "nucleic acid," in its broadest sense, includes any compound and/or substance that comprises a polymer of nucleotides. These polymers are also referred to as polynucleotides.

Nucleic acids may be or may include, for example, ribonucleic acids (RNAs), deoxyribonucleic acids (DNAs), threose nucleic acids (TNAs), glycol nucleic acids (GNAs), peptide nucleic acids (PNAs), locked nucleic acids (LNAs, including LNA having a β- D-ribo configuration, α-LNA having an α-L-ribo configuration (a diastereomer of LNA), 2′-amino-LNA having a 2′-amino functionalization, and 2′-amino- α-LNA having a 2′-amino functionalization), ethylene nucleic acids (ENA), cyclohexenyl nucleic acids (CeNA) or chimeras or combinations thereof.

As a non-limiting example, when a DNA nucleic acid cancer vaccine as described herein is delivered to a cell, the DNA is transcribed into RNA, and the RNA will be processed into a polypeptide by the intracellular machinery which can then process the polypeptide into immunosensitive fragments capable of stimulating an immune response against a tumor or population of cancerous cells. As a non-limiting example, when an RNA

(*e.g.*, mRNA) nucleic acid cancer vaccine as described herein is delivered to a cell, the RNA (*e.g.*, mRNA) will be processed into a polypeptide by the intracellular machinery which can then process the polypeptide into immunosensitive fragments capable of stimulating an immune response against a tumor or population of cancerous cells.

In some embodiments, nucleic acids of the present disclosure function as messenger RNA (mRNA). "Messenger RNA" (mRNA) refers to any nucleic acid that encodes a (at least one) polypeptide (a naturally-occurring, non-naturally-occurring, or modified polymer of amino acids) and can be translated to produce the encoded polypeptide *in vitro*, *in vivo*, *in situ* or *ex vivo*.

The basic components of an mRNA molecule typically include at least one coding region, a 5′ untranslated region (UTR), a 3′ UTR, a 5′ cap and a poly-A tail. Nucleic acids of the present disclosure may function as mRNA but can be distinguished from wild-type mRNA in their functional and/or structural design features which serve to overcome existing problems of effective polypeptide expression using nucleic-acid based therapeutics.

Polynucleotides of the present disclosure, in some embodiments, are codon optimized. Codon optimization methods are known in the art and may be used as provided herein. Codon optimization, in some embodiments, may be used to match codon frequencies in target and host organisms to ensure proper folding; bias GC content to increase mRNA stability or reduce secondary structures; minimize tandem repeat codons or base runs that may impair gene construction or expression; customize transcriptional and translational control regions; insert or remove protein trafficking sequences; remove/add post translation modification sites in encoded protein (*e.g.,* glycosylation sites); add, remove or shuffle protein domains; insert or delete restriction sites; modify ribosome binding sites and mRNA degradation sites; adjust translational rates to allow the various domains of the protein to fold properly; or to reduce or eliminate problem secondary structures within the polynucleotide. Codon optimization tools, algorithms and services are known in the art – non-limiting examples include services from GeneArt (Life Technologies), DNA2.0 (Menlo Park CA) and/or proprietary methods. In some embodiments, the open reading frame (ORF) sequence is optimized using optimization algorithms.

In some embodiments, a codon optimized sequence shares less than 95% sequence identity with a naturally-occurring or wild-type sequence (*e.g.,* a naturally-occurring or wild-type mRNA sequence encoding a polypeptide or protein of interest (*e.g.,* an antigenic protein or polypeptide). In some embodiments, a codon optimized sequence shares less than 90% sequence identity with a naturally-occurring or wild-type sequence (*e.g.,* a naturally-

occurring or wild-type mRNA sequence encoding a polypeptide or protein of interest (*e.g.*, an antigenic protein or polypeptide). In some embodiments, a codon optimized sequence shares less than 85% sequence identity with a naturally-occurring or wild-type sequence (*e.g.*, a naturally-occurring or wild-type mRNA sequence encoding a polypeptide or protein of interest (*e.g.*, an antigenic protein or polypeptide). In some embodiments, a codon optimized sequence shares less than 80% sequence identity with a naturally-occurring or wild-type sequence (*e.g.*, a naturally-occurring or wild-type mRNA sequence encoding a polypeptide or protein of interest (*e.g.*, an antigenic protein or polypeptide). In some embodiments, a codon optimized sequence shares less than 75% sequence identity with a naturally-occurring or wild-type sequence (*e.g.*, a naturally-occurring or wild-type mRNA sequence encoding a polypeptide or protein of interest (*e.g.*, an antigenic protein or polypeptide).

In some embodiments, a codon optimized sequence shares between 65% and 85% (*e.g.*, between about 67% and about 85% or between about 67% and about 80%) sequence identity with a naturally-occurring or wild-type sequence (*e.g.*, a naturally-occurring or wild-type mRNA sequence encoding a polypeptide or protein of interest (*e.g.*, an antigenic protein or polypeptide). In some embodiments, a codon optimized sequence shares between 65% and 75% or about 80% sequence identity with a naturally-occurring or wild-type sequence (*e.g.*, a naturally-occurring or wild-type mRNA sequence encoding a polypeptide or protein of interest (*e.g.*, an antigenic protein or polypeptide).

In some embodiments a codon optimized RNA may, for instance, be one in which the levels of G/C are enhanced. The G/C-content of nucleic acid molecules may influence the stability of the RNA. RNA having an increased amount of guanine (G) and/or cytosine (C) residues may be functionally more stable than nucleic acids containing a large amount of adenine (A) and thymine (T) or uracil (U) nucleotides. WO02/098443 discloses a pharmaceutical composition containing an mRNA stabilized by sequence modifications in the translated region. Due to the degeneracy of the genetic code, the modifications work by substituting existing codons for those that promote greater RNA stability without changing the resulting amino acid. The approach is limited to coding regions of the RNA.

Antigens/Antigenic Polypeptides

In some embodiments, each peptide epitope may be from 5-100 amino acids long (inclusive). In some embodiments the length of at least one of the peptide epitopes is 5-100, 5-95, 5-90, 5-85, 5-80, 5-75, 5-70, 5-65, 5-60, 5-55, 5-50, 5-45, 5-40, 5-39, 5-38, 5-37, 5-36, 5-35, 5-34, 5-33, 5-32, 5-31, 5-30, 5-29, 5-28, 5-27, 5-26, 5-25, 5-24, 5-23, 5-22, 5-21, 5-20,

8-100, 8-95, 8-90, 8-85, 8-80, 8-75, 8-70, 8-65, 8-60, 8-55, 8-50, 8-45, 8-40, 8-39, 8-38, 8-37, 8-36, 8-35, 8-34, 8-33, 8-32, 8-31, 8-30, 8-29, 8-28, 8-27, 8-26, 8-25, 8-24, 8-23, 8-22, 8-21, 8-20, 10-100, 10-95, 10-90, 10-85, 10-80, 10-75, 10-70, 10-65, 10-60, 10-55, 10-50, 10-45, 10-40, 10-39, 10-38, 10-37, 10-36, 10-35, 10-34, 10-33, 10-32, 10-31, 10-30, 10-29, 10-28, 10-27, 10-26, 10-25, 10-24, 10-23, 10-22, 10-21, or 10-20 amino acids.

In some embodiments, each of the peptide epitopes encoded by the nucleic acid cancer vaccine may have a different length. In certain embodiments, at least one of the peptide epitopes has a different length than another peptide epitope encoded by the nucleic acid cancer vaccine. Each peptide epitope may be any length that is reasonable for an epitope.

Polypeptides for use with the instant disclosure include gene products, naturally occurring polypeptides, synthetic polypeptides, homologs, orthologs, paralogs, fragments and other equivalents, variants, and analogs of the foregoing. A polypeptide may be a single molecule or may be a multi-molecular complex such as a dimer, trimer or tetramer. Polypeptides may also comprise single chain or multichain polypeptides such as antibodies or insulin and may be associated or linked. Most commonly, disulfide linkages are found in multichain polypeptides. The term polypeptide may also apply to amino acid polymers in which at least one amino acid residue is an artificial chemical analogue of a corresponding naturally-occurring amino acid.

The term "polypeptide variant" refers to molecules which differ in their amino acid sequence from a native or reference sequence. The amino acid sequence variants may possess substitutions, deletions, and/or insertions at certain positions within the amino acid sequence, as compared to a native or reference sequence. Ordinarily, variants possess at least 50% identity to a native or reference sequence. In some embodiments, variants share at least 80%, or at least 90% identity with a native or reference sequence.

In some embodiments "variant mimics" are provided. As used herein, the term "variant mimic" is one which contains at least one amino acid that would mimic an activated sequence. For example, glutamate may serve as a mimic for phosphoro-threonine and/or phosphoro-serine. Alternatively, variant mimics may result in deactivation or in an inactivated product containing the mimic, for example, phenylalanine may act as an inactivating substitution for tyrosine; or alanine may act as an inactivating substitution for serine.

"Orthologs" refers to genes in different species that evolved from a common ancestral gene by speciation. Normally, orthologs retain the same function in the course of evolution.

Identification of orthologs is critical for reliable prediction of gene function in newly sequenced genomes.

"Analogs" is meant to include polypeptide variants which differ by one or more amino acid alterations including, for example, substitutions, additions, or deletions of amino acid residues that still maintain one or more of the properties of the parent or starting polypeptide.

The present disclosure provides several types of compositions that are polynucleotide or polypeptide based, including variants and derivatives. These include, for example, substitutional, insertional, deletion and covalent variants and derivatives. The term "derivative" is used synonymously with the term "variant" but generally refers to a molecule that has been modified and/or changed in any way relative to a reference molecule or starting molecule.

As such, polynucleotides encoding peptides or polypeptides containing substitutions, insertions and/or additions, deletions and covalent modifications with respect to reference sequences, in particular the polypeptide sequences disclosed herein, are included within the scope of this disclosure. For example, sequence tags or amino acids, such as one or more lysines, can be added to peptide sequences (*e.g.,* at the N-terminal or C-terminal ends). Sequence tags can be used for peptide detection, purification or localization. Lysines can be used to increase peptide solubility or to allow for biotinylation. Alternatively, amino acid residues located at the carboxy and amino terminal regions of the amino acid sequence of a peptide or protein may optionally be deleted providing for truncated sequences. Certain amino acids (*e.g.,* C-terminal or N-terminal residues) may alternatively be deleted depending on the use of the sequence, as for example, expression of the sequence as part of a larger sequence which is soluble, or linked to a solid support.

"Substitutional variants" when referring to polypeptides are those that have at least one amino acid residue in a native or starting sequence removed and a different amino acid inserted in its place at the same position. Substitutions may be single, where only one amino acid in the molecule has been substituted, or they may be multiple, where two or more amino acids have been substituted in the same molecule.

As used herein the term "conservative amino acid substitution" refers to the substitution of an amino acid that is normally present in the sequence with a different amino acid of similar size, charge, or polarity. Examples of conservative substitutions include the substitution of a non-polar (hydrophobic) residue such as isoleucine, valine and leucine for another non-polar residue. Likewise, examples of conservative substitutions include the

substitution of one polar (hydrophilic) residue for another such as between arginine and lysine, between glutamine and asparagine, and between glycine and serine. Additionally, the substitution of a basic residue such as lysine, arginine or histidine for another, or the substitution of one acidic residue such as aspartic acid or glutamic acid for another acidic residue are additional examples of conservative substitutions. Examples of non-conservative substitutions include the substitution of a non-polar (hydrophobic) amino acid residue such as isoleucine, valine, leucine, alanine, methionine for a polar (hydrophilic) residue such as cysteine, glutamine, glutamic acid or lysine and/or a polar residue for a non-polar residue.

"Features" when referring to polypeptide or polynucleotide are defined as distinct amino acid sequence-based or nucleotide-based components of a molecule respectively. Features of the polypeptides encoded by the polynucleotides include surface manifestations, local conformational shape, folds, loops, half-loops, domains, half-domains, sites, termini or any combination thereof.

As used herein when referring to polypeptides the term "domain" refers to a motif of a polypeptide having one or more identifiable structural or functional characteristics or properties (*e.g.,* binding capacity, serving as a site for protein-protein interactions).

As used herein when referring to polypeptides the terms "site" as it pertains to amino acid based embodiments is used synonymously with "amino acid residue" and "amino acid side chain." As used herein when referring to polynucleotides the terms "site" as it pertains to nucleotide based embodiments is used synonymously with "nucleotide." A site represents a position within a peptide or polypeptide or polynucleotide that may be modified, manipulated, altered, derivatized or varied within the polypeptide or polynucleotide based molecules.

As used herein the terms "termini" or "terminus" when referring to polypeptides or polynucleotides refers to an extremity of a polypeptide or polynucleotide respectively. Such extremity is not limited only to the first or final site of the polypeptide or polynucleotide but may include additional amino acids or nucleotides in the terminal regions. Polypeptide-based molecules may be characterized as having both an N-terminus (terminated by an amino acid with a free amino group (NH_2)) and a C-terminus (terminated by an amino acid with a free carboxyl group (COOH)). Proteins are in some cases made up of multiple polypeptide chains brought together by disulfide bonds or by non-covalent forces (multimers, oligomers). These proteins have multiple N- and C-termini. Alternatively, the termini of the polypeptides may be modified such that they begin or end, as the case may be, with a non-polypeptide based moiety such as an organic conjugate.

As recognized by those skilled in the art, protein fragments, functional protein domains, and homologous proteins are also considered to be within the scope of polypeptides of interest. For example, provided herein is any protein fragment (meaning a polypeptide sequence at least one amino acid residue shorter than a reference polypeptide sequence but otherwise identical) of a reference protein 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100 or greater than 100 amino acids in length. In another example, any protein that includes a stretch of 10, 20, 30, 40, 50, or 100 amino acids which are 40%, 50%, 60%, 70%, 80%, 90%, 95%, or 100% identical to any of the sequences described herein can be utilized in accordance with the disclosure. In some embodiments, a polypeptide includes 2, 3, 4, 5, 6, 7, 8, 9, 10, or more mutations as shown in any of the sequences provided or referenced herein. In another example, any protein that includes a stretch of 20, 30, 40, 50, or 100 amino acids that are greater than 80%, 90%, 95%, or 100% identical to any of the sequences described herein, wherein the protein has a stretch of 5, 10, 15, 20, 25, or 30 amino acids that are less than 80%, 75%, 70%, 65%, or 60% identical to any of the sequences described herein can be utilized in accordance with the disclosure.

Polypeptide or polynucleotide molecules of the present disclosure may share a certain degree of sequence similarity or "identity" with the reference molecules (*e.g.,* reference polypeptides or reference polynucleotides), for example, with art-described molecules (*e.g.,* engineered or designed molecules or wild-type molecules). The term "identity" as known in the art, refers to a relationship between the sequences of two or more polypeptides or polynucleotides (*e.g.*, DNA molecules and/or RNA molecules), as determined by comparing the sequences. In the art, identity also means the degree of sequence relatedness between them as determined by the number of matches between strings of two or more amino acid residues or nucleic acid residues. Identity measures the percent of identical matches between the smaller of two or more sequences with gap alignments (if any) addressed by a particular mathematical model or computer program (*e.g.,* "algorithms"). Identity of related peptides can be readily calculated by known methods. "Percent identity" or "% identity" as it applies to polypeptide or polynucleotide sequences is defined as the percentage of residues (amino acid residues or nucleic acid residues) in the candidate amino acid or nucleic acid sequence that are identical with the residues in the amino acid sequence or nucleic acid sequence of a second sequence after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent identity. Methods and computer programs for the alignment are well known in the art. It is understood that identity depends on a calculation of percent identity but may differ in value due to gaps and penalties introduced in the calculation. Calculation of

the percent identity of two polynucleic acid sequences, for example, can be performed by aligning the two sequences for optimal comparison purposes (*e.g.*, gaps can be introduced in one or both of a first and a second nucleic acid sequences for optimal alignment and non-identical sequences can be disregarded for comparison purposes). In certain embodiments, the length of a sequence aligned for comparison purposes is at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, or 100% of the length of the reference sequence. The nucleotides at corresponding nucleotide positions are then compared. When a position in the first sequence is occupied by the same nucleotide as the corresponding position in the second sequence, then the molecules are identical at that position. The percent identity between the two sequences is a function of the number of identical positions shared by the sequences, taking into account the number of gaps, and the length of each gap, which needs to be introduced for optimal alignment of the two sequences. The comparison of sequences and determination of percent identity between two sequences can be accomplished using a mathematical algorithm.

Generally, variants of a particular polynucleotide or polypeptide have at least 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% but less than 100% sequence identity to that particular reference polynucleotide or polypeptide as determined by sequence alignment programs and parameters described herein and known to those skilled in the art. For example, the percent identity between two nucleic acid sequences can be determined using methods such as those described in Computational Molecular Biology, Lesk, A. M., ed., Oxford University Press, New York, 1988; Biocomputing: Informatics and Genome Projects, Smith, D. W., ed., Academic Press, New York, 1993; Sequence Analysis in Molecular Biology, von Heinje, G., Academic Press, 1987; Computer Analysis of Sequence Data, Part I, Griffin, A. M., and Griffin, H. G., eds., Humana Press, New Jersey, 1994; and Sequence Analysis Primer, Gribskov, M. and Devereux, J., eds., M Stockton Press, New York, 1991; each of which is incorporated herein by reference. For example, the percent identity between two nucleic acid sequences can be determined using the algorithm of Meyers and Miller (CABIOS, 1989, 4:11-17), which has been incorporated into the ALIGN program (version 2.0) using a PAM120 weight residue table, a gap length penalty of 12 and a gap penalty of 4. The percent identity between two nucleic acid sequences can, alternatively, be determined using the GAP program in the GCG software package using an NWSgapdna.CMP matrix. Methods commonly employed to determine percent identity between sequences include, but are not limited to those disclosed in Carillo, H., and Lipman, D., SIAM J Applied Math., 48:1073

(1988); incorporated herein by reference. Techniques for determining identity are codified in publicly available computer programs. Exemplary computer software to determine homology between two sequences include, but are not limited to, GCG program package, Devereux, J., et al., Nucleic Acids Research, 12(1), 387 (1984)), BLASTP, BLASTN, and FASTA (Stephen F. Altschul, et al (1997), "Gapped BLAST and PSI-BLAST: a new generation of protein database search programs", Nucleic Acids Res. 25:3389-3402). Another popular local alignment technique is based on the Smith-Waterman algorithm (Smith, T.F. & Waterman, M.S. (1981) "Identification of common molecular subsequences." J. Mol. Biol. 147:195-197). A general global alignment technique based on dynamic programming is the Needleman–Wunsch algorithm (Needleman, S.B. & Wunsch, C.D. (1970) "A general method applicable to the search for similarities in the amino acid sequences of two proteins." J. Mol. Biol. 48:443-453). More recently a Fast Optimal Global Sequence Alignment Algorithm (FOGSAA) has been developed that purportedly produces global alignment of nucleotide and protein sequences faster than other optimal global alignment methods, including the Needleman–Wunsch algorithm.

As used herein, the term "homology" refers to the overall relatedness between polymeric molecules, *e.g.,* between nucleic acid molecules (*e.g.,* DNA molecules and/or RNA molecules) and/or between polypeptide molecules. Polymeric molecules (*e.g.,* nucleic acid molecules (*e.g.,* DNA molecules and/or RNA molecules) and/or polypeptide molecules) that share a threshold level of similarity or identity determined by alignment of matching residues are termed homologous. Homology is a qualitative term that describes a relationship between molecules and can be based upon the quantitative similarity or identity. Similarity or identity is a quantitative term that defines the degree of sequence match between two compared sequences. In some embodiments, polymeric molecules are considered to be "homologous" to one another if their sequences are at least 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 99% identical or similar. The term "homologous" necessarily refers to a comparison between at least two sequences (polynucleotide or polypeptide sequences). Two polynucleotide sequences are considered homologous if the polypeptides they encode are at least 50%, 60%, 70%, 80%, 90%, 95%, or even 99% for at least one stretch of at least 20 amino acids. In some embodiments, homologous polynucleotide sequences are characterized by the ability to encode a stretch of at least 4–5 uniquely specified amino acids. For polynucleotide sequences less than 60 nucleotides in length, homology is determined by the ability to encode a stretch of at least 4–5 uniquely specified amino acids. Two protein sequences are considered homologous if the

proteins are at least 50%, 60%, 70%, 80%, or 90% identical for at least one stretch of at least 20 amino acids.

Homology implies that the compared sequences diverged in evolution from a common origin. The term "homolog" refers to a first amino acid sequence or nucleic acid sequence (*e.g.,* gene (DNA or RNA) or protein sequence) that is related to a second amino acid sequence or nucleic acid sequence by descent from a common ancestral sequence. The term "homolog" may apply to the relationship between genes and/or proteins separated by the event of speciation or to the relationship between genes and/or proteins separated by the event of genetic duplication. "Orthologs" are genes (or proteins) in different species that evolved from a common ancestral gene (or protein) by speciation. Typically, orthologs retain the same function in the course of evolution. "Paralogs" are genes (or proteins) related by duplication within a genome. Orthologs retain the same function in the course of evolution, whereas paralogs evolve new functions, even if these are related to the original one.

Chemical Modifications

Modified Nucleotide Sequences Encoding Epitope Antigen Polypeptides

In some embodiments, the nucleic acid cancer vaccine of the invention comprises one or more chemically modified nucleobases. The invention includes modified polynucleotides comprising a polynucleotide described herein (*e.g.,* a nucleic acid comprising a nucleotide sequence encoding one or more cancer peptide epitopes). The modified nucleic acids can be chemically modified and/or structurally modified. When the nucleic acids of the present invention are chemically and/or structurally modified the polynucleotides can be referred to as "modified nucleic acids."

The present disclosure provides for modified nucleosides and nucleotides of a nucleic acid (*e.g.,* RNA polynucleotides, such as mRNA polynucleotides) encoding one or more cancer peptide epitopes. A "nucleoside" refers to a compound containing a sugar molecule (*e.g.,* a pentose or ribose) or a derivative thereof in combination with an organic base (*e.g.,* a purine or pyrimidine) or a derivative thereof (also referred to herein as "nucleobase"). A "nucleotide" refers to a nucleoside including a phosphate group. Modified nucleotides can by synthesized by any useful method, such as, for example, chemically, enzymatically, or recombinantly, to include one or more modified or non-natural nucleosides. Nucleic acids can comprise a region or regions of linked nucleosides. Such regions can have variable backbone linkages. The linkages can be standard phosphodiester linkages, in which case the polynucleotides would comprise regions of nucleotides.

The modified nucleic acids disclosed herein can comprise various distinct modifications. In some embodiments, the modified polynucleotides contain one, two, or more (optionally different) nucleoside or nucleotide modifications. In some embodiments, a modified polynucleotide introduced to a cell can exhibit one or more desirable properties such as, *e.g.,* improved protein expression, reduced immunogenicity, or reduced degradation in the cell, as compared to an unmodified polynucleotide.

In some embodiments, a nucleic acid disclosed herein (*e.g.,* a nucleic acid encoding one or more peptide epitopes) is structurally modified. As used herein, a "structural" modification is one in which two or more linked nucleosides are inserted, deleted, duplicated, inverted, or randomized in a polynucleotide without significant chemical modification to the nucleotides themselves. Because chemical bonds will necessarily be broken and reformed to effect a structural modification, structural modifications are of a chemical nature and hence are chemical modifications. However, structural modifications will result in a different sequence of nucleotides. For example, the polynucleotide "ATCG" can be chemically modified to "AT-5meC-G." The same polynucleotide can be structurally modified from "ATCG" to "ATCCCG." Here, the dinucleotide "CC" has been inserted, resulting in a structural modification to the nucleic acid.

In some embodiments, the nucleic acids of the instant disclosure are chemically modified. As used herein in reference to a nucleic acid, the terms "chemical modification" or, as appropriate, "chemically modified" refer to modification with respect to adenosine (A), guanosine (G), uridine (U), or cytidine (C) ribo- or deoxyribonucleosides in one or more of their position, pattern, percentage, or population. Generally, herein, these terms are not intended to refer to the ribonucleotide modifications in naturally occurring 5′-terminal mRNA cap moieties.

In some embodiments, the nucleic acids of the instant disclosure can have a uniform chemical modification of all or any of the same nucleoside type or a population of modifications produced by mere downward titration of the same starting modification in all or any of the same nucleoside type, or a measured percent of a chemical modification of all any of the same nucleoside type but with random incorporation, such as where all uridines are replaced by a uridine analog, *e.g.,* pseudouridine or 5-methoxyuridine. In another embodiment, the polynucleotides can have a uniform chemical modification of two, three, or four of the same nucleoside type throughout the entire polynucleotide (such as all uridines and all cytosines, etc. are modified in the same way).

Modified nucleotide base pairing encompasses not only the standard adenosine-thymine, adenosine-uracil, or guanosine-cytosine base pairs, but also base pairs formed between

nucleotides and/or modified nucleotides comprising non-standard or modified bases, wherein the arrangement of hydrogen bond donors and hydrogen bond acceptors permits hydrogen bonding between a non-standard base and a standard base or between two complementary non-standard base structures. One example of such non-standard base pairing is the base pairing between the modified nucleotide inosine and adenine, cytosine, or uracil. Any combination of base/sugar or linker can be incorporated into polynucleotides of the present disclosure.

The skilled artisan will appreciate that, except where otherwise noted, nucleic acid sequences set forth in the instant application will recite "T"s in a representative DNA sequence but where the sequence represents RNA, the "T"s would be substituted for "U"s.

Cancer vaccines of the present disclosure comprise, in some embodiments, at least one nucleic acid (*e.g.*, RNA) having an open reading frame encoding at least one (*e.g.*, 3-200 or 3-130) peptide epitope(s), wherein the nucleic acid comprises nucleotides and/or nucleosides that can be standard (unmodified) or modified as is known in the art. In some embodiments, nucleotides and nucleosides of the present disclosure comprise modified nucleotides or nucleosides. Such modified nucleotides and nucleosides can be naturally-occurring modified nucleotides and nucleosides or non-naturally occurring modified nucleotides and nucleosides. Such modifications can include those at the sugar, backbone, or nucleobase portion of the nucleotide and/or nucleoside as are recognized in the art.

In some embodiments, a naturally-occurring modified nucleotide or nucleotide of the disclosure is one as is generally known or recognized in the art. Non-limiting examples of such naturally occurring modified nucleotides and nucleotides can be found, inter alia, in the widely recognized MODOMICS database.

In some embodiments, a non-naturally occurring modified nucleotide or nucleoside of the disclosure is one as is generally known or recognized in the art. Non-limiting examples of such non-naturally occurring modified nucleotides and nucleosides can be found, inter alia, in published US application Nos. PCT/US2012/058519; PCT/US2013/075177; PCT/US2014/058897; PCT/US2014/058891; PCT/US2014/070413; PCT/US2015/36773; PCT/US2015/36759; PCT/US2015/36771; or PCT/IB2017/051367 all of which are incorporated by reference herein for this purpose.

Hence, nucleic acids of the disclosure (*e.g.*, DNA nucleic acids and RNA nucleic acids, such as mRNA nucleic acids) can comprise standard nucleotides and nucleosides, naturally-occurring nucleotides and nucleosides, non-naturally-occurring nucleotides and nucleosides, or any combination thereof.

Nucleic acids of the disclosure (*e.g.*, DNA nucleic acids and RNA nucleic acids, such as mRNA nucleic acids), in some embodiments, comprise various (more than one) different types of standard and/or modified nucleotides and nucleosides. In some embodiments, a particular region of a nucleic acid contains one, two or more (optionally different) types of standard and/or modified nucleotides and nucleosides.

In some embodiments, a modified RNA nucleic acid (*e.g.*, a modified mRNA nucleic acid), introduced to a cell or organism, exhibits reduced degradation in the cell or organism, respectively, relative to an unmodified nucleic acid comprising standard nucleotides and nucleosides.

In some embodiments, a modified RNA nucleic acid (*e.g.*, a modified mRNA nucleic acid), introduced into a cell or organism, may exhibit reduced immunogenicity in the cell or organism, respectively (*e.g.*, a reduced innate response) relative to an unmodified nucleic acid comprising standard nucleotides and nucleosides.

Nucleic acids (*e.g.*, RNA nucleic acids, such as mRNA nucleic acids), in some embodiments, comprise non-natural modified nucleotides that are introduced during synthesis or post-synthesis of the nucleic acids to achieve desired functions or properties. The modifications may be present on internucleotide linkages, purine or pyrimidine bases, or sugars. The modification may be introduced with chemical synthesis or with a polymerase enzyme at the terminal of a chain or anywhere else in the chain. Any of the regions of a nucleic acid may be chemically modified.

The present disclosure provides for modified nucleosides and nucleotides of a nucleic acid (*e.g.*, DNA nucleic acids or RNA nucleic acids, such as mRNA nucleic acids). A "nucleoside" refers to a compound containing a sugar molecule (*e.g.*, a pentose or ribose) or a derivative thereof in combination with an organic base (*e.g.*, a purine or pyrimidine) or a derivative thereof (also referred to herein as "nucleobase"). A "nucleotide" refers to a nucleoside, including a phosphate group. Modified nucleotides may by synthesized by any useful method, such as, for example, chemically, enzymatically, or recombinantly, to include one or more modified or non-natural nucleosides. Nucleic acids can comprise a region or regions of linked nucleosides. Such regions may have variable backbone linkages. The linkages can be standard phosphodiester linkages, in which case the nucleic acids would comprise regions of nucleotides.

Modified nucleotide base pairing encompasses not only the standard adenosine-thymine, adenosine-uracil, or guanosine-cytosine base pairs, but also base pairs formed between nucleotides and/or modified nucleotides comprising non-standard or modified bases,

wherein the arrangement of hydrogen bond donors and hydrogen bond acceptors permits hydrogen bonding between a non-standard base and a standard base or between two complementary non-standard base structures, such as, for example, in those nucleic acids having at least one chemical modification. One example of such non-standard base pairing is the base pairing between the modified nucleotide inosine and adenine, cytosine or uracil. Any combination of base/sugar or linker may be incorporated into nucleic acids of the present disclosure.

In some embodiments, modified nucleobases in nucleic acids (*e.g.*, RNA nucleic acids, such as mRNA nucleic acids) comprise 1-methyl-pseudouridine (m1ψ), 1-ethyl-pseudouridine (e1ψ), 5-methoxy-uridine (mo5U), 5-methyl-cytidine (m5C), and/or pseudouridine (ψ). In some embodiments, modified nucleobases in nucleic acids (*e.g.*, RNA nucleic acids, such as mRNA nucleic acids) comprise 5-methoxymethyl uridine, 5-methylthio uridine, 1-methoxymethyl pseudouridine, 5-methyl cytidine, and/or 5-methoxy cytidine. In some embodiments, the polyribonucleotide includes a combination of at least two (*e.g.*, 2, 3, 4 or more) of any of the aforementioned modified nucleobases, including but not limited to chemical modifications.

In some embodiments, a RNA nucleic acid of the disclosure comprises 1-methyl-pseudouridine (m1ψ) substitutions at one or more or all uridine positions of the nucleic acid.

In some embodiments, a RNA nucleic acid of the disclosure comprises 1-methyl-pseudouridine (m1ψ) substitutions at one or more or all uridine positions of the nucleic acid and 5-methyl cytidine substitutions at one or more or all cytidine positions of the nucleic acid.

In some embodiments, a RNA nucleic acid of the disclosure comprises pseudouridine (ψ) substitutions at one or more or all uridine positions of the nucleic acid.

In some embodiments, a RNA nucleic acid of the disclosure comprises pseudouridine (ψ) substitutions at one or more or all uridine positions of the nucleic acid and 5-methyl cytidine substitutions at one or more or all cytidine positions of the nucleic acid.

In some embodiments, a RNA nucleic acid of the disclosure comprises uridine at one or more or all uridine positions of the nucleic acid.

In some embodiments, nucleic acids (*e.g.*, RNA nucleic acids, such as mRNA nucleic acids) are uniformly modified (*e.g.*, fully modified, modified throughout the entire sequence) for a particular modification. For example, a nucleic acid can be uniformly modified with 1-methyl-pseudouridine, meaning that all uridine residues in the mRNA sequence are replaced with 1-methyl-pseudouridine. Similarly, a nucleic acid can be uniformly modified for any

type of nucleoside residue present in the sequence by replacement with a modified residue such as those set forth above.

The nucleic acids of the present disclosure may be partially or fully modified along the entire length of the molecule. For example, one or more or all or a given type of nucleotide (*e.g.*, purine or pyrimidine, or any one or more or all of A, G, U, C) may be uniformly modified in a nucleic acid of the disclosure, or in a predetermined sequence region thereof (*e.g.*, in the mRNA including or excluding the poly-A tail). In some embodiments, all nucleotides X in a nucleic acid of the present disclosure (or in a sequence region thereof) are modified nucleotides, wherein X may be any one of nucleotides A, G, U, C, or any one of the combinations A+G, A+U, A+C, G+U, G+C, U+C, A+G+U, A+G+C, G+U+C or A+G+C.

The nucleic acid may contain from about 1% to about 100% modified nucleotides (either in relation to overall nucleotide content, or in relation to one or more types of nucleotide, *i.e.*, any one or more of A, G, U, or C) or any intervening percentage (*e.g.*, from 1% to 20%, from 1% to 25%, from 1% to 50%, from 1% to 60%, from 1% to 70%, from 1% to 80%, from 1% to 90%, from 1% to 95%, from 10% to 20%, from 10% to 25%, from 10% to 50%, from 10% to 60%, from 10% to 70%, from 10% to 80%, from 10% to 90%, from 10% to 95%, from 10% to 100%, from 20% to 25%, from 20% to 50%, from 20% to 60%, from 20% to 70%, from 20% to 80%, from 20% to 90%, from 20% to 95%, from 20% to 100%, from 50% to 60%, from 50% to 70%, from 50% to 80%, from 50% to 90%, from 50% to 95%, from 50% to 100%, from 70% to 80%, from 70% to 90%, from 70% to 95%, from 70% to 100%, from 80% to 90%, from 80% to 95%, from 80% to 100%, from 90% to 95%, from 90% to 100%, and from 95% to 100%). It will be understood that any remaining percentage is accounted for by the presence of unmodified A, G, U, or C.

The nucleic acids may contain at a minimum 1% and at maximum 100% modified nucleotides, or any intervening percentage, such as at least 5% modified nucleotides, at least 10% modified nucleotides, at least 25% modified nucleotides, at least 50% modified nucleotides, at least 80% modified nucleotides, or at least 90% modified nucleotides. For example, the nucleic acids may contain a modified pyrimidine such as a modified uracil or cytosine. In some embodiments, at least 5%, at least 10%, at least 25%, at least 50%, at least 80%, at least 90% or 100% of the uracil in the nucleic acid is replaced with a modified uracil (*e.g.*, a 5-substituted uracil). The modified uracil can be replaced by a compound having a single unique structure, or can be replaced by a plurality of compounds having different structures (*e.g.*, 2, 3, 4 or more unique structures). In some embodiments, at least 5%, at least 10%, at least 25%, at least 50%, at least 80%, at least 90%, or 100% of the cytosine in the

nucleic acid is replaced with a modified cytosine (*e.g.*, a 5-substituted cytosine). The modified cytosine can be replaced by a compound having a single unique structure, or can be replaced by a plurality of compounds having different structures (*e.g.*, 2, 3, 4 or more unique structures).

In some embodiments, the nucleic acid can include any useful linker between the nucleosides. Such linkers, including backbone modifications, that are useful in the composition of the present disclosure include, but are not limited to the following: 3′-alkylene phosphonates, 3′-amino phosphoramidate, alkene containing backbones, aminoalkylphosphoramidates, aminoalkylphosphotriesters, boranophosphates, -CH_2-O-N(CH_3)-CH_2-, -CH_2-N(CH_3)-N(CH_3)-CH_2-, -CH_2-NH-CH_2-, chiral phosphonates, chiral phosphorothioates, formacetyl and thioformacetyl backbones, methylene (methylimino), methylene formacetyl and thioformacetyl backbones, methyleneimino and methylenehydrazino backbones, morpholino linkages, -N(CH_3)-CH_2-CH_2-, oligonucleosides with heteroatom internucleoside linkage, phosphinates, phosphoramidates, phosphorodithioates, phosphorothioate internucleoside linkages, phosphorothioates, phosphotriesters, PNA, siloxane backbones, sulfamate backbones, sulfide sulfoxide and sulfone backbones, sulfonate and sulfonamide backbones, thionoalkylphosphonates, thionoalkylphosphotriesters, and thionophosphoramidates.

The modified nucleosides and nucleotides (*e.g.,* building block molecules), which can be incorporated into a nucleic acid (*e.g.,* RNA or mRNA, as described herein), can be modified on the sugar of the ribonucleic acid. For example, the 2′ hydroxyl group (OH) can be modified or replaced with a number of different substituents. Exemplary substitutions at the 2′-position include, but are not limited to, H, halo, optionally substituted C_{1-6} alkyl; optionally substituted C_{1-6} alkoxy; optionally substituted C_{6-10} aryloxy; optionally substituted C_{3-8} cycloalkyl; optionally substituted C_{3-8} cycloalkoxy; optionally substituted C_{6-10} aryloxy; optionally substituted C_{6-10} aryl-C_{1-6} alkoxy, optionally substituted C_{1-12} (heterocyclyl)oxy; a sugar (*e.g.,* ribose, pentose, or any described herein); a polyethyleneglycol (PEG), -O(CH_2CH_2O)$_n$$CH_2CH_2OR$, where R is H or optionally substituted alkyl, and n is an integer from 0 to 20 (*e.g.,* from 0 to 4, from 0 to 8, from 0 to 10, from 0 to 16, from 1 to 4, from 1 to 8, from 1 to 10, from 1 to 16, from 1 to 20, from 2 to 4, from 2 to 8, from 2 to 10, from 2 to 16, from 2 to 20, from 4 to 8, from 4 to 10, from 4 to 16, and from 4 to 20); “locked” nucleic acids (LNA) in which the 2′-hydroxyl is connected by a C_{1-6} alkylene or C_{1-6} heteroalkylene bridge to the 4′-carbon of the same ribose sugar, where exemplary bridges included methylene, propylene, ether, or amino bridges; aminoalkyl; aminoalkoxy; amino; and amino acid.

Generally, RNA includes the sugar group ribose, which is a 5-membered ring having an oxygen. Exemplary, non-limiting modified nucleotides include replacement of the oxygen in ribose (*e.g.,* with S, Se, or alkylene, such as methylene or ethylene); addition of a double bond (*e.g.,* to replace ribose with cyclopentenyl or cyclohexenyl); ring contraction of ribose (*e.g.,* to form a 4-membered ring of cyclobutane or oxetane); ring expansion of ribose (*e.g.,* to form a 6- or 7-membered ring having an additional carbon or heteroatom, such as for anhydrohexitol, altritol, mannitol, cyclohexanyl, cyclohexenyl, and morpholino that also has a phosphoramidate backbone); multicyclic forms (*e.g.,* tricyclo; and "unlocked" forms, such as glycol nucleic acid (GNA) (*e.g.,* R-GNA or S-GNA, where ribose is replaced by glycol units attached to phosphodiester bonds), threose nucleic acid (TNA, where ribose is replace with α-L-threofuranosyl-(3′→2′)) , and peptide nucleic acid (PNA, where 2-amino-ethyl-glycine linkages replace the ribose and phosphodiester backbone). The sugar group can also contain one or more carbons that possess the opposite stereochemical configuration than that of the corresponding carbon in ribose. Thus, a polynucleotide molecule can include nucleotides containing, *e.g.,* arabinose, as the sugar. Such sugar modifications are described in, for example, International Patent Publication Nos. WO2013052523 and WO2014093924, the contents of each of which are incorporated herein by reference in their entireties for this purpose.

The nucleic acids of the disclosure (*e.g.,* a nucleic acid encoding one or more peptide epitopes or a functional fragment or variant thereof) can include a combination of modifications to the sugar, the nucleobase, and/or the internucleoside linkage. These combinations can include any one or more modifications described herein.

The nucleic acid cancer vaccines disclosed herein are compositions, including pharmaceutical compositions. The disclosure also encompasses methods for the selection, design, preparation, manufacture, formulation, and/or use of nucleic acid cancer vaccines as provided herein. Also provided are systems (*e.g.*, computerized systems), processes, devices and kits for the selection, design, and/or utilization of the nucleic acid cancer vaccines described herein.

In Vitro Transcription of RNA (e.g., mRNA)

Cancer vaccines of the present disclosure may comprise at least one nucleic acid (*e.g.*, an RNA polynucleotide, such as an mRNA (message RNA) or an mmRNA (modified mRNA)). mRNA, for example, is transcribed *in vitro* from template DNA, referred to as an "*in vitro* transcription template." In some embodiments, an *in vitro* transcription template encodes a 5′ untranslated (UTR) region, contains an open reading frame, and encodes a 3′

UTR and a poly-A tail. The particular nucleic acid sequence composition and length of an *in vitro* transcription template will depend on the mRNA encoded by the template.

In some embodiments, a nucleic acid includes 15 to 3,000 nucleotides. For example, a polynucleotide may include 15 to 50, 15 to 100, 15 to 200, 15 to 300, 15 to 400, 15 to 500, 15 to 600, 15 to 700, 15 to 800, 15 to 900, 15 to 1000, 15 to 1200, 15 to 1400, 15 to 1500, 15 to 1800, 15 to 2000, 15 to 2500, 15 to 3000, 50 to 100, 50 to 200, 50 to 300, 50 to 400, 50 to 500, 50 to 600, 50 to 700, 50 to 800, 50 to 900, 50 to 1000, 50 to 1200, 50 to 1400, 50 to 1500, 50 to 1800, 50 to 2000, 50 to 2500, 50 to 3000, 100 to 200, 100 to 300, 100 to 400, 100 to 500, 100 to 600, 100 to 700, 100 to 800, 100 to 900, 100 to 1000, 100 to 1200, 100 to 1400, 100 to 1500, 100 to 1800, 100 to 2000, 100 to 2500, 100 to 3000, 200 to 300, 200 to 400, 200 to 500, 200 to 600, 200 to 700, 200, to 800, 200 to 900, 200 to 1000, 200 to 1500, 200 to 3000, 500 to 1000, 500 to 1500, 500 to 2000, 500 to 2500, 500 to 3000, 1000 to 1500, 1000 to 2000, 1000 to 2500, 1000 to 3000, 1500 to 3000, 2500 to 3000, or 2000 to 3000 nucleotides).

In other aspects, the disclosure relates to a method for preparing a nucleic acid cancer vaccine (*e.g.,* an mRNA cancer vaccine) by IVT methods. *In vitro* transcription (IVT) methods permit template-directed synthesis of RNA molecules of almost any sequence. The size of the RNA molecules that can be synthesized using IVT methods range from short oligonucleotides to long nucleic acid polymers of several thousand bases. IVT methods permit synthesis of large quantities of RNA transcript (*e.g.,* from microgram to milligram quantities). See Beckert *et al.,* Synthesis of RNA by *in vitro* transcription, *Methods Mol Biol.* 703:29-41(2011); Rio *et al.* RNA: A Laboratory Manual. Cold Spring Harbor: Cold Spring Harbor Laboratory Press, 2011, 205-220.; Cooper, Geoffery M. The Cell: A Molecular Approach. 4th ed. Washington D.C.: ASM Press, 2007. 262-299, each of which is herein incorporated by reference for this purpose. Generally, IVT utilizes a DNA template featuring a promoter sequence upstream of a sequence of interest. The promoter sequence is most commonly of bacteriophage origin (*e.g.*, the T7, T3 or SP6 promoter sequence) but many other promotor sequences can be tolerated including those designed *de novo*. Transcription of the DNA template is typically best achieved by using the RNA polymerase corresponding to the specific bacteriophage promoter sequence. Exemplary RNA polymerases include, but are not limited to T7 RNA polymerase, T3 RNA polymerase, or SP6 RNA polymerase, among others. IVT is generally initiated at a dsDNA but can proceed on a single strand.

It will be appreciated that nucleic acid cancer vaccines (*e.g.*, mRNA cancer vaccines) of the present disclosure, *e.g.,* mRNAs encoding the cancer antigen, may be made using any

appropriate synthesis method. For example, in some embodiments, mRNA vaccines of the present disclosure are made using IVT from a single bottom strand DNA as a template and complementary oligonucleotide that serves as promotor. The single bottom strand DNA may act as a DNA template for *in vitro* transcription of RNA, and may be obtained from, for example, a plasmid, a PCR product, or chemical synthesis. In some embodiments, the single bottom strand DNA is linearized from a circular template. The single bottom strand DNA template generally includes a promoter sequence, *e.g.,* a bacteriophage promoter sequence, to facilitate IVT. Methods of making RNA using a single bottom strand DNA and a top strand promoter complementary oligonucleotide are known in the art. An exemplary method includes, but is not limited to, annealing the DNA bottom strand template with the top strand promoter complementary oligonucleotide (*e.g.,* T7 promoter complementary oligonucleotide, T3 promoter complementary oligonucleotide, or SP6 promoter complementary oligonucleotide), followed by IVT using an RNA polymerase corresponding to the promoter sequence, *e.g.,* aT7 RNA polymerase, a T3 RNA polymerase, or an SP6 RNA polymerase.

IVT methods can also be performed using a double-stranded DNA template. For example, in some embodiments, the double-stranded DNA template is made by extending a complementary oligonucleotide to generate a complementary DNA strand using strand extension techniques available in the art. In some embodiments, a single bottom strand DNA template containing a promoter sequence and sequence encoding one or more peptide epitopes of interest is annealed to a top strand promoter complementary oligonucleotide and subjected to a PCR-like process to extend the top strand to generate a double-stranded DNA template. Alternatively or additionally, a top strand DNA containing a sequence complementary to the bottom strand promoter sequence and complementary to the sequence encoding one or more peptide epitopes of interest is annealed to a bottom strand promoter oligonucleotide and subjected to a PCR-like process to extend the bottom strand to generate a double-stranded DNA template. In some embodiments, the number of PCR-like cycles ranges from 1 to 20 cycles, *e.g.,* 3 to 10 cycles. In some embodiments, a double-stranded DNA template is synthesized wholly or in part by chemical synthesis methods. The double-stranded DNA template can be subjected to *in vitro* transcription as described herein.

In another aspect, nucleic acid cancer vaccines of the present disclosure comprising, *e.g.,* mRNAs encoding the peptide epitopes, may be made using two DNA strands that are complementary across an overlapping portion of their sequence, leaving single-stranded overhangs (*i.e.*, sticky ends) when the complementary portions are annealed. These single-stranded overhangs can be made double-stranded by extending using the other strand as a

template, thereby generating double-stranded DNA. In some cases, this primer extension method can permit larger ORFs to be incorporated into the template DNA sequence, *e.g.,* as compared to sizes incorporated into the template DNA sequences obtained by top strand DNA synthesis methods. In the primer extension method, a portion of the 3′-end of a first strand (in the 5′-3′ direction) is complementary to a portion the 3′-end of a second strand (in the 3′-5′ direction). In some such embodiments, the single first strand DNA may include a sequence of a promoter (*e.g.,* T7, T3, or SP6), optionally a 5′-UTR, and some or all of an ORF (*e.g.,* a portion of the 5′-end of the ORF). In some embodiments, the single second strand DNA may include complementary sequences for some or all of an ORF (*e.g.,* a portion complementary to the 3′-end of the ORF), and optionally a 3′-UTR, a stop sequence, and/or a poly-A tail. Methods of making RNA using two synthetic DNA strands may include annealing the two strands with overlapping complementary portions, followed by primer extension using one or more PCR-like cycles to extend the strands to generate a double-stranded DNA template. In some embodiments, the number of PCR-like cycles ranges from 1 to 20 cycles, *e.g.,* 3 to 10 cycles. Such double-stranded DNA can be subjected to *in vitro* transcription as described herein.

In another aspect, nucleic acid vaccines of the present disclosure comprising, *e.g.,* mRNAs encoding the peptide epitopes, may be made using synthetic double-stranded linear DNA molecules, such as gBlocks® (Integrated DNA Technologies, Coralville, Iowa), as the double-stranded DNA template. An advantage to such synthetic double-stranded linear DNA molecules is that they provide a longer template from which to generate mRNAs. For example, gBlocks® can range in size from 45-1000 (*e.g.,* 125-750 nucleotides). In some embodiments, a synthetic double-stranded linear DNA template includes a full length 5′-UTR, a full length 3′-UTR, or both. A full length 5′-UTR may be up to 100 nucleotides in length, *e.g.,* about 40-60 nucleotides. A full length 3′-UTR may be up to 300 nucleotides in length, *e.g.,* about 100-150 nucleotides.

To facilitate generation of longer constructs, two or more double-stranded linear DNA molecules and/or gene fragments that are designed with overlapping sequences on the 3′ strands may be assembled together using methods known in art. For example, the Gibson Assembly™ Method (Synthetic Genomics, Inc., La Jolla, CA) may be performed with the use of a mesophilic exonuclease that cleaves bases from the 5′-end of the double-stranded DNA fragments, followed by annealing of the newly formed complementary single-stranded 3′-ends, polymerase-dependent extension to fill in any single-stranded gaps, and finally, covalent joining of the DNA segments by a DNA ligase.

In another aspect, nucleic acid cancer vaccines of the present disclosure comprising, *e.g.,* mRNAs encoding the peptide epitopes, may be made using chemical synthesis of the RNA. Methods, for instance, involve annealing a first polynucleotide comprising an open reading frame encoding the polypeptide and a second polynucleotide comprising a 5′-UTR to a complementary polynucleotide conjugated to a solid support. The 3′-terminus of the second polynucleotide is then ligated to the 5′-terminus of the first polynucleotide under suitable conditions. Suitable conditions include the use of a DNA Ligase. The ligation reaction produces a first ligation product. The 5′ terminus of a third polynucleotide comprising a 3′-UTR is then ligated to the 3′-terminus of the first ligation product under suitable conditions. Suitable conditions for the second ligation reaction include an RNA Ligase. A second ligation product is produced in the second ligation reaction. The second ligation product is released from the solid support to produce an mRNA encoding a polypeptide of interest. In some embodiments the mRNA is between 30 and 1000 nucleotides.

An mRNA encoding one or more peptide epitopes may also be prepared by binding a first nucleic acid comprising an open reading frame encoding the nucleic acid to a second nucleic acid comprising 3′-UTR to a complementary nucleic acid conjugated to a solid support. The 5′-terminus of the second nucleic acid is ligated to the 3′-terminus of the first nucleic acid under suitable conditions (including, *e.g.*, a DNA Ligase). The method produces a first ligation product. A third nucleic acid comprising a 5′-UTR is ligated to the first ligation product under suitable conditions (including, *e.g.*, an RNA Ligase, such as T4 RNA) to produce a second ligation product. The second ligation product is released from the solid support to produce an mRNA encoding one or more peptide epitopes.

In some embodiments the first nucleic acid features a 5′-triphosphate and a 3′-OH. In other embodiments the second nucleic acid comprises a 3′-OH. In yet other embodiments, the third nucleic acid comprises a 5′-triphosphate and a 3′-OH. The second nucleic acid may also include a 5′-cap structure. The method may also involve the further step of ligating a fourth nucleic acid comprising a poly-A region at the 3′-terminus of the third nucleic acid. The fourth nucleic acid may comprise a 5′-triphosphate.

The method may or may not comprise reverse phase purification. The method may also include a washing step wherein the solid support is washed to remove unreacted nucleic acids. The solid support may be, for instance, a capture resin. In some embodiments the method involves dT purification.

In accordance with the present disclosure, template DNA encoding the nucleic acid (*e.g.*, mRNA) cancer vaccines of the present disclosure includes an open reading frame

(ORF) encoding one or more peptide epitopes. In some embodiments, the template DNA includes an ORF of up to 1000 nucleotides, *e.g.,* about 10-350, 30-300 nucleotides or about 50-250 nucleotides. In some embodiments, the template DNA includes an ORF of about 150 nucleotides. In some embodiments, the template DNA includes an ORF of about 200 nucleotides.

In some embodiments, IVT transcripts are purified from the components of the IVT reaction mixture after the reaction takes place. For example, the crude IVT mix may be treated with RNase-free DNase to digest the original template. The nucleic acid (*e.g.*, mRNA) can be purified using methods known in the art, including but not limited to, precipitation using an organic solvent or column based purification method. Commercial kits are available to purify RNA, *e.g.,* MEGACLEAR™ Kit (Ambion, Austin, TX). The nucleic acid (*e.g.*, mRNA) can be quantified using methods known in the art, including but not limited to, commercially available instruments, *e.g.,* NanoDrop. Purified nucleic acids (*e.g.*, mRNAs) can be analyzed, for example, by agarose gel electrophoresis to confirm the nucleic acid is the proper size and/or to confirm that no degradation of the nucleic acid has occurred.

Untranslated Regions (UTRs)

Untranslated regions (UTRs) are sections of a nucleic acid before a start codon (5′ UTR) and after a stop codon (3′ UTR) that are not translated. In some embodiments, a nucleic acid (*e.g.,* a ribonucleic acid (RNA), *e.g.,* a messenger RNA (mRNA)) of the disclosure comprising an open reading frame (ORF) encoding one or more peptide epitopes further comprises one or more UTR (*e.g.,* a 5′ UTR or functional fragment thereof, a 3′ UTR or functional fragment thereof, or a combination thereof).

A UTR can be homologous or heterologous to the coding region in a nucleic acid. In some embodiments, the UTR is homologous to the ORF encoding the one or more peptide epitopes. In some embodiments, the UTR is heterologous to the ORF encoding the one or more peptide epitopes. In some embodiments, the nucleic acid comprises two or more 5′ UTRs or functional fragments thereof, each of which has the same or different nucleotide sequences. In some embodiments, the nucleic acid comprises two or more 3′ UTRs or functional fragments thereof, each of which has the same or different nucleotide sequences.

In some embodiments, the 5′ UTR or functional fragment thereof, 3′ UTR or functional fragment thereof, or any combination thereof is sequence optimized.

In some embodiments, the 5′ UTR or functional fragment thereof, 3′ UTR or functional fragment thereof, or any combination thereof comprises at least one chemically modified nucleobase, *e.g.,* 5-methoxyuracil.

UTRs can have features that provide a regulatory role, *e.g.,* increased or decreased stability, localization, and/or translation efficiency. A nucleic acid comprising a UTR can be administered to a cell, tissue, or organism, and one or more regulatory features can be measured using routine methods. In some embodiments, a functional fragment of a 5′ UTR or 3′ UTR comprises one or more regulatory features of a full length 5′ or 3′ UTR, respectively.

Natural 5′ UTRs bear features that play roles in translation initiation. They harbor signatures like Kozak sequences that are commonly known to be involved in the process by which the ribosome initiates translation of many genes. 5′ UTRs also have been known to form secondary structures that are involved in elongation factor binding.

By engineering the features typically found in abundantly expressed genes of specific target organs, one can enhance the stability and protein production of a nucleic acid. For example, introduction of 5′ UTR of liver-expressed mRNA, such as albumin, serum amyloid A, Apolipoprotein A/B/E, transferrin, alpha fetoprotein, erythropoietin, or Factor VIII, can enhance expression of nucleic acids in hepatic cell lines or liver. Likewise, use of 5′ UTRs from other tissue-specific mRNA to improve expression in that tissue is possible for muscle (*e.g.,* MyoD, Myosin, Myoglobin, Myogenin, Herculin), for endothelial cells (*e.g.,* Tie-1, CD36), for myeloid cells (*e.g.,* C/EBP, AML1, G-CSF, GM-CSF, CD11b, MSR, Fr-1, i-NOS), for leukocytes (*e.g.,* CD45, CD18), for adipose tissue (*e.g.,* CD36, GLUT4, ACRP30, adiponectin), and for lung epithelial cells (*e.g.,* SP-A/B/C/D).

In some embodiments, UTRs are selected from a family of transcripts whose proteins share a common function, structure, feature, or property. For example, an encoded polypeptide can belong to a family of proteins (*i.e.*, that share at least one function, structure, feature, localization, origin, or expression pattern), which are expressed in a particular cell, tissue or at some time during development. The UTRs from any of the genes or mRNA can be swapped for any other UTR of the same or different family of proteins to create a new nucleic acid.

In some embodiments, the 5′ UTR and the 3′ UTR can be heterologous. In some embodiments, the 5′ UTR can be derived from a different species than the 3′ UTR. In some embodiments, the 3′ UTR can be derived from a different species than the 5′ UTR.

International Patent Application No. PCT/US2014/021522 (Publ. No. WO/2014/164253) provides a listing of exemplary UTRs that may be utilized in the nucleic acids of the present

disclosure as flanking regions to an ORF. This publication is incorporated by reference herein for this purpose.

Additional exemplary UTRs that may be utilized in the nucleic acids of the present disclosure include, but are not limited to, one or more 5′ UTRs and/or 3′ UTRs derived from the nucleic acid sequence of: a globin, such as an α- or β-globin (*e.g.,* a *Xenopus*, mouse, rabbit, or human globin); a strong Kozak translational initiation signal; a CYBA (*e.g.,* human cytochrome b-245 α polypeptide); an albumin (*e.g.,* human albumin7); a HSD17B4 (hydroxysteroid (17-β) dehydrogenase); a virus (*e.g.,* a tobacco etch virus (TEV), a Venezuelan equine encephalitis virus (VEEV), a Dengue virus, a cytomegalovirus (CMV; *e.g.,* CMV immediate early 1 (IE1)), a hepatitis virus (*e.g.,* hepatitis B virus), a sindbis virus, or a PAV barley yellow dwarf virus); a heat shock protein (*e.g.,* hsp70); a translation initiation factor (*e.g.,* elF4G); a glucose transporter (*e.g.,* hGLUT1 (human glucose transporter 1)); an actin (*e.g.,* human α or β actin); a GAPDH; a tubulin; a histone; a citric acid cycle enzyme; a topoisomerase (*e.g.,* a 5′ UTR of a TOP gene lacking the 5′ TOP motif (the oligopyrimidine tract)); a ribosomal protein Large 32 (L32); a ribosomal protein (*e.g.,* human or mouse ribosomal protein, such as, for example, rps9); an ATP synthase (*e.g.,* ATP5A1 or the β subunit of mitochondrial H^+-ATP synthase); a growth hormone (*e.g.,* bovine (bGH) or human (hGH)); an elongation factor (*e.g.,* elongation factor 1 α1 (EEF1A1)); a manganese superoxide dismutase (MnSOD); a myocyte enhancer factor 2A (MEF2A); a β-F1-ATPase, a creatine kinase, a myoglobin, a granulocyte-colony stimulating factor (G-CSF); a collagen (*e.g.,* collagen type I, alpha 2 (Col1A2), collagen type I, alpha 1 (Col1A1), collagen type VI, alpha 2 (Col6A2), collagen type VI, alpha 1 (Col6A1)); a ribophorin (*e.g.,* ribophorin I (RPNI)); a low density lipoprotein receptor-related protein (*e.g.,* LRP1); a cardiotrophin-like cytokine factor (*e.g.,* Nnt1); calreticulin (Calr); a procollagen-lysine, 2-oxoglutarate 5-dioxygenase 1 (Plod1); and a nucleobindin (*e.g.,* Nucb1).

In some embodiments, the 5′ UTR is selected from the group consisting of a β-globin 5′ UTR; a 5′ UTR containing a strong Kozak translational initiation signal; a cytochrome b-245 α polypeptide (CYBA) 5′ UTR; a hydroxysteroid (17-β) dehydrogenase (HSD17B4) 5′ UTR; a Tobacco etch virus (TEV) 5′ UTR; a Venezuelan equine encephalitis virus (TEEV) 5′ UTR; a 5′ proximal open reading frame of rubella virus (RV) RNA encoding nonstructural proteins; a Dengue virus (DEN) 5′ UTR; a heat shock protein 70 (Hsp70) 5′ UTR; a eIF4G 5′ UTR; a GLUT1 5′ UTR; functional fragments thereof and any combination thereof.

In some embodiments, the 3′ UTR is selected from the group consisting of a β-globin 3′ UTR; a CYBA 3′ UTR; an albumin 3′ UTR; a growth hormone (GH) 3′ UTR; a VEEV 3′ UTR; a hepatitis B virus (HBV) 3′ UTR; α-globin 3′ UTR; a DEN 3′ UTR; a PAV barley yellow dwarf

virus (BYDV-PAV) 3′ UTR; an elongation factor 1 α1 (EEF1A1) 3′ UTR; a manganese superoxide dismutase (MnSOD) 3′ UTR; a β subunit of mitochondrial H(+)-ATP synthase (β-mRNA) 3′ UTR; a GLUT1 3′ UTR; a MEF2A 3′ UTR; a β-F1-ATPase 3′ UTR; functional fragments thereof and combinations thereof.

Wild-type UTRs derived from any gene or mRNA can be incorporated into the nucleic acids of the disclosure. In some embodiments, a UTR can be altered relative to a wild type or native UTR to produce a variant UTR, *e.g.,* by changing the orientation or location of the UTR relative to the ORF; or by inclusion of additional nucleotides, deletion of nucleotides, swapping or transposition of nucleotides. In some embodiments, variants of 5′ or 3′ UTRs can be utilized, for example, mutants of wild type UTRs, or variants wherein one or more nucleotides are added to or removed from a terminus of the UTR.

Additionally, one or more synthetic UTRs can be used in combination with one or more non-synthetic UTRs. *See*, *e.g.,* Mandal and Rossi, Nat. Protoc. 2013 8(3):568-82, and sequences available at www.addgene.org/Derrick_Rossi/, the contents of each are incorporated herein by reference in their entirety. UTRs or portions thereof can be placed in the same orientation as in the transcript from which they were selected or can be altered in orientation or location. Hence, a 5′ and/or 3′ UTR can be inverted, shortened, lengthened, or combined with one or more other 5′ UTRs or 3′ UTRs.

In some embodiments, the nucleic acid may comprise multiple UTRs, *e.g.,* a double, a triple or a quadruple 5′ UTR or 3′ UTR. For example, a double UTR comprises two copies of the same UTR either in series or substantially in series. For example, a double beta-globin 3′ UTR can be used (see, for example, US2010/0129877, the contents of which are incorporated herein by reference for this purpose).

The nucleic acids of the disclosure can comprise combinations of features. For example, the ORF can be flanked by a 5′ UTR that comprises a strong Kozak translational initiation signal and/or a 3′ UTR comprising an oligo(dT) sequence for templated addition of a poly-A tail. A 5′ UTR can comprise a first nucleic acid fragment and a second nucleic acid fragment from the same and/or different UTRs (see, *e.g.,* US2010/0293625, herein incorporated by reference in its entirety for this purpose).

Other non-UTR sequences can be used as regions or subregions within the nucleic acids of the disclosure. For example, introns or portions of intron sequences can be incorporated into the nucleic acids of the disclosure. Incorporation of intronic sequences can increase protein production as well as nucleic acid expression levels. In some embodiments, the nucleic acid of the disclosure comprises an internal ribosome entry site (IRES) instead of or in addition to a UTR

(see, *e.g.,* Yakubov et al., Biochem. Biophys. Res. Commun. 2010 394(1):189-193, the contents of which are incorporated herein by reference in their entirety). In some embodiments, the nucleic acid comprises an IRES instead of a 5′ UTR sequence. In some embodiments, the nucleic acid comprises an ORF and a viral capsid sequence. In some embodiments, the nucleic acid comprises a synthetic 5′ UTR in combination with a non-synthetic 3′ UTR.

In some embodiments, the UTR can also include at least one translation enhancer nucleic acid, translation enhancer element, or translational enhancer elements (collectively, "TEE," which refers to nucleic acid sequences that increase the amount of polypeptide or protein produced from a polynucleotide. As a non-limiting example, the TEE can include those described in US2009/0226470, incorporated herein by reference in its entirety for this purpose, and others known in the art. As a non-limiting example, the TEE can be located between the transcription promoter and the start codon. In some embodiments, the 5′ UTR comprises a TEE. In one aspect, a TEE is a conserved element in a UTR that can promote translational activity of a nucleic acid such as, but not limited to, cap-dependent or cap-independent translation. In one non-limiting example, the TEE comprises the TEE sequence in the 5′-leader of the Gtx homeodomain protein. See Chappell et al., PNAS 2004 101:9590-9594, incorporated herein by reference in its entirety for this purpose.

The terms "translational enhancer polynucleotide" or "translation enhancer polynucleotide sequence" refer to a nucleic acid that includes one or more of the TEE provided herein and/or known in the art (see, *e.g.*, US6310197, US6849405, US7456273, US7183395, US2009/0226470, US2007/0048776, US2011/0124100, US2009/0093049, US2013/0177581, WO2009/075886, WO2007/025008, WO2012/009644, WO2001/055371, WO1999/024595, EP2610341A1, and EP2610340A1; the contents of each of which are incorporated herein by reference in their entirety for this purpose), or their variants, homologs, or functional derivatives. In some embodiments, the nucleic acid of the disclosure comprises one or multiple copies of a TEE. The TEE in a translational enhancer nucleic acid can be organized in one or more sequence segments. A sequence segment can harbor one or more of the TEEs provided herein, with each TEE being present in one or more copies. When multiple sequence segments are present in a translational enhancer nucleic acid, they can be homogenous or heterogeneous. Thus, the multiple sequence segments in a translational enhancer nucleic acid can harbor identical or different types of the TEE provided herein, identical or different number of copies of each of the TEE, and/or identical or different organization of the TEE within each sequence segment. In one embodiment, the nucleic acid of the disclosure comprises a translational enhancer nucleic acid sequence.

In some embodiments, a 5′ UTR and/or 3′ UTR comprising at least one TEE described herein can be incorporated in a monocistronic sequence such as, but not limited to, a vector system or a nucleic acid vector. In some embodiments, a 5′ UTR and/or 3′ UTR of a polynucleotide of the disclosure comprises a TEE or portion thereof described herein. In some embodiments, the TEEs in the 3′ UTR can be the same and/or different from the TEE located in the 5′ UTR.

In some embodiments, a 5′ UTR and/or 3′ UTR of a nucleic acid of the disclosure can include at least 1, at least 2, at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, at least 10, at least 11, at least 12, at least 13, at least 14, at least 15, at least 16, at least 17, at least 18 at least 19, at least 20, at least 21, at least 22, at least 23, at least 24, at least 25, at least 30, at least 35, at least 40, at least 45, at least 50, at least 55, or more than 60 TEE sequences. In one embodiment, the 5′ UTR of a nucleic acid of the disclosure can include 1-60, 1-55, 1-50, 1-45, 1-40, 1-35, 1-30, 1-25, 1-20, 1-15, 1-10, 9, 8, 7, 6, 5, 4, 3, 2, or 1 TEE sequences. The TEE sequences in the 5′ UTR of the nucleic acid of the disclosure can be the same or different TEE sequences. A combination of different TEE sequences in the 5′ UTR of the nucleic acid of the disclosure can include combinations in which more than one copy of any of the different TEE sequences are incorporated.

In some embodiments, the 5′ UTR and/or 3′ UTR comprises a spacer to separate two TEE sequences. As a non-limiting example, the spacer can be a 15 nucleotide spacer and/or other spacers known in the art (*e.g.*, in multiples of three nucleotides). As another non-limiting example, the 5′ UTR and/or 3′ UTR comprises a TEE sequence-spacer module repeated at least once, at least twice, at least 3 times, at least 4 times, at least 5 times, at least 6 times, at least 7 times, at least 8 times, at least 9 times, at least 10 times, or more than 10 times in the 5′ UTR and/or 3′ UTR, respectively. In some embodiments, the 5′ UTR and/or 3′ UTR comprises a TEE sequence-spacer module repeated 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 times.

3′ UTR and the AU Rich Elements

In certain embodiments, a nucleic acid of the present disclosure (*e.g.,* a nucleic acid encoding a peptide epitope of the disclosure) further comprises a 3′ UTR.

A 3′-UTR is the section of mRNA that immediately follows the translation termination codon and often contains regulatory regions that post-transcriptionally influence gene expression. Regulatory regions within the 3′-UTR can influence polyadenylation, translation efficiency, localization, and stability of the mRNA. In one embodiment, the 3′-UTR useful for the disclosure comprises a binding site for regulatory proteins or microRNAs.

In some embodiments, the 3′-UTR has a silencer region, which binds to repressor proteins and inhibits the expression of the mRNA. In other embodiments, the 3′-UTR comprises an AU-rich element (AREs). Proteins bind AREs to affect the stability or decay rate of transcripts in a localized manner or affect translation initiation. In other embodiments, the 3′-UTR comprises the sequence AAUAAA that directs addition of several hundred adenine residues called the poly-A tail to the end of the mRNA transcript.

Natural or wild type 3′ UTRs are known to have stretches of Adenosines and Uridines embedded in them. These AU rich signatures are particularly prevalent in genes with high rates of turnover. Based on their sequence features and functional properties, the AU rich elements (AREs) can be separated into three classes (Chen et al, 1995): Class I AREs contain several dispersed copies of an AUUUA motif within U-rich regions. C-Myc and MyoD contain class I AREs. Class II AREs possess two or more overlapping UUAUUUA(U/A)(U/A) nonamers. Molecules containing this type of AREs include GM-CSF and TNF-a. Class III ARES do not contain an AUUUA motif. c-Jun and Myogenin are two well-studied examples of this class. Most proteins binding to the AREs are known to destabilize the messenger, whereas members of the ELAV family, most notably HuR, have been documented to increase the stability of mRNA. HuR binds to AREs of all the three classes. Engineering the HuR specific binding sites into the 3′ UTR of nucleic acid molecules will lead to HuR binding and thus, stabilization of the message *in vivo*.

Introduction, removal or modification of 3′ UTR AU rich elements (AREs) can be used to modulate the stability of nucleic acids of the disclosure. When engineering specific nucleic acids, one or more copies of an ARE can be introduced to make nucleic acids of the disclosure less stable and thereby curtail translation and decrease production of the resultant protein. Likewise, AREs can be identified and removed or mutated to increase the intracellular stability and thus increase translation and production of the resultant protein. Transfection experiments can be conducted in relevant cell lines, using nucleic acids of the disclosure and protein production can be assayed at various time points post-transfection. For example, cells can be transfected with different ARE-engineering molecules and by using an ELISA kit to the relevant protein and assaying protein produced at 6 hour, 12 hour, 24 hour, 48 hour, and 7 days post-transfection.

Regions having a 5′ Cap

The nucleic acid cancer vaccine described herein may be an mRNA cancer vaccine comprising one or more mRNA having open reading frames that encode peptide epitopes. Each of these mRNA may have a 5′ Cap.

The 5′ cap structure of a natural mRNA is involved in nuclear export, increasing mRNA stability and binds the mRNA Cap Binding Protein (CBP), which is responsible for mRNA stability in the cell and translation competency through the association of CBP with poly-A binding protein to form the mature cyclic mRNA species. The cap further assists the removal of 5′ proximal introns during mRNA splicing.

Endogenous mRNA molecules can be 5′-end capped generating a 5′-ppp-5′-triphosphate linkage between a terminal guanosine cap residue and the 5′-terminal transcribed sense nucleotide of the mRNA molecule (cap). This 5′-guanylate cap can then be methylated to generate an N7-methyl-guanylate residue (cap-0). The ribose sugars of the terminal and/or anteterminal transcribed nucleotides of the 5′ end of the mRNA can optionally also be 2′-O-methylated (*e.g.*, with a 2′-hydroxy group on the first ribose sugar (cap-1); or with a 2′-hydroxy group on the first two ribose sugars (cap-2)). 5′-decapping through hydrolysis and cleavage of the guanylate cap structure can target a nucleic acid molecule, such as an mRNA molecule, for degradation.

In some embodiments, nucleic acids of the present disclosure (*e.g.*, a nucleic acid encoding a peptide epitope) incorporate a cap moiety.

In some embodiments, nucleic acids of the present disclosure (*e.g.*, a nucleic acid encoding a peptide epitope) comprise a non-hydrolyzable cap structure preventing decapping and thus increasing mRNA half-life. Because cap structure hydrolysis requires cleavage of 5′-ppp-5′ phosphorodiester linkages, modified nucleotides can be used during the capping reaction. For example, a Vaccinia Capping Enzyme from New England Biolabs (Ipswich, MA) can be used with α-thio-guanosine nucleotides according to the manufacturer's instructions to create a phosphorothioate linkage in the 5′-ppp-5′ cap. Additional modified guanosine nucleotides can be used such as α-methyl-phosphonate and seleno-phosphate nucleotides.

Additional modifications include, but are not limited to, 2′-O-methylation of the ribose sugars of 5′-terminal and/or 5′-anteterminal nucleotides of the polynucleotide (as mentioned above) on the 2′-hydroxyl group of the sugar ring. Multiple distinct 5′-cap structures can be used to generate the 5′-cap of a nucleic acid molecule, such as a polynucleotide that functions as an mRNA molecule. Cap analogs, which herein are also

referred to as synthetic cap analogs, chemical caps, chemical cap analogs, or structural or functional cap analogs, differ from natural (*i.e.*, endogenous, wild-type or physiological) 5′-caps in their chemical structure, while retaining cap function. Cap analogs can be chemically (*i.e.*, non-enzymatically) or enzymatically synthesized and/or linked to the polynucleotides of the disclosure.

For example, the Anti-Reverse Cap Analog (ARCA) cap contains two guanines linked by a 5′-5′-triphosphate group, wherein one guanine contains an N7 methyl group as well as a 3′-O-methyl group (*i.e.*, N7,3′-O-dimethyl-guanosine-5′-triphosphate-5′-guanosine (m7G-3′mppp-G); which can equivalently be designated 3′ O-Me-m7G(5′)ppp(5′)G). The 3′-O atom of the other, unmodified, guanine becomes linked to the 5′-terminal nucleotide of the capped polynucleotide. The N7- and 3′-O-methlyated guanine provides the terminal moiety of the capped polynucleotide.

Another exemplary cap is mCAP, which is similar to ARCA but has a 2′-O-methyl group on guanosine (*i.e.*, N7,2′-O-dimethyl-guanosine-5′-triphosphate-5′-guanosine, m7Gm-ppp-G).

In some embodiments, the cap is a dinucleotide cap analog. As a non-limiting example, the dinucleotide cap analog can be modified at different phosphate positions with a boranophosphate group or a phophoroselenoate group such as the dinucleotide cap analogs described in U.S. Patent No. US 8,519,110, the contents of which are herein incorporated by reference in its entirety for this purpose.

In another embodiment, the cap is a cap analog is a N7-(4-chlorophenoxyethyl) substituted dicucleotide form of a cap analog known in the art and/or described herein. Non-limiting examples of a N7-(4-chlorophenoxyethyl) substituted dicucleotide form of a cap analog include a N7-(4-chlorophenoxyethyl)-G(5′)ppp(5′)G and a N7-(4-chlorophenoxyethyl)-m3′-OG(5′)ppp(5′)G cap analog (see, *e.g.*, the various cap analogs and the methods of synthesizing cap analogs described in Kore et al. Bioorganic & Medicinal Chemistry 2013 21:4570-4574; the contents of which are herein incorporated by reference in its entirety for this purpose). In another embodiment, a cap analog of the present disclosure is a 4-chloro/bromophenoxyethyl analog.

While cap analogs allow for the concomitant capping of a polynucleotide or a region thereof, in an *in vitro* transcription reaction, up to 20% of transcripts can remain uncapped. This, as well as the structural differences of a cap analog from an endogenous 5′-cap structures of nucleic acids produced by the endogenous, cellular transcription machinery, can lead to reduced translational competency and reduced cellular stability.

Nucleic acids of the disclosure (*e.g.,* a nucleic acids encoding peptide antigens) can also be capped post-manufacture (whether through IVT or chemical synthesis), using enzymes, in order to generate more authentic 5′-cap structures. As used herein, the phrase "more authentic" refers to a feature that closely mirrors or mimics, either structurally or functionally, an endogenous or wild type feature. That is, a "more authentic" feature is better representative of an endogenous, wild-type, natural or physiological cellular function and/or structure as compared to synthetic features or analogs, etc., or which outperforms the corresponding endogenous, wild-type, natural or physiological feature in one or more respects. Non-limiting examples of more authentic 5′cap structures are those that, among other things, have enhanced binding of cap binding proteins, increased half-life, reduced susceptibility to 5′ endonucleases and/or reduced 5′decapping, as compared to synthetic 5′cap structures known in the art (or to a wild-type, natural or physiological 5′cap structure). For example, recombinant Vaccinia Virus Capping Enzyme and recombinant 2′-O-methyltransferase enzyme can create a canonical 5′-5′-triphosphate linkage between the 5′-terminal nucleotide of a polynucleotide and a guanine cap nucleotide wherein the cap guanine contains an N7 methylation and the 5′-terminal nucleotide of the mRNA contains a 2′-O-methyl. Such a structure is termed the cap-1 structure. This cap results in a higher translational-competency and cellular stability and a reduced activation of cellular pro-inflammatory cytokines, as compared, *e.g.,* to other 5′cap analog structures known in the art. Cap structures include, but are not limited to, 7mG(5′)ppp(5′)N,pN2p (cap-0), 7mG(5′)ppp(5′)NlmpNp (cap-1), and 7mG(5′)-ppp(5′)NlmpN2mp (cap-2).

As a non-limiting example, capping chimeric nucleic acids post-manufacture can be more efficient as nearly 100% of the chimeric nucleic acids can be capped. This is in contrast to ~80% when a cap analog is linked to a chimeric nucleic acids in the course of an *in vitro* transcription reaction.

According to the present disclosure, 5′ terminal caps can include endogenous caps or cap analogs. According to the present disclosure, a 5′ terminal cap can comprise a guanine analog. Useful guanine analogs include, but are not limited to, inosine, N1-methyl-guanosine, 2′fluoro-guanosine, 7-deaza-guanosine, 8-oxo-guanosine, 2-amino-guanosine, LNA-guanosine, and 2-azido-guanosine.

Poly-A Tails

In some embodiments, the nucleic acids of the present disclosure (*e.g.,* a nucleic acid encoding peptide epitopes) further comprise a poly-A tail. In further embodiments, terminal

groups on the poly-A tail can be incorporated for stabilization. In other embodiments, a poly-A tail comprises des-3′ hydroxyl tails.

During RNA processing, a long chain of adenine nucleotides (poly-A tail) can be added to a nucleic acid such as an mRNA molecule in order to increase stability. Immediately after transcription, the 3′ end of the transcript can be cleaved to free a 3′ hydroxyl. Then poly-A polymerase adds a chain of adenine nucleotides to the RNA. The process, called polyadenylation, adds a poly-A tail that can be between, for example, approximately 80 to approximately 250 residues long, including approximately 80, 90, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 210, 220, 230, 240, or 250 residues long. In some embodiments, the poly-A tail comprises about 100 nucleotides.

Poly-A tails can also be added after the construct is exported from the nucleus.

According to the present disclosure, terminal groups on the poly-A tail can be incorporated for stabilization. Polynucleotides of the present disclosure can include des-3′ hydroxyl tails. They can also include structural moieties or 2′-Omethyl modifications as taught by Junjie Li, et al. (Current Biology, Vol. 15, 1501–1507, August 23, 2005, the contents of which are incorporated herein by reference in its entirety for this purpose).

The nucleic acids of the present disclosure can be designed to encode transcripts with alternative poly-A tail structures including histone mRNA. According to Norbury, "[t]erminal uridylation has also been detected on human replication-dependent histone mRNAs. The turnover of these mRNAs is thought to be important for the prevention of potentially toxic histone accumulation following the completion or inhibition of chromosomal DNA replication. These mRNAs are distinguished by their lack of a 3′ poly-A tail, the function of which is instead assumed by a stable stem–loop structure and its cognate stem–loop binding protein (SLBP); the latter carries out the same functions as those of PABP on polyadenylated mRNAs" (Norbury, "Cytoplasmic RNA: a case of the tail wagging the dog," Nature Reviews Molecular Cell Biology; AOP, published online 29 August 2013; doi:10.1038/nrm3645) the contents of which are incorporated herein by reference in its entirety for this purpose.

Unique poly-A tail lengths provide certain advantages to the nucleic acids of the present disclosure. Generally, the length of a poly-A tail, when present, is greater than 30 nucleotides in length. In another embodiment, the poly-A tail is greater than 35 nucleotides in length (*e.g.,* at least or greater than about 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 70, 80, 90, 100, 120, 140, 160, 180, 200, 250, 300, 350, 400, 450, 500, 600, 700, 800, 900, 1,000, 1,100, 1,200, 1,300, 1,400, 1,500, 1,600, 1,700, 1,800, 1,900, 2,000, 2,500, or 3,000 nucleotides).

In some embodiments, the nucleic acid or region thereof includes from about 15 to about 3,000 nucleotides (*e.g.*, from 15 to 50, 15 to 100, 15 to 200, 15 to 300, 15 to 400, 15 to 500, 15 to 600, 15 to 700, 15 to 800, 15 to 900, 15 to 1000, 15 to 1200, 15 to 1400, 15 to 1500, 15 to 1800, 15 to 2000, 15 to 2500, 15 to 3000, 50 to 100, 50 to 200, 50 to 300, 50 to 400, 50 to 500, 50 to 600, 50 to 700, 50 to 800, 50 to 900, 50 to 1000, 50 to 1200, 50 to 1400, 50 to 1500, 50 to 1800, 50 to 2000, 50 to 2500, 50 to 3000, 100 to 200, 100 to 300, 100 to 400, 100 to 500, 100 to 600, 100 to 700, 100 to 800, 100 to 900, 100 to 1000, 100 to 1200, 100 to 1400, 100 to 1500, 100 to 1800, 100 to 2000, 100 to 2500, 100 to 3000, 200 to 300, 200 to 400, 200 to 500, 200 to 600, 200 to 700, 200, to 800, 200 to 900, 200 to 1000, 200 to 1500, 200 to 3000, 500 to 1000, 500 to 1500, 500 to 2000, 500 to 2500, 500 to 3000, 1000 to 1500, 1000 to 2000, 1000 to 2500, 1000 to 3000, 1500 to 3000, 2500 to 3000, or 2000 to 3000 nucleotides).

In some embodiments, the poly-A tail is designed relative to the length of the overall nucleic acid or the length of a particular region of the nucleic acid. This design can be based on the length of a coding region, the length of a particular feature or region or based on the length of the ultimate product expressed from the nucleic acids.

In this context, the poly-A tail can be 10, 20, 30, 40, 50, 60, 70, 80, 90, or 100% greater in length than the nucleic acid or feature thereof. The poly-A tail can also be designed as a fraction of the nucleic acid to which it belongs. In this context, the poly-A tail can be 10, 20, 30, 40, 50, 60, 70, 80, or 90% or more of the total length of the construct, a construct region or the total length of the construct minus the poly-A tail. Further, engineered binding sites and conjugation of nucleic acids for Poly-A binding protein can enhance expression.

Additionally, multiple distinct nucleic acids can be linked together via the PABP (Poly-A binding protein) through the 3′-end using modified nucleotides at the 3′-terminus of the poly-A tail. Transfection experiments can be conducted in relevant cell lines at and protein production can be assayed by ELISA at 12hr, 24hr, 48hr, 72hr, and/or day 7 post-transfection.

In some embodiments, the nucleic acids of the present disclosure are designed to include a poly-A-G Quartet region. The G-quartet is a cyclic hydrogen bonded array of four guanine nucleotides that can be formed by G-rich sequences in both DNA and RNA. In this embodiment, the G-quartet is incorporated at the end of the poly-A tail. The resultant nucleic acid is assayed for stability, protein production, and other parameters including half-life at various time points. It has been discovered that the poly-A-G quartet results in protein

production from an mRNA equivalent to at least 75% of that seen using a poly-A tail of 120 nucleotides alone.

Start codon region

The disclosure also includes a nucleic acid that comprises both a start codon region and the nucleic acid described herein (*e.g.,* a nucleic acid comprising a nucleotide sequence encoding peptide epitopes). In some embodiments, the nucleic acids of the present disclosure can have regions that are analogous to or function like a start codon region.

In some embodiments, the translation of a nucleic acid can initiate on a codon that is not the start codon AUG. Translation of the nucleic acid can initiate on an alternative start codon such as, but not limited to, ACG, AGG, AAG, CTG/CUG, GTG/GUG, ATA/AUA, ATT/AUU, TTG/UUG (see Touriol et al. Biology of the Cell 95 (2003) 169-178 and Matsuda and Mauro PLoS ONE, 2010 5:11; the contents of each of which are herein incorporated by reference in its entirety for this purpose).

As a non-limiting example, the translation of a nucleic acid begins on the alternative start codon ACG. As another non-limiting example, nucleic acid translation begins on the alternative start codon CTG or CUG. As yet another non-limiting example, the translation of a nucleic acid begins on the alternative start codon GTG or GUG.

Nucleotides flanking a codon that initiates translation such as, but not limited to, a start codon or an alternative start codon, are known to affect the translation efficiency, the length and/or the structure of the nucleic acid. (See, *e.g.,* Matsuda and Mauro PLoS ONE, 2010 5:11; the contents of which are herein incorporated by reference in its entirety for this purpose). Masking any of the nucleotides flanking a codon that initiates translation can be used to alter the position of translation initiation, translation efficiency, length, and/or structure of a polynucleotide.

In some embodiments, a masking agent can be used near the start codon or alternative start codon in order to mask or hide the codon to reduce the probability of translation initiation at the masked start codon or alternative start codon. Non-limiting examples of masking agents include antisense locked nucleic acids (LNA) nucleic acids and exon-junction complexes (EJCs) (See, *e.g.,* Matsuda and Mauro describing masking agents LNA polynucleotides and EJCs (PLoS ONE, 2010 5:11); the contents of which are herein incorporated by reference in its entirety for this purpose).

In another embodiment, a masking agent can be used to mask a start codon of a nucleic acid in order to increase the likelihood that translation will initiate on an alternative

start codon. In some embodiments, a masking agent can be used to mask a first start codon or alternative start codon in order to increase the chance that translation will initiate on a start codon or alternative start codon downstream to the masked start codon or alternative start codon.

In another embodiment, the start codon of a nucleic acid can be removed from the nucleic acid sequence in order to have the translation of the nucleic acid begin on a codon that is not the start codon. Translation of the nucleic acid can begin on the codon following the removed start codon or on a downstream start codon or an alternative start codon. In a non-limiting example, the start codon ATG or AUG is removed as the first 3 nucleotides of the nucleic acid sequence in order to have translation initiate on a downstream start codon or alternative start codon. The nucleic acid sequence where the start codon was removed can further comprise at least one masking agent for the downstream start codon and/or alternative start codons in order to control or attempt to control the initiation of translation, the length of the nucleic acid and/or the structure of the nucleic acid.

Stop Codon Region

The disclosure also includes a nucleic acid that comprises both a stop codon region and the nucleic acid described herein (*e.g.,* a nucleic acid encoding peptide epitopes). In some embodiments, the nucleic acids of the present disclosure can include at least two stop codons before the 3′ untranslated region (UTR). The stop codon can be selected from TGA, TAA and TAG in the case of DNA, or from UGA, UAA and UAG in the case of RNA. In some embodiments, the nucleic acids of the present disclosure include the stop codon TGA in the case or DNA, or the stop codon UGA in the case of RNA, and one additional stop codon. In a further embodiment the addition stop codon can be TAA or UAA. In another embodiment, the nucleic acids of the present disclosure include three consecutive stop codons, four stop codons, or more.

Insertions and Substitutions

The disclosure also includes a nucleic acid of the present disclosure that further comprises insertions and/or substitutions.

In some embodiments, the 5′ UTR of the nucleic acid can be replaced by the insertion of at least one region and/or string of nucleosides of the same base. The region and/or string of nucleotides can include, but is not limited to, at least 3, at least 4, at least 5, at least 6, at least 7, or at least 8 nucleotides and the nucleotides can be natural and/or unnatural. As a

non-limiting example, the group of nucleotides can include 5-8 adenine, cytosine, thymine, a string of any of the other nucleotides disclosed herein and/or combinations thereof.

In some embodiments, the 5′ UTR of the nucleic acid can be replaced by the insertion of at least two regions and/or strings of nucleotides of two different bases such as, but not limited to, adenine, cytosine, thymine, any of the other nucleotides disclosed herein, and/or combinations thereof. For example, the 5′ UTR can be replaced by inserting 5-8 adenine bases followed by the insertion of 5-8 cytosine bases. In another example, the 5′ UTR can be replaced by inserting 5-8 cytosine bases followed by the insertion of 5-8 adenine bases.

In some embodiments, the nucleic acid can include at least one substitution and/or insertion downstream of the transcription start site that can be recognized by an RNA polymerase. As a non-limiting example, at least one substitution and/or insertion can occur downstream of the transcription start site by substituting at least one nucleic acid in the region just downstream of the transcription start site (such as, but not limited to, +1 to +6). Changes to region of nucleotides just downstream of the transcription start site can affect initiation rates, increase apparent nucleotide triphosphate (NTP) reaction constant values, and increase the dissociation of short transcripts from the transcription complex curing initial transcription (Brieba et al, Biochemistry (2002) 41: 5144-5149; herein incorporated by reference in its entirety for this purpose). The modification, substitution, and/or insertion of at least one nucleoside can cause a silent mutation of the sequence or can cause a mutation in the amino acid sequence.

In some embodiments, the nucleic acid can include the substitution of at least 1, at least 2, at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, at least 10, at least 11, at least 12, or at least 13 guanine bases downstream of the transcription start site.

In some embodiments, the nucleic acid can include the substitution of at least 1, at least 2, at least 3, at least 4, at least 5, or at least 6 guanine bases in the region just downstream of the transcription start site. As a non-limiting example, if the nucleotides in the region are GGGAGA, the guanine bases can be substituted by at least 1, at least 2, at least 3, or at least 4 adenine nucleotides. In another non-limiting example, if the nucleotides in the region are GGGAGA the guanine bases can be substituted by at least 1, at least 2, at least 3, or at least 4 cytosine bases. In another non-limiting example, if the nucleotides in the region are GGGAGA the guanine bases can be substituted by at least 1, at least 2, at least 3, or at least 4 thymine, and/or any of the nucleotides described herein.

In some embodiments, the nucleic acid can include at least one substitution and/or insertion upstream of the start codon. For the purpose of clarity, one of skill in the art would

appreciate that the start codon is the first codon of the protein coding region whereas the transcription start site is the site where transcription begins. The nucleic acid can include, but is not limited to, at least 1, at least 2, at least 3, at least 4, at least 5, at least 6, at least 7, or at least 8 substitutions and/or insertions of nucleotide bases. The nucleotide bases can be inserted or substituted at 1, at least 1, at least 2, at least 3, at least 4, or at least 5 locations upstream of the start codon. The nucleotides inserted and/or substituted can be the same base (*e.g.,* all A, or all C, or all T, or all G), two different bases (*e.g.,* A and C, A and T, or C and T), three different bases (*e.g.,* A, C and T, or A, C and T) or at least four different bases.

As a non-limiting example, the guanine base upstream of the coding region in the nucleic acid can be substituted with adenine, cytosine, thymine, or any of the nucleotides described herein. In another non-limiting example, the substitution of guanine bases in the nucleic acid can be designed so as to leave one guanine base in the region downstream of the transcription start site and before the start codon (see Esvelt et al. Nature (2011) 472(7344): 499-503; the contents of which is herein incorporated by reference in its entirety for this purpose). As a non-limiting example, at least 5 nucleotides can be inserted at 1 location downstream of the transcription start site but upstream of the start codon and the at least 5 nucleotides can be the same base type.

According to the present disclosure, two regions or parts of a chimeric nucleic acid may be joined or ligated, for example, using triphosphate chemistry. In some embodiments, a first region or part of 100 nucleotides or less is chemically synthesized with a 5′-monophosphate and terminal 3′-desOH or blocked OH. If the region is longer than 80 nucleotides, it may be synthesized as two or more strands that will subsequently be chemically linked by ligation. If the first region or part is synthesized as a non-positionally modified region or part using IVT, conversion to the 5′-monophosphate with subsequent capping of the 3′-terminus may follow. Monophosphate protecting groups may be selected from any of those known in the art. A second region or part of the chimeric nucleic acid may be synthesized using either chemical synthesis or IVT methods, *e.g.,* as described herein. IVT methods may include use of an RNA polymerase that can utilize a primer with a modified cap. Alternatively, a cap may be chemically synthesized and coupled to the IVT region or part.

It is noted that for ligation methods, ligation with DNA T4 ligase followed by DNAse treatment (to eliminate the DNA splint required for DNA T4 Ligase activity) should readily prevent the undesirable formation of concatenation products.

The entire chimeric polynucleotide need not be manufactured with a phosphate-sugar backbone. If one of the regions or parts encodes a polypeptide, then it is preferable that such region or part comprise a phosphate-sugar backbone.

Ligation may be performed using any appropriate technique, such as enzymatic ligation, click chemistry, orthoclick chemistry, solulink, or other bioconjugate chemistries known to those in the art. In some embodiments, the ligation is directed by a complementary oligonucleotide splint. In some embodiments, the ligation is performed without a complementary oligonucleotide splint.

Computerized Systems

The above-described embodiments can be implemented in any of numerous ways. For example, the embodiments may be implemented using hardware, software or a combination thereof. When implemented in software, the software code can be executed on any suitable processor or collection of processors, whether provided in a single computer or distributed among multiple computers. It should be appreciated that any component or collection of components that perform the functions described above can be generically considered as one or more controllers that control the above-discussed functions. The one or more controllers can be implemented in numerous ways, such as with dedicated hardware, or with general purpose hardware (*e.g.,* one or more processors) that is programmed using microcode or software to perform the functions recited above.

In this respect, it should be appreciated that one implementation comprises at least one computer-readable storage medium (*i.e.*, at least one tangible, non-transitory computer-readable medium), such as a computer memory (*e.g.,* hard drive, flash memory, processor working memory, etc.), a floppy disk, an optical disk, a magnetic tape, or other tangible, non-transitory computer-readable medium, encoded with a computer program (*i.e.*, a plurality of instructions), which, when executed on one or more processors, performs above-discussed functions. The computer-readable storage medium can be transportable such that the program stored thereon can be loaded onto any computer resource to implement techniques discussed herein. In addition, it should be appreciated that the reference to a computer program which, when executed, performs above-discussed functions, is not limited to an application program running on a host computer. Rather, the term "computer program" is used herein in a generic sense to reference any type of computer code (*e.g.,* software or

microcode) that can be employed to program one or more processors to implement above-techniques.

As a non-limiting example, in one aspect, the instant disclosure provides a computerized system for selecting nucleic acids to include in a nucleic acid cancer vaccine having a maximum length, the system comprising: a communication interface configured to receive a plurality of sequences of nucleic acids encoding a plurality of peptide epitopes, wherein each of the peptide epitopes are portions of personalized cancer antigens; and at least one computer processor programmed to: for each of the plurality of peptide epitopes, calculate a score for each of a plurality of nucleic acids in the peptide, each of which includes at least one of the one or more peptide epitopes, wherein at least two of the nucleic acid sequences have different lengths; and ranking based on the calculated scores, the plurality of nucleic acid sequences in the plurality of peptides; and selecting based on the ranking and the maximum length of the vaccine, nucleic acid sequences for inclusion in the vaccine. The score may be calculated by any means known in the art. As a set of non-limiting examples, the score may be calculated at least in part based on one or more factors selected from the group consisting of gene expression, RNA Seq, transcript abundance, DNA allele frequency, amino acid conservation, physiochemical similarity, oncogene, predicted binding affinity to a specific HLA allele, clonality, binding efficiency and presence in an indel. In some embodiments, the variant allele frequency (VAF) may be used. In one embodiment, the VAF cutoff is selected to be at a level where the addition subclonal mutations is avoided, as contamination of a tumor sample with adjacent normal tissues both reduces the tumor purity and results in a reduced (apparent) VAF. Accordingly, in instances in which the tumor purity is low (*e.g.,* when the average VAF is less than 20%), the VAF cutoff is lowered (*e.g.,* from 10% to 5%). In some embodiments, the VAF cutoff is less than 15%, 14%, 13%, 12%, 11%, 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, 1%, or less. In certain embodiments, the one or more factors are inputted into a statistical model. In some embodiments, the statistical model may be a regression model (*e.g.*, a linear regression model, a logistic regression model, a generalized linear model, *etc.*). In some embodiments, the statistical model may be a generalized linear model (*e.g.*, a logistic regression model, a probit regression model, *etc.*). In some embodiments, the statistical model may be, for example, a random forest regression model, a neural network, a support vector machine, a Gaussian mixture model, a hierarchical Bayesian model, and/or any other suitable statistical model.

Methods of Treatment

Provided herein are compositions (*e.g.*, pharmaceutical compositions), methods, kits, and reagents for prevention and/or treatment of cancer in humans (*e.g.*, subjects or patients) and other mammals. Nucleic acid cancer vaccines may be used as therapeutic or prophylactic agents in medicine to prevent and/or treat cancer. In exemplary aspects, the cancer vaccines of the present disclosure are used to provide prophylactic protection from cancer. Prophylactic protection from cancer can be achieved following administration of a cancer vaccine of the present disclosure. Vaccines can be administered once, twice, three times, four times, or more but it may be sufficient to administer the vaccine once (optionally followed by a single booster). It may also be desirable to administer the vaccine to an individual having cancer to achieve a therapeutic response. Dosing may need to be adjusted accordingly.

Once a cancer vaccine (*e.g.*, a nucleic acid cancer vaccine) is synthesized, it is administered to the patient. In some embodiments the vaccine is administered on a schedule for up to two months, up to three months, up to four month, up to five months, up to six months, up to seven months, up to eight months, up to nine months, up to ten months, up to eleven months, up to 1 year, up to 1 and ½ years, up to two years, up to three years, or up to four years. The schedule may be the same or varied. In some embodiments the schedule is weekly for the first 3 weeks and then monthly thereafter. The schedule may be determined or varied by one of skill in the art (*e.g.*, a medical doctor) depending on the individual patient or subject's criteria (*e.g.*, weight, age, type of cancer, *etc.*).

The vaccine may be administered by any route. In some embodiments the vaccine is administered by an intradermal, intramuscular, intravascular, intratumoral, and/or subcutaneous route.

In some embodiments, the nucleic acid cancer vaccine may also be administered with an anti-cancer therapeutic agent. The nucleic acid cancer vaccine and other therapeutic agent may be administered simultaneously or sequentially. When the other therapeutic agents are administered simultaneously they can be administered in the same or separate formulations, but are administered at the same time. The other therapeutic agents are administered sequentially with one another and with the nucleic acid cancer vaccine, when the administration of the other therapeutic agents and the nucleic acid cancer vaccine is temporally separated. The separation in time between administrations of these compounds may be a matter of minutes or it may be longer, *e.g.*, hours, days, weeks, months. Other therapeutic agents include but are not limited to anti-cancer therapeutic, adjuvants, cytokines, antibodies, antigens, etc.

At any point in the treatment the patient may be examined to determine whether the mutations in the vaccine are still appropriate. Based on that analysis the vaccine may be adjusted or reconfigured to include one or more different mutations or to remove one or more mutations.

In exemplary embodiments, a cancer vaccine containing RNA polynucleotides as described herein can be administered to a subject (*e.g.,* a mammalian subject, such as a human subject), and the RNA polynucleotides are translated *in vivo* to produce an antigenic polypeptide.

The cancer vaccines may be induced for translation of a polypeptide (*e.g.,* antigen or immunogen) in a cell, tissue or organism. In exemplary embodiments, such translation occurs *in vivo*, although there can be envisioned embodiments where such translation occurs *ex vivo*, in culture or *in vitro*. In exemplary embodiments, the cell, tissue or organism is contacted with an effective amount of a composition containing a cancer vaccine that contains a polynucleotide that has at least one a translatable region encoding an antigenic polypeptide.

An "effective amount" of a cancer RNA vaccine may be provided based, at least in part, on the target tissue, target cell type, means of administration, physical characteristics of the polynucleotide (*e.g.,* size, and extent of modified nucleosides) and other components of the cancer vaccine, and other determinants. In general, an effective amount of the cancer vaccine composition provides an induced or boosted immune response as a function of antigen production in the cell, preferably more efficient than a composition containing a corresponding unmodified polynucleotide encoding the same antigen or a peptide antigen. Increased antigen production may be demonstrated by increased cell transfection (the percentage of cells transfected with the cancer vaccine), increased protein translation from the polynucleotide, decreased nucleic acid degradation (as demonstrated, for example, by increased duration of protein translation from a modified polynucleotide), or altered antigen specific immune response of the host cell.

Cancer vaccines may be administered prophylactically or therapeutically as part of an active immunization scheme to healthy individuals or early in cancer or during active cancer after onset of symptoms. In some embodiments, the amount of RNA vaccines of the present disclosure provided to a cell, a tissue or a subject may be an amount effective for immune prophylaxis.

Cancer vaccines may be administered with other prophylactic or therapeutic compounds. As a non-limiting example, a prophylactic or therapeutic compound may be an

immune potentiator or a booster. As used herein, when referring to a composition, such as a vaccine, the term "booster" refers to an extra administration of the prophylactic (vaccine) composition. A booster (or booster vaccine) may be given after an earlier administration of the prophylactic composition. The time of administration between the initial administration of the prophylactic composition and the booster may be, but is not limited to, 1 minute, 2 minutes, 3 minutes, 4 minutes, 5 minutes, 6 minutes, 7 minutes, 8 minutes, 9 minutes, 10 minutes, 15 minutes, 20 minutes 35 minutes, 40 minutes, 45 minutes, 50 minutes, 55 minutes, 1 hour, 2 hours, 3 hours, 4 hours, 5 hours, 6 hours, 7 hours, 8 hours, 9 hours, 10 hours, 11 hours, 12 hours, 13 hours, 14 hours, 15 hours, 16 hours, 17 hours, 18 hours, 19 hours, 20 hours, 21 hours, 22 hours, 23 hours, 1 day, 36 hours, 2 days, 3 days, 4 days, 5 days, 6 days, 1 week, 10 days, 2 weeks, 3 weeks, 1 month, 2 months, 3 months, 4 months, 5 months, 6 months, 7 months, 8 months, 9 months, 10 months, 11 months, 1 year, 18 months, 2 years, 3 years, 4 years, 5 years, 6 years, 7 years, 8 years, 9 years, 10 years, 11 years, 12 years, 13 years, 14 years, 15 years, 16 years, 17 years, 18 years, 19 years, 20 years, 25 years, 30 years, 35 years, 40 years, 45 years, 50 years, 55 years, 60 years, 65 years, 70 years, 75 years, 80 years, 85 years, 90 years, 95 years or more than 99 years. In exemplary embodiments, the time of administration between the initial administration of the prophylactic composition and the booster may be, but is not limited to, 1 week, 2 weeks, 3 weeks, 1 month, 2 months, 3 months, 6 months or 1 year.

The cancer vaccines may be utilized in various settings depending on the severity of the cancer or the degree or level of unmet medical need. As a non-limiting example, the cancer vaccines may be utilized to treat any stage of cancer.

A non-limiting list of cancers that the cancer vaccines may treat is presented below. Peptide epitopes or antigens may be derived from any antigen of these cancers or tumors. Such epitopes may be referred to as cancer or tumor antigens. Cancer cells may differentially express cell surface molecules during different phases of tumor progression. For example, a cancer cell may express a cell surface antigen in a benign state, yet down-regulate that particular cell surface antigen upon metastasis. As such, it is envisioned that the tumor or cancer antigen may encompass antigens produced during any stage of cancer progression. The methods of the disclosure may be adjusted to accommodate for these changes. For instance, several different cancer vaccines may be generated for a particular patient. For instance, a first vaccine may be used at the start of the treatment. At a later time point, a new cancer vaccine may be generated and administered to the patient to account for different antigens being expressed.

In some embodiments, the tumor antigen is one of the following antigens: CD2, CD19, CD20, CD22, CD27, CD33, CD37, CD38, CD40, CD44, CD47, CD52, CD56, CD70, CD79, CD137, 4- IBB, 5T4, AGS-5 , AGS-16, Angiopoietin 2, B7.1, B7.2, B7DC, B7H1, B7H2, B7H3, BT-062, BTLA, CAIX, Carcinoembryonic antigen, CTLA4, Cripto, ED-B, ErbBl, ErbB2, ErbB3, ErbB4, EGFL7, EpCAM, EphA2, EphA3, EphB2, FAP, Fibronectin, Folate Receptor, Ganglioside GM3, GD2, glucocorticoid-induced tumor necrosis factor receptor (GITR), gplOO, gpA33, GPNMB, ICOS, IGF1R, Integrin av, Integrin αvβ , LAG-3, Lewis Y, Mesothelin, c-MET, MN Carbonic anhydrase IX, MUC1, MUC16, Nectin-4, NKGD2, NOTCH, OX40, OX40L, PD-1, PDL1, PSCA, PSMA, RANKL, ROR1, ROR2, SLC44A4, Syndecan-1, TACI, TAG-72, Tenascin, TIM3, TRAILRl , TRAILR2,VEGFR- 1 , VEGFR-2, VEGFR-3, and variants thereof.

Cancers or tumors include but are not limited to neoplasms, malignant tumors, metastases, or any disease or disorder characterized by uncontrolled cell growth such that it would be considered cancerous. The cancer may be a primary or metastatic cancer. Specific cancers that can be treated according to the present disclosure include, but are not limited to, those listed below (for a review of such disorders, see Fishman et al., 1985, Medicine, 2d Ed., J.B. Lippincott Co., Philadelphia). Cancers for use with the instantly described methods and compositions may include, but are not limited to, biliary tract cancer; bladder cancer; brain cancer including glioblastomas and medulloblastomas; breast cancer; cervical cancer; choriocarcinoma; colon cancer; endometrial cancer; esophageal cancer; gastric cancer; hematological neoplasms including acute lymphocytic and myelogenous leukemia; multiple myeloma; AIDS-associated leukemias and adult T-cell leukemia lymphoma; intraepithelial neoplasms including Bowen's disease and Paget's disease; liver cancer; lung cancer; lymphomas including Hodgkin's disease and lymphocytic lymphomas; neuroblastomas; oral cancer including squamous cell carcinoma; ovarian cancer including those arising from epithelial cells, stromal cells, germ cells and mesenchymal cells; pancreatic cancer; prostate cancer; rectal cancer; sarcomas including leiomyosarcoma, rhabdomyosarcoma, liposarcoma, fibrosarcoma, and osteosarcoma; skin cancer including melanoma, Kaposi's sarcoma, basocellular cancer, and squamous cell cancer; testicular cancer including germinal tumors such as seminoma, non-seminoma, teratomas; tumor mutational burden high tumors; choriocarcinomas; stromal tumors and germ cell tumors; thyroid cancer including thyroid adenocarcinoma and medullar carcinoma; and renal cancer including adenocarcinoma and Wilms' tumor. In some embodiments that cancer is any one of melanoma, bladder

carcinoma, HPV negative HNSCC, NSCLC, SCLC, MSI-High tumors, or TMB (tumor mutational burden) High cancers.

In some embodiments, the cancer is selected from the group consisting of non-small cell lung cancer (NSCLC), small cell lung cancer, melanoma, bladder urothelial carcinoma, HPV-negative head and neck squamous cell carcinoma (HNSCC), and a solid malignancy that is microsatellite high (MSI H) / mismatch repair (MMR) deficient. In some embodiments, the NSCLC lacks an EGFR sensitizing mutation and/or an ALK translocation. In some embodiments, the solid malignancy that is microsatellite high (MSI H) / mismatch repair (MMR) deficient is selected from the group consisting of colorectal cancer, stomach adenocarcinoma, esophageal adenocarcinoma, and endometrial cancer.

Provided herein are pharmaceutical compositions including cancer vaccines and RNA vaccine compositions and/or complexes optionally in combination with one or more pharmaceutically acceptable excipients. Cancer vaccines may be formulated or administered alone or in conjunction with one or more other components as described herein.

In other embodiments the cancer vaccines described herein may be combined with any other therapy useful for treating the patient. For instance a patient may be treated with the cancer vaccine and an anti-cancer agent. Thus, in one embodiment, the methods of the disclosure can be used in conjunction with one or more cancer therapeutics, for example, in conjunction with an anti-cancer agent, a traditional cancer vaccine, chemotherapy, radiotherapy, etc. (*e.g.,* simultaneously, or as part of an overall treatment procedure). Parameters of cancer treatment that may vary include, but are not limited to, dosages, timing of administration or duration or therapy; and the cancer treatment can vary in dosage, timing, or duration. Another treatment for cancer is surgery, which can be utilized either alone or in combination with any of the previous treatment methods. Any agent or therapy (*e.g.,* traditional cancer vaccines, chemotherapies, radiation therapies, surgery, hormonal therapies, and/or biological therapies/immunotherapies) which is known to be useful, or which has been used or is currently being used for the prevention or treatment of cancer can be used in combination with a composition of the disclosure in accordance with the disclosure described herein. One of ordinary skill in the medical arts can determine an appropriate treatment for a subject.

Examples of such agents (*i.e.,* , anti-cancer agents) include, but are not limited to, DNA-interactive agents including, but not limited to, the alkylating agents (*e.g.,* nitrogen mustards, *e.g.,* Chlorambucil, Cyclophosphamide, Isofamide, Mechlorethamine, Melphalan, Uracil mustard; Aziridine such as Thiotepa; methanesulphonate esters such as Busulfan;

nitroso ureas, such as Carmustine, Lomustine, Streptozocin; platinum complexes, such as Cisplatin, Carboplatin; bioreductive alkylator, such as Mitomycin, and Procarbazine, Dacarbazine and Altretamine); the DNA strand-breakage agents, *e.g.,* Bleomycin; the intercalating topoisomerase II inhibitors, *e.g.,* Intercalators, such as Amsacrine, Dactinomycin, Daunorubicin, Doxorubicin, Idarubicin, Mitoxantrone, and nonintercalators, such as Etoposide and Teniposide; the nonintercalating topoisomerase II inhibitors, *e.g.,* Etoposide and Teniposde; and the DNA minor groove binder, *e.g.,* Plicamydin; the antimetabolites including, but not limited to, folate antagonists such as Methotrexate and trimetrexate; pyrimidine antagonists, such as Fluorouracil, Fluorodeoxyuridine, CB3717, Azacitidine and Floxuridine; purine antagonists such as Mercaptopurine, 6-Thioguanine, Pentostatin; sugar modified analogs such as Cytarabine and Fludarabine; and ribonucleotide reductase inhibitors such as hydroxyurea; tubulin Interactive agents including, but not limited to, colchicine, Vincristine and Vinblastine, both alkaloids and Paclitaxel and cytoxan; hormonal agents including, but not limited to, estrogens, conjugated estrogens and Ethinyl Estradiol and Diethylstilbesterol, Chlortrianisen and Idenestrol; progestins such as Hydroxyprogesterone caproate, Medroxyprogesterone, and Megestrol; and androgens such as testosterone, testosterone propionate; fluoxymesterone, methyltestosterone; adrenal corticosteroid, *e.g.,* Prednisone, Dexamethasone, Methylprednisolone, and Prednisolone; leutinizing hormone releasing hormone agents or gonadotropin-releasing hormone antagonists, *e.g.,* leuprolide acetate and goserelin acetate; antihormonal antigens including, but not limited to, antiestrogenic agents such as Tamoxifen, antiandrogen agents such as Flutamide; and antiadrenal agents such as Mitotane and Aminoglutethimide; cytokines including, but not limited to, IL-1.alpha., IL-1 β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-13, IL-18, TGF-β, GM-CSF, M-CSF, G-CSF, TNF-α, TNF-β, LAF, TCGF, BCGF, TRF, BAF, BDG, MP, LIF, OSM, TMF, PDGF, IFN-α, IFN-β, IFN-.γ, and Uteroglobins (U.S. Pat. No. 5,696,092); anti-angiogenics including, but not limited to, agents that inhibit VEGF (*e.g.,* other neutralizing antibodies), soluble receptor constructs, tyrosine kinase inhibitors, antisense strategies, RNA aptamers and ribozymes against VEGF or VEGF receptors, immunotoxins and coaguligands, tumor vaccines, and antibodies.

Specific examples of anti-cancer agents which can be used in accordance with the methods of the disclosure include, but not limited to: acivicin; aclarubicin; acodazole hydrochloride; acronine; adozelesin; aldesleukin; altretamine; ambomycin; ametantrone acetate; aminoglutethimide; amsacrine; anastrozole; anthramycin; asparaginase; asperlin; azacitidine; azetepa; azotomycin; batimastat; benzodepa; bicalutamide; bisantrene

hydrochloride; bisnafide dimesylate; bizelesin; bleomycin sulfate; brequinar sodium; bropirimine; busulfan; cactinomycin; calusterone; caracemide; carbetimer; carboplatin; carmustine; carubicin hydrochloride; carzelesin; cedefingol; chlorambucil; cirolemycin; cisplatin; cladribine; crisnatol mesylate; cyclophosphamide; cytarabine; dacarbazine; dactinomycin; daunorubicin hydrochloride; decitabine; dexormaplatin; dezaguanine; dezaguanine mesylate; diaziquone; docetaxel; doxorubicin; doxorubicin hydrochloride; droloxifene; droloxifene citrate; dromostanolone propionate; duazomycin; edatrexate; eflomithine hydrochloride; elsamitrucin; enloplatin; enpromate; epipropidine; epirubicin hydrochloride; erbulozole; esorubicin hydrochloride; estramustine; estramustine phosphate sodium; etanidazole; etoposide; etoposide phosphate; etoprine; fadrozole hydrochloride; fazarabine; fenretinide; floxuridine; fludarabine phosphate; fluorouracil; flurocitabine; fosquidone; fostriecin sodium; gemcitabine; gemcitabine hydrochloride; hydroxyurea; idarubicin hydrochloride; ifosfamide; ilmofosine; interleukin II (including recombinant interieukin II, or rIL2), interferon alpha-2a; interferon alpha-2b; interferon alpha-n1; interferon alpha-n3; interferon beta-Ia; interferon gamma-Ib; iproplatin; irinotecan hydrochloride; lanreotide acetate; letrozole; leuprolide acetate; liarozole hydrochloride; lometrexol sodium; lomustine; losoxantrone hydrochloride; masoprocol; maytansine; mechlorethamine hydrochloride; megestrol acetate; melengestrol acetate; melphalan; menogaril; mercaptopurine; methotrexate; methotrexate sodium; metoprine; meturedepa; mitindomide; mitocarcin; mitocromin; mitogillin; mitomalcin; mitomycin; mitosper; mitotane; mitoxantrone hydrochloride; mycophenolic acid; nocodazole; nogalamycin; ormaplatin; oxisuran; paclitaxel; pegaspargase; peliomycin; pentamustine; peplomycin sulfate; perfosfamide; pipobroman; piposulfan; piroxantrone hydrochloride; plicamycin; plomestane; porfimer sodium; porfiromycin; prednimustine; procarbazine hydrochloride; puromycin; puromycin hydrochloride; pyrazofurin; riboprine; rogletimide; safingol; safingol hydrochloride; semustine; simtrazene; sparfosate sodium; sparsomycin; spirogermanium hydrochloride; spiromustine; spiroplatin; streptonigrin; streptozocin; sulofenur; talisomycin; tecogalan sodium; tegafur; teloxantrone hydrochloride; temoporfin; teniposide; teroxirone; testolactone; thiamiprine; thioguanine; thiotepa; tiazofurin; tirapazamine; toremifene citrate; trestolone acetate; triciribine phosphate; trimetrexate; trimetrexate glucuronate; triptorelin; tubulozole hydrochloride; uracil mustard; uredepa; vapreotide; verteporfin; vinblastine sulfate; vincristine sulfate; vindesine; vindesine sulfate; vinepidine sulfate; vinglycinate sulfate; vinleurosine sulfate; vinorelbine tartrate; vinrosidine sulfate; vinzolidine sulfate; vorozole; zeniplatin; zinostatin; and zorubicin hydrochloride.

Other anti-cancer drugs which may be used with the instant compositions and methods include, but are not limited to: 20-epi-1,25 dihydroxyvitamin D3; 5-ethynyluracil; angiogenesis inhibitors; anti-dorsalizing morphogenetic protein-1; ara-CDP-DL-PTBA; BCR/ABL antagonists; CaRest M3; CARN 700; casein kinase inhibitors (ICOS); clotrimazole; collismycin A; collismycin B; combretastatin A4; crambescidin 816; cryptophycin 8; curacin A; dehydrodidemnin B; didemnin B; dihydro-5-azacytidine; dihydrotaxol, duocarmycin SA; kahalalide F; lamellarin-N triacetate; leuprolide+estrogen+progesterone; lissoclinamide 7; monophosphoryl lipid A+myobacterium cell wall sk; N-acetyldinaline; N-substituted benzamides; O6-benzylguanine; placetin A; placetin B; platinum complex; platinum compounds; platinum-triamine complex; rhenium Re 186 etidronate; RII retinamide; rubiginone B 1; SarCNU; sarcophytol A; sargramostim; senescence derived inhibitor 1; spicamycin D; tallimustine; 5-fluorouracil; thrombopoietin; thymotrinan; thyroid stimulating hormone; variolin B; thalidomide; velaresol; veramine; verdins; verteporfin; vinorelbine; vinxaltine; vitaxin; zanoterone; zeniplatin; and zilascorb.

The disclosure also encompasses administration of a composition comprising a cancer vaccine in combination with radiation therapy comprising the use of x-rays, gamma rays and other sources of radiation to destroy the cancer cells. In certain embodiments, the radiation treatment is administered as external beam radiation or teletherapy wherein the radiation is directed from a remote source. In other embodiments, the radiation treatment is administered as internal therapy or brachytherapy wherein a radioactive source is placed inside the body close to cancer cells or a tumor mass.

In specific embodiments, an appropriate anti-cancer regimen is selected depending on the type of cancer (*e.g.*, by a physician). For instance, a patient with ovarian cancer may be administered a prophylactically or therapeutically effective amount of a composition comprising a cancer vaccine in combination with a prophylactically or therapeutically effective amount of one or more other agents useful for ovarian cancer therapy, including but not limited to, intraperitoneal radiation therapy, such as P32 therapy, total abdominal and pelvic radiation therapy, cisplatin, the combination of paclitaxel (Taxol) or docetaxel (Taxotere) and cisplatin or carboplatin, the combination of cyclophosphamide and cisplatin, the combination of cyclophosphamide and carboplatin, the combination of 5-FU and leucovorin, etoposide, liposomal doxorubicin, gemcitabine or topotecan. Cancer therapies and their dosages, routes of administration and recommended usage are known in the art and have been described in such literature as the Physician's Desk Reference (56th ed., 2002).

In some embodiments of the disclosure the cancer vaccines are administered with a T cell activator such as an immune checkpoint modulator. Immune checkpoint modulators include both stimulatory checkpoint molecules and inhibitory checkpoint molecules (*e.g.*, an anti-CTLA4 and/or an anti-PD1 antibody).

Stimulatory checkpoint inhibitors function by promoting the checkpoint process. Several stimulatory checkpoint molecules are members of the tumor necrosis factor (TNF) receptor superfamily (*e.g.*, CD27, CD40, OX40, GITR, or CD137), while others belong to the B7-CD28 superfamily (*e.g.*, CD28 or ICOS0. OX40 (CD134), is involved in the expansion of effector and memory T cells. Anti-OX40 monoclonal antibodies have been shown to be effective in treating advanced cancer. MEDI0562 is a humanized OX40 agonist. GITR, Glucocorticoid-Induced TNFR family Related gene, is involved in T cell expansion. Several antibodies to GITR have been shown to promote an anti-tumor responses. ICOS, Inducible T-cell costimulator, is important in T cell effector function. CD27 supports antigen-specific expansion of naïve T cells and is involved in the generation of T and B cell memory. Several agonistic anti-CD27 antibodies are in development. CD122 is the Interleukin-2 receptor beta sub-unit. NKTR-214 is a CD122-biased immune-stimulatory cytokine.

Inhibitory checkpoint molecules include, but are not limited to: PD-1, TIM-3, VISTA, A2AR, B7-H3, B7-H4, BTLA, CTLA-4, IDO, KIR and LAG3. CTLA-4, PD-1, and ligands thereof are members of the CD28-B7 family of co-signaling molecules that play important roles throughout all stages of T-cell function and other cell functions. CTLA-4, Cytotoxic T-Lymphocyte-Associated protein 4 (CD152), is involved in controlling T cell proliferation.

The PD-1 receptor is expressed on the surface of activated T cells (and B cells) and, under normal circumstances, binds to its ligands (PD-L1 and PD-L2) that are expressed on the surface of antigen-presenting cells, such as dendritic cells or macrophages. This interaction sends a signal into the T cell and inhibits it. Cancer cells take advantage of this system by driving high levels of expression of PD-L1 on their surface. This allows them to gain control of the PD-1 pathway and switch off T cells expressing PD-1 that may enter the tumor microenvironment, thus suppressing the anticancer immune response. Pembrolizumab (formerly MK-3475 and lambrolizumab, trade name Keytruda) is a human antibody used in cancer immunotherapy and targets the PD-1 receptor.

The checkpoint inhibitor is a molecule such as a monoclonal antibody, a humanized antibody, a fully human antibody, a fusion protein or a combination thereof or a small molecule. For instance, the checkpoint inhibitor inhibits a checkpoint protein which may be CTLA-4, PDL1, PDL2, PD1, B7-H3, B7-H4, BTLA, HVEM, TIM3, GAL9, LAG3, VISTA,

KIR, 2B4, CD160, CGEN-15049, CHK1, CHK2, A2aR, B-7 family ligands or a combination thereof. Ligands of checkpoint proteins include but are not limited to CTLA-4, PDL1, PDL2, PD1, B7-H3, B7-H4, BTLA, HVEM, TIM3, GAL9, LAG3, VISTA, KIR, 2B4, CD160, CGEN-15049, CHK 1, CHK2, A2aR, and B-7 family ligands. In some embodiments the anti-PD-1 antibody is BMS-936558 (nivolumab). In other embodiments the anti-CTLA-4 antibody is ipilimumab (trade name Yervoy, formerly known as MDX-010 and MDX-101).

In some embodiments the cancer therapeutic agents, including the checkpoint modulators, are delivered in the form of mRNA encoding the cancer therapeutic agents.

In some embodiments the cancer therapeutic agent is a targeted therapy. The targeted therapy may be a BRAF inhibitor such as vemurafenib (PLX4032) or dabrafenib. The BRAF inhibitor may be PLX 4032, PLX 4720, PLX 4734, GDC-0879, PLX 4032, PLX-4720, PLX 4734 and Sorafenib Tosylate. BRAF is a human gene that makes a protein called B-Raf, also referred to as proto-oncogene B-Raf and v-Raf murine sarcoma viral oncogene homolog B1. The B-Raf protein is involved in sending signals inside cells, which are involved in directing cell growth. Vemurafenib, a BRAF inhibitor, was approved by FDA for treatment of late-stage melanoma.

In other embodiments the cancer therapeutic agent is a cytokine. In yet other embodiments the cancer therapeutic agent is a vaccine comprising a population based tumor specific antigen. In yet other embodiments, the cancer therapeutic agent is vaccine containing one or more traditional antigens expressed by cancer-germline genes (antigens common to tumors found in multiple patients, also referred to as "shared cancer antigens"). In some embodiments, a traditional antigen is one that is known to be found in cancers or tumors generally or in a specific type of cancer or tumor. In some embodiments, a traditional cancer antigen is a non-mutated tumor antigen. In some embodiments, a traditional cancer antigen is a mutated tumor antigen.

The p53 gene (official symbol TP53) is mutated more frequently than any other gene in human cancers. Large cohort studies have shown that, for most p53 mutations, the genomic position is unique to one or only a few patients and the mutation cannot be used as recurrent neoantigens for therapeutic vaccines designed for a specific population of patients. A small subset of p53 loci do, however, exhibit a "hotspot" pattern (described elsewhere herein), in which several positions in the gene are mutated with relatively high frequency. Strikingly, a large portion of these recurrently mutated regions occur near exon-intron boundaries, disrupting the canonical nucleotide sequence motifs recognized by the mRNA splicing machinery.

Mutation of a splicing motif can alter the final mRNA sequence even if no change to the local amino acid sequence is predicted (*i.e.,* for synonymous or intronic mutations). Therefore, these mutations are often annotated as "noncoding" by common annotation tools and neglected for further analysis, even though they may alter mRNA splicing in unpredictable ways and exert severe functional impact on the translated protein. If an alternatively spliced isoform produces an in-frame sequence change (*i.e.*, no pretermination codon (PTC) is produced), it can escape depletion by nonsense-mediated mRNA decay (NMD) and be readily expressed, processed, and presented on the cell surface by the HLA system. Further, mutation-derived alternative splicing is usually "cryptic", *i.e.*, not expressed in normal tissues, and therefore may be recognized by T-cells as non-self neoantigens.

In some instances, the cancer therapeutic agent is a vaccine which includes one or more neoantigens which are recurrent polymorphisms ("hot spot mutations"). For example, among other things, the present disclosure provides neoantigen peptide sequences resulting from certain recurrent somatic cancer mutations in p53.

Formulations

Cancer vaccines (*e.g.*, nucleic acid cancer vaccines such as mRNA cancer vaccines) may be formulated or administered in combination with one or more pharmaceutically-acceptable excipients. As a non-limiting set of examples, cancer vaccines can be formulated using one or more excipients to: (1) increase stability; (2) increase cell transfection; (3) permit the sustained or delayed release (*e.g.*, from a depot formulation); (4) alter the biodistribution (*e.g.*, target to specific tissues or cell types); (5) increase the translation of encoded protein *in vivo*; and/or (6) alter the release profile of encoded protein (antigen) *in vivo*. In addition to traditional excipients such as any and all solvents, dispersion media, diluents, or other liquid vehicles, dispersion or suspension aids, surface active agents, isotonic agents, thickening or emulsifying agents, preservatives, excipients can include, without limitation, lipidoids, liposomes, lipid nanoparticles, polymers, lipoplexes, core-shell nanoparticles, peptides, proteins, cells transfected with cancer vaccines (*e.g.*, for transplantation into a subject), hyaluronidase, nanoparticle mimics and combinations thereof.

In some embodiments, vaccine compositions comprise at least one additional active substance, such as, for example, a therapeutically-active substance, a prophylactically-active substance, or a combination of both. Vaccine compositions may be sterile, pyrogen-free or both sterile and pyrogen-free. General considerations in the formulation and/or manufacture of pharmaceutical agents, such as vaccine compositions, may be found, for example, in

Remington: The Science and Practice of Pharmacy 21st ed., Lippincott Williams & Wilkins, 2005 (incorporated herein by reference in its entirety for this purpose).

In some embodiments, cancer vaccines are administered to humans, human patients or subjects. For the purposes of the present disclosure, the phrase "active ingredient" generally refers to the cancer vaccines or the nucleic acids contained therein, for example, RNA (*e.g.*, mRNA) encoding antigenic polypeptides.

Formulations of the vaccine compositions described herein may be prepared by any method known or hereafter developed in the art of pharmacology. In general, such preparatory methods include the step of bringing the active ingredient (*e.g.*, nucleic acids such as mRNA) into association with an excipient and/or one or more other accessory ingredients, and then, if necessary and/or desirable, dividing, shaping and/or packaging the product into a desired single- or multi-dose unit.

The formulation of any of the compositions disclosed herein can include one or more components in addition to those described above. For example, the lipid composition can include one or more permeability enhancer molecules, carbohydrates, polymers, surface altering agents (*e.g.,* surfactants), or other components. For example, a permeability enhancer molecule can be a molecule described by U.S. Patent Application Publication No. 2005/0222064. Carbohydrates can include simple sugars (*e.g.,* glucose) and polysaccharides (*e.g.,* glycogen and derivatives and analogs thereof).

A polymer can be included in and/or used to encapsulate or partially encapsulate a pharmaceutical composition disclosed herein (*e.g.,* a pharmaceutical composition in lipid nanoparticle form). A polymer can be biodegradable and/or biocompatible. A polymer can be selected from, but is not limited to, polyamines, polyethers, polyamides, polyesters, polycarbamates, polyureas, polycarbonates, polystyrenes, polyimides, polysulfones, polyurethanes, polyacetylenes, polyethylenes, polyethyleneimines, polyisocyanates, polyacrylates, polymethacrylates, polyacrylonitriles, and polyarylates.

In some embodiments, the compositions disclosed herein may be formulated as lipid nanoparticles (LNP). Accordingly, the present disclosure also provides nanoparticle compositions comprising (i) a lipid composition comprising a delivery agent, and (ii) a nucleic acid encoding one or more peptide epitopes. In such nanoparticle composition, the lipid composition disclosed herein can encapsulate the nucleic acid encoding one or more peptide epitopes.

Nanoparticle compositions are typically sized on the order of micrometers or smaller and can include a lipid bilayer. Nanoparticle compositions encompass lipid nanoparticles

(LNPs), liposomes (*e.g.,* lipid vesicles), and lipoplexes. For example, a nanoparticle composition can be a liposome having a lipid bilayer with a diameter of 500 nm or less.

Nanoparticle compositions include, for example, lipid nanoparticles (LNPs), liposomes, and lipoplexes. In some embodiments, nanoparticle compositions are vesicles including one or more lipid bilayers. In certain embodiments, a nanoparticle composition includes two or more concentric bilayers separated by aqueous compartments. Lipid bilayers can be functionalized and/or crosslinked to one another. Lipid bilayers can include one or more ligands, proteins, or channels.

In one embodiment, a lipid nanoparticle comprises an ionizable lipid, a structural lipid, a phospholipid, and mRNA. In some embodiments, the LNP comprises an ionizable lipid, a PEG-modified lipid, a phospholipid and a structural lipid.

The ratio between the lipid composition and the cancer vaccine may be from about 10:1 to about 60:1 (wt/wt). In some embodiments, the ratio between the lipid composition and the nucleic acid may be about 10:1, 11:1, 12:1, 13:1, 14:1, 15:1, 16:1, 17:1, 18:1, 19:1, 20:1, 21:1, 22:1, 23:1, 24:1, 25:1, 26:1, 27:1, 28:1, 29:1, 30:1, 31:1, 32:1, 33:1, 34:1, 35:1, 36:1, 37:1, 38:1, 39:1, 40:1, 41:1, 42:1, 43:1, 44:1, 45:1, 46:1, 47:1, 48:1, 49:1, 50:1, 51:1, 52:1, 53:1, 54:1, 55:1, 56:1, 57:1, 58:1, 59:1 or 60:1 (wt/wt). In some embodiments, the wt/wt ratio of the lipid composition to the cancer vaccine is about 20:1 or about 15:1.

In one embodiment, the cancer vaccine (*e.g.,* the nucleic acid cancer vaccine) may be comprised in lipid nanoparticles such that the lipid:polynucleotide weight ratio is 5:1, 10:1, 15:1, 20:1, 25:1, 30:1, 35:1, 40:1, 45:1, 50:1, 55:1, 60:1 or 70:1, or a range or any of these ratios such as, but not limited to, 5:1 to about 10:1, from about 5:1 to about 15:1, from about 5:1 to about 20:1, from about 5:1 to about 25:1, from about 5:1 to about 30:1, from about 5:1 to about 35:1, from about 5:1 to about 40:1, from about 5:1 to about 45:1, from about 5:1 to about 50:1, from about 5:1 to about 55:1, from about 5:1 to about 60:1, from about 5:1 to about 70:1, from about 10:1 to about 15:1, from about 10:1 to about 20:1, from about 10:1 to about 25:1, from about 10:1 to about 30:1, from about 10:1 to about 35:1, from about 10:1 to about 40:1, from about 10:1 to about 45:1, from about 10:1 to about 50:1, from about 10:1 to about 55:1, from about 10:1 to about 60:1, from about 10:1 to about 70:1, from about 15:1 to about 20:1, from about 15:1 to about 25:1,from about 15:1 to about 30:1, from about 15:1 to about 35:1, from about 15:1 to about 40:1, from about 15:1 to about 45:1, from about 15:1 to about 50:1, from about 15:1 to about 55:1, from about 15:1 to about 60:1 or from about 15:1 to about 70:1.

In one embodiment, the cancer vaccine (*e.g.,* the nucleic acid cancer vaccine) may be comprised in lipid nanoparticles in a concentration from approximately 0.1 mg/ml to 2 mg/ml such as, but not limited to, 0.1 mg/ml, 0.2 mg/ml, 0.3 mg/ml, 0.4 mg/ml, 0.5 mg/ml, 0.6 mg/ml, 0.7 mg/ml, 0.8 mg/ml, 0.9 mg/ml, 1.0 mg/ml, 1.1 mg/ml, 1.2 mg/ml, 1.3 mg/ml, 1.4 mg/ml, 1.5 mg/ml, 1.6 mg/ml, 1.7 mg/ml, 1.8 mg/ml, 1.9 mg/ml, 2.0 mg/ml or greater than 2.0 mg/ml.

As generally defined herein, the term "lipid" refers to a small molecule that has hydrophobic or amphiphilic properties. Lipids may be naturally occurring or synthetic. Examples of classes of lipids include, but are not limited to, fats, waxes, sterol-containing metabolites, vitamins, fatty acids, glycerolipids, glycerophospholipids, sphingolipids, saccharolipids, and polyketides, and prenol lipids. In some instances, the amphiphilic properties of some lipids lead them to form liposomes, vesicles, or membranes in aqueous media.

In some embodiments, a lipid nanoparticle (LNP) may comprise an ionizable lipid. As used herein, the term "ionizable lipid" has its ordinary meaning in the art and may refer to a lipid comprising one or more charged moieties. In some embodiments, an ionizable lipid may be positively charged or negatively charged. An ionizable lipid may be positively charged, in which case it can be referred to as "cationic lipid". In certain embodiments, an ionizable lipid molecule may comprise an amine group, and can be referred to as an ionizable amino lipids. As used herein, a "charged moiety" is a chemical moiety that carries a formal electronic charge, *e.g.,* monovalent (+1, or -1), divalent (+2, or -2), trivalent (+3, or -3), etc. The charged moiety may be anionic (*i.e.,* negatively charged) or cationic (*i.e.,* positively charged). Examples of positively-charged moieties include amine groups (*e.g.,* primary, secondary, and/or tertiary amines), ammonium groups, pyridinium group, guanidine groups, and imidizolium groups. In a particular embodiment, the charged moieties comprise amine groups. Examples of negatively- charged groups or precursors thereof, include carboxylate groups, sulfonate groups, sulfate groups, phosphonate groups, phosphate groups, hydroxyl groups, and the like. The charge of the charged moiety may vary, in some cases, with the environmental conditions, for example, changes in pH may alter the charge of the moiety, and/or cause the moiety to become charged or uncharged. In general, the charge density of the molecule may be selected as desired. Ionizable lipids can also be the compounds disclosed in International Publication Nos.: WO2017075531, WO2015199952, WO2013086354, or WO2013116126, or selected from formulae CLI-CLXXXXII of US

Patent No.7,404,969; each of which is hereby incorporated by reference in its entirety for this purpose.

It should be understood that the terms "charged" or "charged moiety" does not refer to a "partial negative charge" or "partial positive charge" on a molecule. The terms "partial negative charge" and "partial positive charge" are given its ordinary meaning in the art. A "partial negative charge" may result when a functional group comprises a bond that becomes polarized such that electron density is pulled toward one atom of the bond, creating a partial negative charge on the atom. Those of ordinary skill in the art will, in general, recognize bonds that can become polarized in this way.

In some embodiments, the ionizable lipid is an ionizable amino lipid, sometimes referred to in the art as an "ionizable cationic lipid". In one embodiment, the ionizable amino lipid may have a positively charged hydrophilic head and a hydrophobic tail that are connected via a linker structure. In addition to these, an ionizable lipid may also be a lipid including a cyclic amine group.

Vaccines of the present disclosure are typically formulated into lipid nanoparticles. In some embodiments, the lipid nanoparticle comprises at least one ionizable amino lipid, at least one non-cationic lipid, at least one sterol, and/or at least one polyethylene glycol (PEG)-modified lipid.

In some embodiments, the lipid nanoparticle comprises a molar ratio of 20-60% ionizable amino lipid. For example, the lipid nanoparticle may comprise a molar ratio of 20-50%, 20-40%, 20-30%, 30-60%, 30-50%, 30-40%, 40-60%, 40-50%, or 50-60% ionizable amino lipid. In some embodiments, the lipid nanoparticle comprises a molar ratio of 20%, 30%, 40%, 50, or 60% ionizable amino lipid.

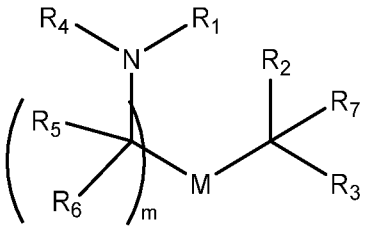
In some embodiments, the lipid nanoparticle comprises a molar ratio of 5-25% non-cationic lipid. For example, the lipid nanoparticle may comprise a molar ratio of 5-20%, 5-15%, 5-10%, 10-25%, 10-20%, 10-25%, 15-25%, 15-20%, or 20-25% non-cationic lipid. In some embodiments, the lipid nanoparticle comprises a molar ratio of 5%, 10%, 15%, 20%, or25% non-cationic lipid.

In some embodiments, the lipid nanoparticle comprises a molar ratio of 25-55% sterol. For example, the lipid nanoparticle may comprise a molar ratio of 25-50%, 25-45%, 25-40%, 25-35%, 25-30%, 30-55%, 30-50%, 30-45%, 30-40%, 30-35%, 35-55%, 35-50%, 35-45%, 35-40%, 40-55%, 40-50%, 40-45%, 45-55%, 45-50%, or 50-55% sterol. In some embodiments, the lipid nanoparticle comprises a molar ratio of 25%, 30%, 35%, 40%, 45%, 50%, or 55% sterol.

In some embodiments, the lipid nanoparticle comprises a molar ratio of 0.5-15% PEG-modified lipid. For example, the lipid nanoparticle may comprise a molar ratio of 0.5-10%, 0.5-5%, 1-15%, 1-10%, 1-5%, 2-15%, 2-10%, 2-5%, 5-15%, 5-10%, or 10-15%. In some embodiments, the lipid nanoparticle comprises a molar ratio of 0.5%, 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, or 15% PEG-modified lipid.

In some embodiments, the lipid nanoparticle comprises a molar ratio of 20-60% ionizable amino lipid, 5-25% non-cationic lipid, 25-55% sterol, and 0.5-15% PEG-modified lipid.

In some embodiments, an ionizable amino lipid of the disclosure comprises a compound of Formula (I):



(I),

or a salt or isomer thereof, wherein:

R_1 is selected from the group consisting of C_{5-30} alkyl, C_{5-20} alkenyl, -R*YR", -YR", and -R"M'R';

R_2 and R_3 are independently selected from the group consisting of H, C_{1-14} alkyl, C_{2-14} alkenyl, -R*YR", -YR", and -R*OR", or R_2 and R_3, together with the atom to which they are attached, form a heterocycle or carbocycle;

R_4 is selected from the group consisting of a C_{3-6} carbocycle, $-(CH_2)_nQ$, $-(CH_2)_nCHQR$, -CHQR, $-CQ(R)_2$, and unsubstituted C_{1-6} alkyl, where Q is selected from a carbocycle, heterocycle, -OR, $-O(CH_2)_nN(R)_2$, -C(O)OR, -OC(O)R, $-CX_3$, $-CX_2H$, $-CXH_2$, -CN, $-N(R)_2$, $-C(O)N(R)_2$, $-N(R)C(O)R$, $-N(R)S(O)_2R$, $-N(R)C(O)N(R)_2$, $-N(R)C(S)N(R)_2$, $-N(R)R_8$, $-O(CH_2)_nOR$, $-N(R)C(=NR_9)N(R)_2$, $-N(R)C(=CHR_9)N(R)_2$, $-OC(O)N(R)_2$, -N(R)C(O)OR, -N(OR)C(O)R, $-N(OR)S(O)_2R$, -N(OR)C(O)OR, $-N(OR)C(O)N(R)_2$, $-N(OR)C(S)N(R)_2$, $-N(OR)C(=NR_9)N(R)_2$, $-N(OR)C(=CHR_9)N(R)_2$, $-C(=NR_9)N(R)_2$, $-C(=NR_9)R$, -C(O)N(R)OR, and $-C(R)N(R)_2C(O)OR$, and each n is independently selected from 1, 2, 3, 4, and 5;

each R_5 is independently selected from the group consisting of C_{1-3} alkyl, C_{2-3} alkenyl, and H;

each R_6 is independently selected from the group consisting of C_{1-3} alkyl, C_{2-3} alkenyl, and H;

M and M' are independently selected from -C(O)O-, -OC(O)-, -C(O)N(R')-, -N(R')C(O)-, -C(O)-, -C(S)-, -C(S)S-, -SC(S)-, -CH(OH)-, -P(O)(OR')O-, -$S(O)_2$-, -S-S-, an aryl group, and a heteroaryl group;

R_7 is selected from the group consisting of C_{1-3} alkyl, C_{2-3} alkenyl, and H;

R_8 is selected from the group consisting of C_{3-6} carbocycle and heterocycle;

R_9 is selected from the group consisting of H, CN, NO_2, C_{1-6} alkyl, -OR, -$S(O)_2$R, -$S(O)_2N(R)_2$, C_{2-6} alkenyl, C_{3-6} carbocycle and heterocycle;

each R is independently selected from the group consisting of C_{1-3} alkyl, C_{2-3} alkenyl, and H;

each R' is independently selected from the group consisting of C_{1-18} alkyl, C_{2-18} alkenyl, -R*YR", -YR", and H;

each R" is independently selected from the group consisting of C_{3-14} alkyl and C_{3-14} alkenyl;

each R* is independently selected from the group consisting of C_{1-12} alkyl and C_{2-12} alkenyl;

each Y is independently a C_{3-6} carbocycle;

each X is independently selected from the group consisting of F, Cl, Br, and I; and

m is selected from 5, 6, 7, 8, 9, 10, 11, 12, and 13.

In some embodiments, a subset of compounds of Formula (I) includes those in which when R_4 is -$(CH_2)_nQ$, -$(CH_2)_nCHQR$, –CHQR, or -$CQ(R)_2$, then (i) Q is not -$N(R)_2$ when n is 1, 2, 3, 4 or 5, or (ii) Q is not 5, 6, or 7-membered heterocycloalkyl when n is 1 or 2.

In some embodiments, another subset of compounds of Formula (I) includes those in which

R_1 is selected from the group consisting of C_{5-30} alkyl, C_{5-20} alkenyl, -R*YR", -YR", and -R"M'R';

R_2 and R_3 are independently selected from the group consisting of H, C_{1-14} alkyl, C_{2-14} alkenyl, -R*YR", -YR", and -R*OR", or R_2 and R_3, together with the atom to which they are attached, form a heterocycle or carbocycle;

R_4 is selected from the group consisting of a C_{3-6} carbocycle, -$(CH_2)_nQ$, -$(CH_2)_nCHQR$, -CHQR, -$CQ(R)_2$, and unsubstituted C_{1-6} alkyl, where Q is selected from a C_{3-6} carbocycle, a 5- to 14-membered heteroaryl having one or more heteroatoms selected from N, O, and S, -OR, -$O(CH_2)_nN(R)_2$, -C(O)OR, -OC(O)R, -CX_3, -CX_2H, -CXH_2, -CN, -$C(O)N(R)_2$, -N(R)C(O)R, -$N(R)S(O)_2$R, -N(R)C(O)$N(R)_2$, -N(R)C(S)$N(R)_2$, -$CRN(R)_2C(O)OR$, -N(R)R_8, -$O(CH_2)_nOR$, -N(R)C(=NR_9)$N(R)_2$, -N(R)C(=CHR_9)$N(R)_2$,

$-OC(O)N(R)_2$, $-N(R)C(O)OR$, $-N(OR)C(O)R$, $-N(OR)S(O)_2R$, $-N(OR)C(O)OR$, $-N(OR)C(O)N(R)_2$, $-N(OR)C(S)N(R)_2$, $-N(OR)C(=NR_9)N(R)_2$, $-N(OR)C(=CHR_9)N(R)_2$, $-C(=NR_9)N(R)_2$, $-C(=NR_9)R$, $-C(O)N(R)OR$, and a 5- to 14-membered heterocycloalkyl having one or more heteroatoms selected from N, O, and S which is substituted with one or more substituents selected from oxo (=O), OH, amino, mono- or di-alkylamino, and C_{1-3} alkyl, and each n is independently selected from 1, 2, 3, 4, and 5;

each R_5 is independently selected from the group consisting of C_{1-3} alkyl, C_{2-3} alkenyl, and H;

each R_6 is independently selected from the group consisting of C_{1-3} alkyl, C_{2-3} alkenyl, and H;

M and M' are independently selected from -C(O)O-, -OC(O)-, -C(O)N(R')-, -N(R')C(O)-, -C(O)-, -C(S)-, -C(S)S-, -SC(S)-, -CH(OH)-, -P(O)(OR')O-, $-S(O)_2$-, -S-S-, an aryl group, and a heteroaryl group;

R_7 is selected from the group consisting of C_{1-3} alkyl, C_{2-3} alkenyl, and H;

R_8 is selected from the group consisting of C_{3-6} carbocycle and heterocycle;

R_9 is selected from the group consisting of H, CN, NO_2, C_{1-6} alkyl, -OR, $-S(O)_2R$, $-S(O)_2N(R)_2$, C_{2-6} alkenyl, C_{3-6} carbocycle and heterocycle;

each R is independently selected from the group consisting of C_{1-3} alkyl, C_{2-3} alkenyl, and H;

each R' is independently selected from the group consisting of C_{1-18} alkyl, C_{2-18} alkenyl, -R*YR", -YR", and H;

each R" is independently selected from the group consisting of C_{3-14} alkyl and C_{3-14} alkenyl;

each R* is independently selected from the group consisting of C_{1-12} alkyl and C_{2-12} alkenyl;

each Y is independently a C_{3-6} carbocycle;

each X is independently selected from the group consisting of F, Cl, Br, and I; and

m is selected from 5, 6, 7, 8, 9, 10, 11, 12, and 13,

or salts or isomers thereof.

In some embodiments, another subset of compounds of Formula (I) includes those in which

R_1 is selected from the group consisting of C_{5-30} alkyl, C_{5-20} alkenyl, -R*YR", -YR", and -R"M'R';

R_2 and R_3 are independently selected from the group consisting of H, C_{1-14} alkyl, C_{2-14} alkenyl, -R*YR", -YR", and -R*OR", or R_2 and R_3, together with the atom to which they are attached, form a heterocycle or carbocycle;

R_4 is selected from the group consisting of a C_{3-6} carbocycle, $-(CH_2)_nQ$, $-(CH_2)_nCHQR$, -CHQR, $-CQ(R)_2$, and unsubstituted C_{1-6} alkyl, where Q is selected from a C_{3-6} carbocycle, a 5- to 14-membered heterocycle having one or more heteroatoms selected from N, O, and S, -OR, $-O(CH_2)_nN(R)_2$, -C(O)OR, -OC(O)R, $-CX_3$, $-CX_2H$, $-CXH_2$, -CN, $-C(O)N(R)_2$,$-N(R)C(O)R$, $-N(R)S(O)_2R$, $-N(R)C(O)N(R)_2$, $-N(R)C(S)N(R)_2$, $-CRN(R)_2C(O)OR$, $-N(R)R_8$, $-O(CH_2)_nOR$, $-N(R)C(=NR_9)N(R)_2$, $-N(R)C(=CHR_9)N(R)_2$, $-OC(O)N(R)_2$, $-N(R)C(O)OR$, $-N(OR)C(O)R$, $-N(OR)S(O)_2R$, $-N(OR)C(O)OR$, $-N(OR)C(O)N(R)_2$, $-N(OR)C(S)N(R)_2$, $-N(OR)C(=NR_9)N(R)_2$, $-N(OR)C(=CHR_9)N(R)_2$, $-C(=NR_9)R$, -C(O)N(R)OR, and $-C(=NR_9)N(R)_2$, and each n is independently selected from 1, 2, 3, 4, and 5; and when Q is a 5- to 14-membered heterocycle and (i) R_4 is $-(CH_2)_nQ$ in which n is 1 or 2, or (ii) R_4 is $-(CH_2)_nCHQR$ in which n is 1, or (iii) R_4 is -CHQR, and $-CQ(R)_2$, then Q is either a 5- to 14-membered heteroaryl or 8- to 14-membered heterocycloalkyl;

each R_5 is independently selected from the group consisting of C_{1-3} alkyl, C_{2-3} alkenyl, and H;

each R_6 is independently selected from the group consisting of C_{1-3} alkyl, C_{2-3} alkenyl, and H;

M and M' are independently selected from -C(O)O-, -OC(O)-, -C(O)N(R')-, -N(R')C(O)-, -C(O)-, -C(S)-, -C(S)S-, -SC(S)-, -CH(OH)-, -P(O)(OR')O-, $-S(O)_2-$, -S-S-, an aryl group, and a heteroaryl group;

R_7 is selected from the group consisting of C_{1-3} alkyl, C_{2-3} alkenyl, and H;

R_8 is selected from the group consisting of C_{3-6} carbocycle and heterocycle;

R_9 is selected from the group consisting of H, CN, NO_2, C_{1-6} alkyl, -OR, $-S(O)_2R$, $-S(O)_2N(R)_2$, C_{2-6} alkenyl, C_{3-6} carbocycle and heterocycle;

each R is independently selected from the group consisting of C_{1-3} alkyl, C_{2-3} alkenyl, and H;

each R' is independently selected from the group consisting of C_{1-18} alkyl, C_{2-18} alkenyl, -R*YR", -YR", and H;

each R" is independently selected from the group consisting of C_{3-14} alkyl and C_{3-14} alkenyl;

each R* is independently selected from the group consisting of C_{1-12} alkyl and C_{2-12} alkenyl;

each Y is independently a C_{3-6} carbocycle;

each X is independently selected from the group consisting of F, Cl, Br, and I; and

m is selected from 5, 6, 7, 8, 9, 10, 11, 12, and 13,

or salts or isomers thereof.

In some embodiments, another subset of compounds of Formula (I) includes those in which

R_1 is selected from the group consisting of C_{5-30} alkyl, C_{5-20} alkenyl, -R*YR", -YR", and -R"M'R';

R_2 and R_3 are independently selected from the group consisting of H, C_{1-14} alkyl, C_{2-14} alkenyl, -R*YR", -YR", and -R*OR", or R_2 and R_3, together with the atom to which they are attached, form a heterocycle or carbocycle;

R_4 is selected from the group consisting of a C_{3-6} carbocycle, $-(CH_2)_nQ$, $-(CH_2)_nCHQR$, -CHQR, $-CQ(R)_2$, and unsubstituted C_{1-6} alkyl, where Q is selected from a C_{3-6} carbocycle, a 5- to 14-membered heteroaryl having one or more heteroatoms selected from N, O, and S, -OR, $-O(CH_2)_nN(R)_2$, -C(O)OR, -OC(O)R, $-CX_3$, $-CX_2H$, $-CXH_2$, -CN, $-C(O)N(R)_2$, -N(R)C(O)R, $-N(R)S(O)_2R$, $-N(R)C(O)N(R)_2$, $-N(R)C(S)N(R)_2$, $-CRN(R)_2C(O)OR$, $-N(R)R_8$, $-O(CH_2)_nOR$, $-N(R)C(=NR_9)N(R)_2$, $-N(R)C(=CHR_9)N(R)_2$, $-OC(O)N(R)_2$, -N(R)C(O)OR, -N(OR)C(O)R, $-N(OR)S(O)_2R$, -N(OR)C(O)OR, $-N(OR)C(O)N(R)_2$, $-N(OR)C(S)N(R)_2$, $-N(OR)C(=NR_9)N(R)_2$, $-N(OR)C(=CHR_9)N(R)_2$, $-C(=NR_9)R$, -C(O)N(R)OR, and $-C(=NR_9)N(R)_2$, and each n is independently selected from 1, 2, 3, 4, and 5;

each R_5 is independently selected from the group consisting of C_{1-3} alkyl, C_{2-3} alkenyl, and H;

each R_6 is independently selected from the group consisting of C_{1-3} alkyl, C_{2-3} alkenyl, and H;

M and M' are independently selected from -C(O)O-, -OC(O)-, -C(O)N(R')-, -N(R')C(O)-, -C(O)-, -C(S)-, -C(S)S-, -SC(S)-, -CH(OH)-, -P(O)(OR')O-, $-S(O)_2-$, -S-S-, an aryl group, and a heteroaryl group;

R_7 is selected from the group consisting of C_{1-3} alkyl, C_{2-3} alkenyl, and H;

R_8 is selected from the group consisting of C_{3-6} carbocycle and heterocycle;

R_9 is selected from the group consisting of H, CN, NO_2, C_{1-6} alkyl, -OR, $-S(O)_2R$, $-S(O)_2N(R)_2$, C_{2-6} alkenyl, C_{3-6} carbocycle and heterocycle;

each R is independently selected from the group consisting of C_{1-3} alkyl, C_{2-3} alkenyl, and H;

each R' is independently selected from the group consisting of C_{1-18} alkyl, C_{2-18} alkenyl, -R*YR", -YR", and H;

each R" is independently selected from the group consisting of C_{3-14} alkyl and C_{3-14} alkenyl;

each R* is independently selected from the group consisting of C_{1-12} alkyl and C_{2-12} alkenyl;

each Y is independently a C_{3-6} carbocycle;

each X is independently selected from the group consisting of F, Cl, Br, and I; and

m is selected from 5, 6, 7, 8, 9, 10, 11, 12, and 13,

or salts or isomers thereof.

In some embodiments, another subset of compounds of Formula (I) includes those in which

R_1 is selected from the group consisting of C_{5-30} alkyl, C_{5-20} alkenyl, -R*YR", -YR", and -R"M'R';

R_2 and R_3 are independently selected from the group consisting of H, C_{2-14} alkyl, C_{2-14} alkenyl, -R*YR", -YR", and -R*OR", or R_2 and R_3, together with the atom to which they are attached, form a heterocycle or carbocycle;

R_4 is $-(CH_2)_nQ$ or $-(CH_2)_nCHQR$, where Q is $-N(R)_2$, and n is selected from 3, 4, and 5;

each R_5 is independently selected from the group consisting of C_{1-3} alkyl, C_{2-3} alkenyl, and H;

each R_6 is independently selected from the group consisting of C_{1-3} alkyl, C_{2-3} alkenyl, and H;

M and M' are independently selected from -C(O)O-, -OC(O)-, -C(O)N(R')-, -N(R')C(O)-, -C(O)-, -C(S)-, -C(S)S-, -SC(S)-, -CH(OH)-, -P(O)(OR')O-, $-S(O)_2-$, -S-S-, an aryl group, and a heteroaryl group;

R_7 is selected from the group consisting of C_{1-3} alkyl, C_{2-3} alkenyl, and H;

each R is independently selected from the group consisting of C_{1-3} alkyl, C_{2-3} alkenyl, and H;

each R' is independently selected from the group consisting of C_{1-18} alkyl, C_{2-18} alkenyl, -R*YR", -YR", and H;

each R" is independently selected from the group consisting of C_{3-14} alkyl and C_{3-14} alkenyl;

each R* is independently selected from the group consisting of C_{1-12} alkyl and C_{1-12} alkenyl;

each Y is independently a C_{3-6} carbocycle;

each X is independently selected from the group consisting of F, Cl, Br, and I; and

m is selected from 5, 6, 7, 8, 9, 10, 11, 12, and 13,

or salts or isomers thereof.

In some embodiments, another subset of compounds of Formula (I) includes those in which

R_1 is selected from the group consisting of C_{5-30} alkyl, C_{5-20} alkenyl, -R*YR", -YR", and -R"M'R';

R_2 and R_3 are independently selected from the group consisting of C_{1-14} alkyl, C_{2-14} alkenyl, -R*YR", -YR", and -R*OR", or R_2 and R_3, together with the atom to which they are attached, form a heterocycle or carbocycle;

R_4 is selected from the group consisting of $-(CH_2)_nQ$, $-(CH_2)_nCHQR$, -CHQR, and $-CQ(R)_2$, where Q is $-N(R)_2$, and n is selected from 1, 2, 3, 4, and 5;

each R_5 is independently selected from the group consisting of C_{1-3} alkyl, C_{2-3} alkenyl, and H;

each R_6 is independently selected from the group consisting of C_{1-3} alkyl, C_{2-3} alkenyl, and H;

M and M' are independently selected from -C(O)O-, -OC(O)-, -C(O)N(R')-, -N(R')C(O)-, -C(O)-, -C(S)-, -C(S)S-, -SC(S)-, -CH(OH)-, -P(O)(OR')O-, $-S(O)_2-$, -S-S-, an aryl group, and a heteroaryl group;

R_7 is selected from the group consisting of C_{1-3} alkyl, C_{2-3} alkenyl, and H;

each R is independently selected from the group consisting of C_{1-3} alkyl, C_{2-3} alkenyl, and H;

each R' is independently selected from the group consisting of C_{1-18} alkyl, C_{2-18} alkenyl, -R*YR", -YR", and H;

each R" is independently selected from the group consisting of C_{3-14} alkyl and C_{3-14} alkenyl;

each R* is independently selected from the group consisting of C_{1-12} alkyl and C_{1-12} alkenyl;

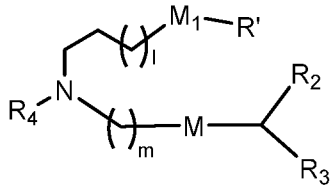
each Y is independently a C_{3-6} carbocycle;

each X is independently selected from the group consisting of F, Cl, Br, and I; and

m is selected from 5, 6, 7, 8, 9, 10, 11, 12, and 13,

or salts or isomers thereof.

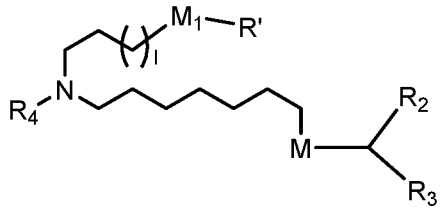
In some embodiments, a subset of compounds of Formula (I) includes those of Formula (IA):



(IA),

or a salt or isomer thereof, wherein l is selected from 1, 2, 3, 4, and 5; m is selected from 5, 6, 7, 8, and 9; M_1 is a bond or M'; R_4 is unsubstituted C_{1-3} alkyl, or $-(CH_2)_nQ$, in which Q is OH, $-NHC(S)N(R)_2$, $-NHC(O)N(R)_2$, $-N(R)C(O)R$, $-N(R)S(O)_2R$, $-N(R)R_8$, $-NHC(=NR_9)N(R)_2$, $-NHC(=CHR_9)N(R)_2$, $-OC(O)N(R)_2$, $-N(R)C(O)OR$, heteroaryl or heterocycloalkyl; M and M' are independently selected from -C(O)O-, -OC(O)-, -C(O)N(R')-, -P(O)(OR')O-, -S-S-, an aryl group, and a heteroaryl group; and R_2 and R_3 are independently selected from the group consisting of H, C_{1-14} alkyl, and C_{2-14} alkenyl.

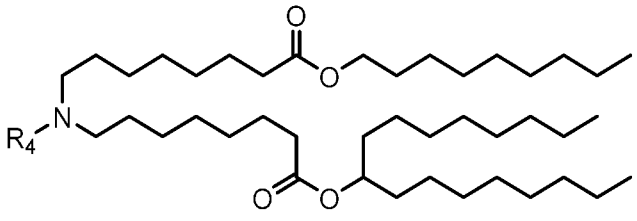
In some embodiments, a subset of compounds of Formula (I) includes those of Formula (II):

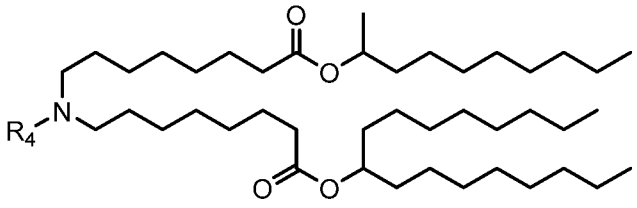


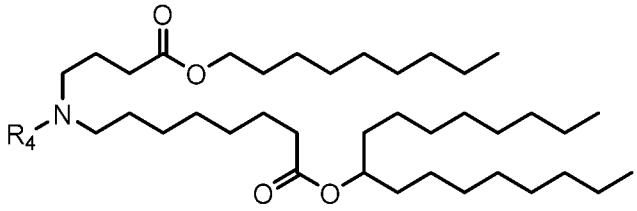
(II) or a salt or isomer thereof,

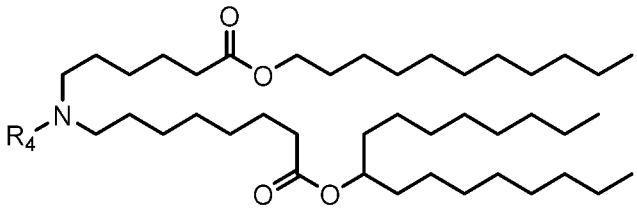
wherein l is selected from 1, 2, 3, 4, and 5; M_1 is a bond or M'; R_4 is unsubstituted C_{1-3} alkyl, or $-(CH_2)_nQ$, in which n is 2, 3, or 4, and Q is OH, $-NHC(S)N(R)_2$, $-NHC(O)N(R)_2$, $-N(R)C(O)R$, $-N(R)S(O)_2R$, $-N(R)R_8$, $-NHC(=NR_9)N(R)_2$, $-NHC(=CHR_9)N(R)_2$, $-OC(O)N(R)_2$, $-N(R)C(O)OR$, heteroaryl or heterocycloalkyl; M and M' are independently selected from -C(O)O-, -OC(O)-, -C(O)N(R')-, -P(O)(OR')O-, -S-S-, an aryl group, and a heteroaryl group; and R_2 and R_3 are independently selected from the group consisting of H, C_{1-14} alkyl, and C_{2-14} alkenyl.

In some embodiments, a subset of compounds of Formula (I) includes those of Formula (IIa), (IIb), (IIc), or (IIe):


(IIa),

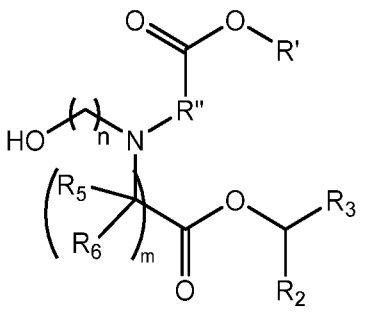

(IIb),


(IIc), or


(IIe),

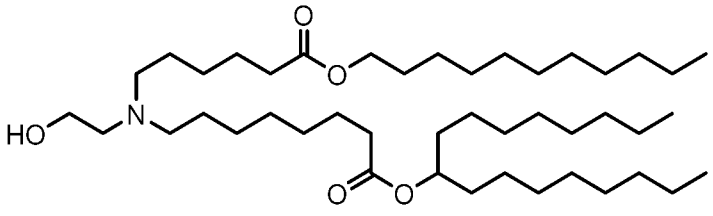
or a salt or isomer thereof, wherein R_4 is as described herein.

In some embodiments, a subset of compounds of Formula (I) includes those of Formula (IId):


(IId),

or a salt or isomer thereof, wherein n is 2, 3, or 4; and m, R', R", and R_2 through R_6 are as described herein. For example, each of R_2 and R_3 may be independently selected from the group consisting of C_{5-14} alkyl and C_{5-14} alkenyl.

In some embodiments, an ionizable cationic lipid of the disclosure comprises a compound having structure:



(Compound 1).

In some embodiments, a non-cationic lipid of the disclosure comprises 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), 1,2-dilinoleoyl-sn-glycero-3-phosphocholine (DLPC), 1,2-dimyristoyl-sn-gly cero-phosphocholine (DMPC), 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), 1,2-diundecanoyl-sn-glycero-phosphocholine (DUPC), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), 1,2-di-O-octadecenyl-sn-glycero-3-phosphocholine (18:0 Diether PC), 1-oleoyl-2 cholesterylhemisuccinoyl-sn-glycero-3-phosphocholine (OChemsPC), 1-hexadecyl-sn-glycero-3-phosphocholine (C16 Lyso PC), 1,2-dilinolenoyl-sn-glycero-3-phosphocholine,1,2-diarachidonoyl-sn-glycero-3-phosphocholine, 1,2-didocosahexaenoyl-sn-glycero-3-phosphocholine, 1,2-diphytanoyl-sn-glycero-3-phosphoethanolamine (ME 16.0 PE), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine, 1,2-dilinoleoyl-sn-glycero-3-phosphoethanolamine, 1,2-dilinolenoyl-sn-glycero-3-phosphoethanolamine, 1,2-diarachidonoyl-sn-glycero-3-phosphoethanolamine, 1,2-didocosahexaenoyl-sn-glycero-3-phosphoethanolamine, 1,2-dioleoyl-sn-glycero-3-phospho-rac-(1-glycerol) sodium salt (DOPG), sphingomyelin, and mixtures thereof.

In some embodiments, a PEG modified lipid of the disclosure comprises a PEG-modified phosphatidylethanolamine, a PEG-modified phosphatidic acid, a PEG-modified ceramide, a PEG-modified dialkylamine, a PEG-modified diacylglycerol, a PEG-modified dialkylglycerol, and mixtures thereof. In some embodiments, the PEG-modified lipid is PEG-DMG, PEG-c-DOMG (also referred to as PEG-DOMG), PEG-DSG and/or PEG-DPG.

In some embodiments, a sterol of the disclosure comprises cholesterol, fecosterol, sitosterol, ergosterol, campesterol, stigmasterol, brassicasterol, tomatidine, ursolic acid, alpha-tocopherol, and mixtures thereof.

In some embodiments, a LNP of the disclosure comprises an ionizable amino lipid of Compound 1, wherein the non-cationic lipid is DSPC, the structural lipid is cholesterol, and the PEG lipid is PEG-DMG.

In some embodiments, a LNP of the disclosure comprises an N:P ratio of from about 2:1 to about 30:1.

In some embodiments, a LNP of the disclosure comprises an N:P ratio of about 6:1.

In some embodiments, a LNP of the disclosure comprises an N:P ratio of about 3:1.

In some embodiments, a LNP of the disclosure comprises a wt/wt ratio of the ionizable cationic lipid component to the RNA of from about 10:1 to about 100:1.

In some embodiments, a LNP of the disclosure comprises a wt/wt ratio of the ionizable cationic lipid component to the RNA of about 20:1.

In some embodiments, a LNP of the disclosure comprises a wt/wt ratio of the ionizable cationic lipid component to the RNA of about 10:1.

In some embodiments, a LNP of the disclosure has a mean diameter from about 50 nm to about 150 nm.

In some embodiments, a LNP of the disclosure has a mean diameter from about 70 nm to about 120 nm.

In one embodiment, the lipid may be a cleavable lipid such as those described in International Publication No. WO2012170889, herein incorporated by reference in its entirety for this purpose. In one embodiment, the lipid may be synthesized by methods known in the art and/or as described in International Publication Nos. WO2013086354; the contents of which is herein incorporated by reference in its entirety for this purpose.

Nanoparticle compositions can be characterized by a variety of methods. For example, microscopy (*e.g.,* transmission electron microscopy or scanning electron microscopy) can be used to examine the morphology and size distribution of a nanoparticle composition. Dynamic light scattering or potentiometry (*e.g.,* potentiometric titrations) can be used to measure zeta potentials. Dynamic light scattering can also be utilized to determine particle sizes. Instruments such as the Zetasizer Nano ZS (Malvern Instruments Ltd, Malvern, Worcestershire, UK) can also be used to measure multiple characteristics of a nanoparticle composition, such as particle size, polydispersity index, and zeta potential.

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The size of the nanoparticles can help counter biological reactions such as, but not limited to, inflammation, or can increase the biological effect of the polynucleotide. As used herein, "size" or "mean size" in the context of nanoparticle compositions refers to the mean diameter of a nanoparticle composition.

Kits

Kits for accomplishing these methods are also provided in other aspects of the disclosure. The kit includes a container housing a formulation, a container housing a vaccine formulation, and instructions for adding a cancer vaccine to the vaccine formulation to produce a cancer vaccine formulation, mixing the cancer vaccine formulation within 24 hours of administration to a subject. In some embodiments the kit includes a mRNA having an open reading frame encoding 3-200 (*e.g.*, 3-130) cancer antigens.

The articles include pharmaceutical or diagnostic grade compounds of the disclosure in one or more containers. The article may include instructions or labels promoting or describing the use of the compounds of the disclosure.

As used herein, "promoted" includes all methods of doing business including methods of education, hospital and other clinical instruction, pharmaceutical industry activity including pharmaceutical sales, and any advertising or other promotional activity including written, oral and electronic communication of any form, associated with compositions of the disclosure in connection with treatment of cancer.

"Instructions" can define a component of promotion, and typically involve written instructions on or associated with packaging of compositions of the disclosure. Instructions also can include any oral or electronic instructions provided in any manner.

Thus the agents described herein may, in some embodiments, be assembled into pharmaceutical or diagnostic or research kits to facilitate their use in therapeutic, diagnostic or research applications. A kit may include one or more containers housing the components of the disclosure and instructions for use. Specifically, such kits may include one or more agents described herein, along with instructions describing the intended therapeutic application and the proper administration of these agents. In certain embodiments agents in a kit may be in a pharmaceutical formulation and dosage suitable for a particular application and for a method of administration of the agents.

The kit may be designed to facilitate use of the methods described herein by physicians and can take many forms. Each of the compositions of the kit, where applicable, may be provided in liquid form (*e.g.*, in solution), or in solid form (*e.g.*, a dry powder). In

certain cases, some of the compositions may be constitutable or otherwise processable (*e.g.,* to an active form), for example, by the addition of a suitable solvent or other species (for example, water or a cell culture medium), which may or may not be provided with the kit. As used herein, "instructions" can define a component of instruction and/or promotion, and typically involve written instructions on or associated with packaging of the disclosure. Instructions also can include any oral or electronic instructions provided in any manner such that a user will clearly recognize that the instructions are to be associated with the kit, for example, audiovisual (*e.g.,* videotape, DVD, etc.), Internet, and/or web-based communications, etc. The written instructions may be in a form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals or biological products, which instructions can also reflects approval by the agency of manufacture, use or sale for human administration.

In certain aspects, the disclosure relates to kits for preparing a nucleic acid cancer vaccine (*e.g.*, an RNA cancer vaccine) by IVT methods. In personalized cancer vaccines, it is important to identify patient specific mutations and vaccinate the patient with one or more neoepitopes. In such vaccines, the antigen(s) encoded by the ORFs of such a nucleic acid will be specific to the patient. The 5′- and 3′-ends of nucleic acids (*e.g.*, RNAs) encoding the antigen(s) may be more broadly applicable, as they include untranslated regions and stabilizing regions that are common to many nucleic acids (*e.g.*, RNAs). Among other things, the present disclosure provides kits that include one or parts of a chimeric nucleic acid, such as one or more 5′- and/or 3′-regions of RNA, which may be combined with an ORF encoding a patient-specific epitope. For example, a kit may include a nucleic acid containing one or more of a 5′-ORF, a 3′-ORF, and a poly-A tail. In some embodiments, each nucleic acid component is in an individual container. In other embodiments, more than one nucleic acid component is present together in a single container. In some embodiments, the kit includes a ligase enzyme. In some embodiments, provided kits include instructions for use. In some embodiments, the instructions include an instruction to ligate the peptide epitope encoding ORF to one or more other components from the kit, *e.g.,* 5′-ORF, a 3′-ORF, and/or a poly-A tail.

The kit may contain any one or more of the components described herein in one or more containers. As an example, in one embodiment, the kit may include instructions for mixing one or more components of the kit and/or isolating and mixing a sample and applying to a subject. The kit may include a container housing agents described herein. The agents may be prepared sterilely, packaged in syringe and shipped refrigerated. Alternatively it may

be housed in a vial or other container for storage. A second container may have other agents prepared sterilely. Alternatively the kit may include the active agents premixed and shipped in a syringe, vial, tube, or other container.

The kit may have a variety of forms, such as a blister pouch, a shrink wrapped pouch, a vacuum sealable pouch, a sealable thermoformed tray, or a similar pouch or tray form, with the accessories loosely packed within the pouch, one or more tubes, containers, a box or a bag. The kit may be sterilized after the accessories are added, thereby allowing the individual accessories in the container to be otherwise unwrapped. The kits can be sterilized using any appropriate sterilization techniques, such as radiation sterilization, heat sterilization, or other sterilization methods known in the art. The kit may also include other components, depending on the specific application, for example, containers, cell media, salts, buffers, reagents, syringes, needles, a fabric, such as gauze, for applying or removing a disinfecting agent, disposable gloves, a support for the agents prior to administration etc.

The compositions of the kit may be provided as any suitable form, for example, as liquid solutions or as dried powders. When the composition provided is a dry powder, the powder may be reconstituted by the addition of a suitable solvent, which may also be provided. In embodiments where liquid forms of the composition are sued, the liquid form may be concentrated or ready to use. The solvent will depend on the compound and the mode of use or administration. Suitable solvents for drug compositions are well known and are available in the literature. The solvent will depend on the compound and the mode of use or administration.

The kits, in one set of embodiments, may comprise a carrier means being compartmentalized to receive in close confinement one or more container means such as vials, tubes, and the like, each of the container means comprising one of the separate elements to be used in the method. For example, one of the containers may comprise a positive control for an assay. Additionally, the kit may include containers for other components, for example, buffers useful in the assay.

The present disclosure also encompasses a finished packaged and labeled pharmaceutical product. This article of manufacture includes the appropriate unit dosage form in an appropriate vessel or container such as a glass vial or other container that is hermetically sealed. In the case of dosage forms suitable for parenteral administration the active ingredient is sterile and suitable for administration as a particulate free solution. In other words, the disclosure encompasses both parenteral solutions and lyophilized powders, each being sterile, and the latter being suitable for reconstitution prior to injection.

Alternatively, the unit dosage form may be a solid suitable for oral, transdermal, topical or mucosal delivery.

In a preferred embodiment, the unit dosage form is suitable for intravenous, intramuscular or subcutaneous delivery. Thus, the disclosure encompasses solutions, preferably sterile, suitable for each delivery route.

In another preferred embodiment, compositions of the disclosure are stored in containers with biocompatible detergents, including but not limited to, lecithin, taurocholic acid, and cholesterol; or with other proteins, including but not limited to, gamma globulins and serum albumins. More preferably, compositions of the disclosure are stored with human serum albumins for human uses, and stored with bovine serum albumins for veterinary uses.

As with any pharmaceutical product, the packaging material and container are designed to protect the stability of the product during storage and shipment. Further, the products of the disclosure include instructions for use or other informational material that advise the physician, technician or patient on how to appropriately prevent or treat the disease or disorder in question. In other words, the article of manufacture includes instruction means indicating or suggesting a dosing regimen including, but not limited to, actual doses, monitoring procedures (such as methods for monitoring mean absolute lymphocyte counts, tumor cell counts, and tumor size) and other monitoring information.

More specifically, the disclosure provides an article of manufacture comprising packaging material, such as a box, bottle, tube, vial, container, sprayer, insufflator, intravenous (i.v.) bag, envelope and the like; and at least one unit dosage form of a pharmaceutical agent contained within said packaging material. The disclosure also provides an article of manufacture comprising packaging material, such as a box, bottle, tube, vial, container, sprayer, insufflator, intravenous (i.v.) bag, envelope and the like; and at least one unit dosage form of each pharmaceutical agent contained within said packaging material. The disclosure further provides an article of manufacture comprising packaging material, such as a box, bottle, tube, vial, container, sprayer, insufflator, intravenous (i.v.) bag, envelope and the like; and at least one unit dosage form of each pharmaceutical agent contained within said packaging material. The disclosure further provides an article of manufacture comprising a needle or syringe, preferably packaged in sterile form, for injection of the formulation, and/or a packaged alcohol pad.

Relative amounts of the active ingredient (*e.g.*, the nucleic acid cancer vaccine), the pharmaceutically acceptable excipient, and/or any additional ingredients in a vaccine composition may vary, depending upon the identity, size, and/or condition of the subject

being treated and further depending upon the route by which the composition is to be administered. For example, the composition may comprise between 0.1% and 99% (w/w) of the active ingredient. By way of example, the composition may comprise between 0.1% and 100%, *e.g.,* between .5 and 50%, between 1-30%, between 5-80%, at least 80% (w/w) active ingredient.

In some embodiments, the package containing the pharmaceutical product contains 0.1 mg to 1 mg of nucleic acid (*e.g.*, mRNA). In some embodiments, the package containing the pharmaceutical product contains 0.35 mg of nucleic acid (*e.g.*, mRNA). In some embodiments, the concentration of the nucleic acid (*e.g.*, mRNA) is 1 mg/mL.

In some embodiments, the nucleic acid (*e.g.*, mRNA) vaccine compositions may be administered at dosage levels sufficient to deliver 0.0001 mg/kg to 100 mg/kg, 0.001 mg/kg to 0.05 mg/kg, 0.005 mg/kg to 0.05 mg/kg, 0.001 mg/kg to 0.005 mg/kg, 0.05 mg/kg to 0.5 mg/kg, 0.01 mg/kg to 50 mg/kg, 0.1 mg/kg to 40 mg/kg, 0.5 mg/kg to 30 mg/kg, 0.01 mg/kg to 10 mg/kg, 0.1 mg/kg to 10 mg/kg, or 1 mg/kg to 25 mg/kg, of subject body weight per day, one or more times a day, per week, per month, etc., to obtain the desired therapeutic, diagnostic, prophylactic, or imaging effect (see *e.g.,* the range of unit doses described in International Publication No. WO2013078199, herein incorporated by reference in its entirety). In some embodiments, the nucleic acid (*e.g.*, mRNA) vaccine is administered at a dosage level sufficient to deliver 0.0100 mg, 0.025 mg, 0.050 mg, 0.075 mg, 0.100 mg, 0.125 mg, 0.150 mg, 0.175 mg, 0.200 mg, 0.225 mg, 0.250 mg, 0.275 mg, 0.300 mg, 0.325 mg, 0.350 mg, 0.375 mg, 0.400 mg, 0.425 mg, 0.450 mg, 0.475 mg, 0.500 mg, 0.525 mg, 0.550 mg, 0.575 mg, 0.600 mg, 0.625 mg, 0.650 mg, 0.675 mg, 0.700 mg, 0.725 mg, 0.750 mg, 0.775 mg, 0.800 mg, 0.825 mg, 0.850 mg, 0.875 mg, 0.900 mg, 0.925 mg, 0.950 mg, 0.975 mg, or 1.0 mg. In some embodiments, the nucleic acid (*e.g.*, mRNA) vaccine is administered at a dosage level sufficient to deliver between 10 µg and 400 µg of the mRNA vaccine to the subject. In some embodiments, the nucleic acid (*e.g.*, mRNA) vaccine is administered at a dosage level sufficient to deliver 0.033mg, 0.1 mg, 0.2 mg, or 0.4 mg to the subject.

The desired dosage may be delivered three times a day, two times a day, once a day, every other day, every third day, every week, every two weeks, every three weeks, every four weeks, every 2 months, every three months, every 6 months, etc. In certain embodiments, the desired dosage may be delivered using multiple administrations (*e.g.,* two, three, four, five, six, seven, eight, nine, ten, eleven, twelve, thirteen, fourteen, or more administrations). When multiple administrations are employed, split dosing regimens such as those described herein may be used. In some embodiments, the nucleic acid (*e.g.*, mRNA) vaccine compositions

may be administered at dosage levels sufficient to deliver 0.0005 mg/kg to 0.01 mg/kg, *e.g.*, about 0.0005 mg/kg to about 0.0075 mg/kg, *e.g.*, about 0.0005 mg/kg, about 0.001 mg/kg, about 0.002 mg/kg, about 0.003 mg/kg, about 0.004 mg/kg or about 0.005 mg/kg. In some embodiments, the nucleic acid (*e.g.*, mRNA) vaccine compositions may be administered once or twice (or more) at dosage levels sufficient to deliver 0.025 mg/kg to 0.250 mg/kg, 0.025 mg/kg to 0.500 mg/kg, 0.025 mg/kg to 0.750 mg/kg, or 0.025 mg/kg to 1.0 mg/kg.

In some embodiments, the nucleic acid (*e.g.*, mRNA) vaccine compositions may be administered twice (*e.g.*, Day 0 and Day 7, Day 0 and Day 14, Day 0 and Day 21, Day 0 and Day 28, Day 0 and Day 60, Day 0 and Day 90, Day 0 and Day 120, Day 0 and Day 150, Day 0 and Day 180, Day 0 and 3 months later, Day 0 and 6 months later, Day 0 and 9 months later, Day 0 and 12 months later, Day 0 and 18 months later, Day 0 and 2 years later, Day 0 and 5 years later, or Day 0 and 10 years later) at a total dose of or at dosage levels sufficient to deliver a total dose of 0.0100 mg, 0.025 mg, 0.050 mg, 0.075 mg, 0.100 mg, 0.125 mg, 0.150 mg, 0.175 mg, 0.200 mg, 0.225 mg, 0.250 mg, 0.275 mg, 0.300 mg, 0.325 mg, 0.350 mg, 0.375 mg, 0.400 mg, 0.425 mg, 0.450 mg, 0.475 mg, 0.500 mg, 0.525 mg, 0.550 mg, 0.575 mg, 0.600 mg, 0.625 mg, 0.650 mg, 0.675 mg, 0.700 mg, 0.725 mg, 0.750 mg, 0.775 mg, 0.800 mg, 0.825 mg, 0.850 mg, 0.875 mg, 0.900 mg, 0.925 mg, 0.950 mg, 0.975 mg, or 1.0 mg. Higher and lower dosages and frequency of administration are encompassed by the present disclosure. For example, a nucleic acid (*e.g.*, mRNA) vaccine composition may be administered three or four times, or more. In some embodiments, the mRNA vaccine composition is administered once a day every three weeks.

In some embodiments, the nucleic acid (*e.g.*, mRNA) vaccine compositions may be administered twice (*e.g.*, Day 0 and Day 7, Day 0 and Day 14, Day 0 and Day 21, Day 0 and Day 28, Day 0 and Day 60, Day 0 and Day 90, Day 0 and Day 120, Day 0 and Day 150, Day 0 and Day 180, Day 0 and 3 months later, Day 0 and 6 months later, Day 0 and 9 months later, Day 0 and 12 months later, Day 0 and 18 months later, Day 0 and 2 years later, Day 0 and 5 years later, or Day 0 and 10 years later) at a total dose of or at dosage levels sufficient to deliver a total dose of 0.010 mg, 0.025 mg, 0.100 mg or 0.400 mg.

In some embodiments the nucleic acid (*e.g.*, mRNA) vaccine for use in a method of vaccinating a subject is administered the subject a single dosage of between 10 μg/kg and 400 μg/kg of the nucleic acid vaccine in an effective amount to vaccinate the subject. In some embodiments the RNA vaccine for use in a method of vaccinating a subject is administered the subject a single dosage of between 10 μg and 400 μg of the nucleic acid vaccine in an effective amount to vaccinate the subject.

The methods and compositions described herein are not limited in its application to the details of construction and the arrangement of components set forth in the following description. The methods and compositions described herein are capable of other embodiments and of being practiced or of being carried out in various ways. Also, the phraseology and terminology used herein is for the purpose of description and should not be regarded as limiting. The use of "including," "comprising," or "having," "containing," "involving," and variations thereof herein, is meant to encompass the items listed thereafter and equivalents thereof as well as additional items.

EXAMPLES

Example 1. Manufacture of Polynucleotides

According to the present disclosure, the manufacture of nucleic acids and/or parts or regions thereof may be accomplished utilizing the methods taught in the art including those detailed in International Application WO2014/152027 entitled "Manufacturing Methods for Production of RNA Transcripts", the contents of which is incorporated herein by reference in its entirety for this purpose.

Purification methods may include those taught in International Application Nos.: WO2014/152030 and WO2014/152031, each of which is incorporated herein by reference in its entirety for this purpose.

Detection and characterization methods for use with the nucleic acids may be performed using any methods known in the art including those taught in WO2014/144039, which is incorporated herein by reference in its entirety for this purpose.

Characterization of the polynucleotides of the disclosure may be accomplished using, for example, a procedure selected from the group consisting of polynucleotide mapping, reverse transcriptase sequencing, charge distribution analysis, and detection of RNA impurities, wherein characterizing comprises determining the RNA transcript sequence, determining the purity of the RNA transcript, or determining the charge heterogeneity of the RNA transcript. Such methods are taught in, for example, WO2014/144711 and WO2014/144767, the contents of each of which is incorporated herein by reference in its entirety for this purpose.

Example 2 Chimeric polynucleotide synthesis

Introduction

According to the present disclosure, two regions or parts of a chimeric nucleic acid may be joined or ligated using triphosphate chemistry.

According to this method, a first region or part of 100 nucleotides or less is chemically synthesized with a 5′ monophosphate and terminal 3′desOH or blocked OH. If the region is longer than 80 nucleotides, it may be synthesized as two strands for ligation.

If the first region or part is synthesized as a non-positionally modified region or part using in vitro transcription (IVT), conversion the 5′ monophosphate with subsequent capping of the 3′ terminus may follow.

Monophosphate protecting groups may be selected from any of those known in the art.

The second region or part of the chimeric polynucleotide may be synthesized using either chemical synthesis or IVT methods. IVT methods may include an RNA polymerase that can utilize a primer with a modified cap. Alternatively, a cap of up to 130 nucleotides may be chemically synthesized and coupled to the IVT region or part.

The entire chimeric polynucleotide need not be manufactured with a phosphate-sugar backbone. If one of the regions or parts encodes a polypeptide, then it is preferable that such region or part comprise a phosphate-sugar backbone.

Ligation is then performed using any known click chemistry, orthoclick chemistry, solulink, or other bioconjugate chemistries known to those in the art.

Synthetic route

The chimeric nucleic acid is made using a series of starting segments. Such segments include:

(a) Capped and protected 5′ segment comprising a normal 3′ OH (SEG. 1)

(b) 5′ triphosphate segment which may include the coding region of a polypeptide and comprising a normal 3′ OH (SEG. 2)

(c) 5′ monophosphate segment for the 3′ end of the chimeric polynucleotide (*e.g.,* the tail) comprising cordycepin or no 3′ OH (SEG. 3)

After synthesis (chemical or IVT), segment 3 (SEG. 3) is treated with cordycepin and then with pyrophosphatase to create the 5′ monophosphate.

Segment 2 (SEG. 2) is then ligated to SEG. 3 using RNA ligase. The ligated polynucleotide is then purified and treated with pyrophosphatase to cleave the diphosphate.

The treated SEG.2-SEG. 3 construct is then purified and SEG. 1 is ligated to the 5′ terminus. A further purification step of the chimeric polynucleotide may be performed.

The yields of each step may be as much as 90-95%.

Example 3: PCR for cDNA Production

PCR procedures for the preparation of cDNA are performed using 2x KAPA HIFI™ HotStart ReadyMix by Kapa Biosystems (Woburn, MA). This system includes 2x KAPA ReadyMix12.5 µl; Forward Primer (10 µM) 0.75 µl; Reverse Primer (10 µM) 0.75 µl; Template cDNA -100 ng; and dH_20 diluted to 25.0 µl. The reaction conditions are at 95° C for 5 min. and 25 cycles of 98° C for 20 sec, then 58° C for 15 sec, then 72° C for 45 sec, then 72° C for 5 min., then 4° C to termination.

The reaction is cleaned up using Invitrogen's PURELINK™ PCR Micro Kit (Carlsbad, CA) per manufacturer's instructions (up to 5 µg). Larger reactions will require a cleanup using a product with a larger capacity. Following the cleanup, the cDNA is quantified using the NANODROP™ and analyzed by agarose gel electrophoresis to confirm the cDNA is the expected size. The cDNA is then submitted for sequencing analysis before proceeding to the *in vitro* transcription reaction.

Example 4. *In vitro* Transcription (IVT)

The *in vitro* transcription reaction generates nucleic acids containing uniformly modified nucleic acids. Such uniformly modified nucleic acids may comprise a region or part of the nucleic acids of the disclosure. The input nucleotide triphosphate (NTP) mix is made in-house using natural and un-natural NTPs.

A typical *in vitro* transcription reaction includes the following:

1 Template cDNA 1.0 µg
2 10x transcription buffer (400 mM Tris-HCl pH 8.0, 190 mM $MgCl_2$, 50 mM DTT, 10 mM Spermidine) 2.0 µl
3 Custom NTPs (25mM each) 7.2 µl
4 RNase Inhibitor 20 U
5 T7 RNA polymerase 3000 U
6 dH_20 Up to 20.0 µl. and
7 Incubation at 37° C for 3 hr-5 hrs.

The crude IVT mix may be stored at 4° C overnight for cleanup the next day. 1 U of RNase-free DNase is then used to digest the original template. After 15 minutes of

incubation at 37° C, the mRNA is purified using Ambion's MEGACLEAR™ Kit (Austin, TX) following the manufacturer's instructions. This kit can purify up to 500 µg of RNA. Following the cleanup, the RNA is quantified using the NanoDrop and analyzed by agarose gel electrophoresis to confirm the RNA is the proper size and that no degradation of the RNA has occurred.

Example 5: *In vivo* Study of Construct and Flank Length

An *in vivo* immunogenicity study was performed to examine the effects of vaccines with different numbers of epitopes and flank lengths. The studies were performed using three constructs, as shown in the table below. The murine vaccines encode predicted neoepitopes (single nucleotide variants) present in the mouse colon (MC38) tumor line as determined by a bioinformatics algorithm. MC38S-1a contains 15 class I and 5 class II epitopes, MC38S-2b contains 26 class I and 8 class II epitopes, and MC38S-3b contains 30 class I and 10 class II epitopes. In the table below three different vaccines were made, in 1a – all the epitopes are surrounded by flanking amino acids for total length of 31 amino acids, for 2b the epitope was surrounded by amino acids to total 25 amino acids for epitope + flanks and then for 3b the epitope was surrounded by amino acids for total length of each epitope + flanks equaling 21 aa. The epitope may vary slightly in length depending on the MHC molecule it is predicted to bind to, but total length was adjusted in this example to account for this slight change to keep the total length at 31, 25 or 21.

mRNA	**MC38S-1a**	**MC38S-2b**	**MC38S-3b**
Epitope number	20	34	40
Flank length	31	25	21
Total nt	1993	2680	2662

Mice were dosed on day 1 (d1; prime) and on day 8 (d8; boost) with 3 µg or 10µg of the test mRNA vaccine. Splenocytes were harvested on day 15 for ELIspot analysis. Briefly, 400,000 cells per well were incubated with 1 µg/mL peptide for 16-18 hours and then IFNγ spot forming units (SFUs) were counted. Minimal peptides corresponding to the epitopes contained in all three vaccines were used for restimulation. A statistical comparison of the different groups is shown in the tables below:

		MC38S-1a		MC38S-2b		MC38S-3b	
Dose		**3 ug**	**10 ug**	**3 ug**	**10 ug**	**3 ug**	**10 ug**
Class I	**8**	2.90	203.60	5.40	79.60	1.90	2.60
	10	1298.4***	2000***	1123.3***	1640***	274.4***	1131.5***
	12	11.50	2.10	77.40	231.23	4.10	152.90
	13	18.30	2.00	89.60	135.60	1.20	10.00
	15	4.70	13.30	1.20	16.50	3.10	0.40
	19	10.40	26.70	1.00	49.40	1.80	5.90
Class II	**37**	8.90	4.50	0.90	8.20	2.50	0.60

Restimulations

		MC38S-1a		MC38S-2b		MC38S-3b	
Dose		**3 ug**	**10 ug**	**3 ug**	**10 ug**	**3 ug**	**10 ug**
Class I	**8**	1.30	58.40	0.80	64.10	3.10	0.30
	10	1394.7*	1232.2***	1034.7*	1589.9***	537.6*	347.6***
	12	211.90	148.50	211.60	422.6**	14.10	3.8**
	13	3.30	4.40	1.00	129.70	0.70	0.60
	15						
	19	1.80	2.40	0.90	54.80	2.00	1.40
Class II	**37**	13.50	24.40	1.20	1.90	0.70	6.30

Note: * = all significant vs. each other; ** = 34mer vs. 40 mer; *** = (20mer and 34mer) vs. 40mer

As shown in FIGs. 4A-4C, a comparable immune response to class I epitopes was detected between the 20mer/31 flank and the 34mer/25 flank vaccines, but not the 30mer/21 flank at both the 3 μg and the 10 μg doses. The 34mer construct demonstrated the only detected response for some of the restimulations.

Example 6: Epitope Selection

The mRNA epitope selection process may involve the following:

1) Neoantigen Prediction steps generate a list of mutation-derived peptides specifically expressed in the tumor and not in normal tissues and select a subset of neoantigens with the highest likelihood to generate a robust, tumor-specific T-cell response based on their predicted ability to be presented by the patient's HLA molecules and their abundance and frequency in the tumor transcriptome.

2) Selfness Analysis may be used to minimize the risk of molecular mimicry between neoantigens and other sequences in the patient's genome by excluding peptides that match others potentially expressed in the patient's normal tissues. Neoantigens are arranged in the concatemer to minimize the creation of pseudo-epitopes at neoantigen junctions.

3) Vaccine Design involves designing the selected neoantigens into a concatemeric construct that generates nucleic acid sequences optimized for ease of synthesis.

Neoantigen Prediction

The core algorithms for neoantigen prediction and selection determine the mRNA abundance and frequency of the variant and its predicted binding to the patient's HLA targets. Peptides are generated by mapping the location of somatic DNA variants to the amino acid (AA) sequences from the high-confidence human genome annotation, GENCODE. RNA-Seq data is used to support mutation calls at the level of single nucleotide variants and to determine the variant frequency in the genome and transcriptome.

The majority of neoantigens in mRNA may consist of a peptide with a single mutated AA in the center with 12 flanking AA's at the C- and N-termini, leading to a length of 25 amino acids per neoantigen (75 nucleotides in an mRNA sequence). Indels which have multiple mutated AAs will consist of an AA sequence 25 AA long that contains at least 1 or more mutant AA up to the entire 25mer being mutant AA. In cases where a mutation occurs < 12 AA away from a protein terminus the peptide and corresponding nucleotide length may be shorter. In some embodiments a preferred peptide length will be 13 AA, which will be rare based on extensive analysis of mutanomes across all tumor types.

Several features relevant to anti-tumor T-cell responses are evaluated for each neoantigen, including the following: 1) confidence in the variant call from WES and RNA-Seq data; 2) mRNA transcript abundance from RNA-Seq data; 3) variant allele frequency from WES and RNA-Seq data; 4) predicted HLA binding affinity from NetMHCpan and NetMHCIIpan.

The HLA allotypes of the patient may be targeted since they present neoantigens to the patient's T-cells. HLA genes are the most polymorphic in the human genome and codominant expression leads to most individuals being heterozygous at some loci. HLA-A, -B and -C loci encode for Class I allotypes and HLA-DR, DP and DQ encode for Class II allotypes. More weight may be assigned in some embodiments to predicted binders of HLA-A, -B and DR (core targets), and lower (although non-zero) weight to other HLA allotypes of the patient (supplementary targets). Nearly all individuals have at least one HLA-A, -B and DR functional allotype (i.e. core MHC alleles) and these are the restricting elements for ~90% of all known human epitopes (FIG. 5). HLA-C-restricted or alloreactive T-cells are rarely observed and HLA-C's cell surface expression is 10% of that seen for HLA-A and B. The remaining supplementary targets encode for class II molecules and individuals can be null for genes encoding them. Moreover, 4-digit precision typing of these supplementary Class II targets is often ambiguous even when using state-of-the-art NGS and other sequence-

based typing methods. If the NGS-based allele typing for either core or supplemental HLA targets is ambiguous, the allele(s) may not be considered when ranking neoantigens.

Selfness Check

A selfness check of each neoantigen may be performed. A patient-specific set of transcripts are created using protein-coding transcript amino acid sequences from a reference human genome annotation, by tailoring the sequences to the patient's own set of germline protein-coding variants. This patient-specific exome (excluding the gene containing the neoantigen) may be used to check each HLA class I binding neoantigen epitope (8- to 11-mer) for 100% exact self-matches. Any neoantigen identified as 100% self-matches elsewhere in the genome and/or transcriptome using this tool may be excluded from the mRNA construct.

Neoantigen Selection

All variants that are not excluded by the selfness check may be evaluated for inclusion in the patient-specific mRNA construct design. Pre-defined weights may be used rather than hard filters based on the knowledge that MHC binding predictions are imperfect and RNA-Seq sensitivity may be limited by tumor content of the biopsy and depth of sequencing.

In some embodiments each mRNA construct may be designed to have up to 34 neoantigens (with peptides of up to 25 amino acids/ 75 nucleotides in length) or an optional range of 13 to 34 neoantigens. This range corresponds to 1,235 – 2,924 nucleotides for the mRNA sequence length. In an exemplary embodiment of a construct comprising 34 neoantigens, the composition may be determined by first selecting the top 29 HLA Class I neoantigens and then the top 5 HLA Class II neoantigens. If a particular neoantigen is selected as both a Class I and II neoantigen it may be counted as one of the 5 Class II neoantigens. The resulting neoantigen slot created by these dual Class I and II predicted binders is automatically filled with the next highest scoring Class I neoantigen.

Low Mutation Burden Tumors

Given the inherent variability of tumor mutanomes, rare cases of tumors with low mutational burden may be treated with the cancer vaccines described herein. In these embodiments it may be desirable for fewer than 34 neoantigens to be used to create an individual mRNA construct. For instance as few as 7 tumor neoantigens may be used. For cases where less than an optimal 34 antigens but greater than or equal to 13 neoantigens are identified, a construct can be generated in which each neoantigen will be included once in the

mRNA construct. In the embodiments where less than 13 neoantigens are found in a tumor mutanome, neoantigens may be duplicated to meet the desirable 13 neoantigen slot.

Pseudoepitopes

Neoantigens may be ordered in the concatemer to minimize the creation pseudo-epitopes at their junctions. Alternatively a spacer such as a single amino acid spacer may be used to disrupt the epitope and reduce the predicted HLA binding affinity.

Population and End-to-End Tests

NGS was performed on 15 tumor and blood samples obtained from several biobanking repositories. The samples were from a variety of tumor types in different formats (e.g. formalin fixed paraffin embedded [FFPE] and fresh frozen). The methods described herein were executed on the NGS data for each of these representative samples, as part of the complete qualification protocol. In addition, a test was performed using 4 related tumor samples. Three tumor lines and a primary tumor sample derived from a single patient were subjected to WES and RNA-Seq and the results were analyzed.

When the four independent outputs were compared, strong concordance was observed between the variants called, the neoantigen ranking and those selected for inclusion in the vaccines (FIGs. 7A-7D). Differences were found, but were explained by divergence of the lines propagated in vitro from the primary tumor and each other. Out of 369 variants identified across the four samples 90.5% were common to all samples. Using raw neoantigen scores, there were strong correlations between all lines compared to the tumor and when the scores diverged substantially it was due to lack of RNA-Seq data in a line or the tumor. When neoantigens were selected independently from each tumor sample by the analysis methods described herein 34 were common for all 4 vaccine designs, and 5 more were common in 3 vaccine designs. Overall the analysis shows that the NGS process, variant calling and the mRNA analysis system are robust, reproducible and generate reasonable outputs.

EQUIVALENTS

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the disclosure described herein. Such equivalents are intended to be encompassed by the following claims.

All references, including patent documents, disclosed herein are incorporated by reference in their entirety.

What is claimed is:

CLAIMS

1. A nucleic acid cancer vaccine, comprising:

one or more nucleic acids each having one or more open reading frames encoding 3-130 peptide epitopes,

wherein each of the peptide epitopes are portions of personalized cancer antigens or portions of cancer hotspot antigens, and

wherein at least two of the peptide epitopes have different lengths.

2. The nucleic acid cancer vaccine of claim 1, wherein 1-34 of the peptide epitopes are portions of cancer hotspot antigens.

3. The nucleic acid cancer vaccine of claim 1, wherein 5-34 of the peptide epitopes are portions of cancer hotspot antigens.

4. The nucleic acid cancer vaccine of any one of claims 1-3, wherein the cancer hotspot antigens comprise a KRAS G12 mutation or a KRAS G13 mutation or both mutations.

5. The nucleic acid cancer vaccine of any one of claims 1-4, wherein the portions of the cancer hotspot neoantigens comprises at least one of the following mutations: a KRAS G12 mutation, a KRAS G13 mutation, a NRAS Q61 mutation, a BRAF V600 mutation, a PIK3CA R88 mutation, a PIK3CA E545 mutation, a PIK3CA H1047 mutation, a TP53 R175 mutation, a TP53 R282 mutation, an EGFR L858 mutation, a FGFR3 S249 mutation, an ERBB2 S310 mutation, a PTEN R130 mutation, and a BCOR N1459 mutation.

6. The nucleic acid cancer vaccine of claim 1, wherein the length of each peptide epitope is determined such that the anti-cancer efficacy of the nucleic acid cancer vaccine has a maximal T-cell activation value based on the length of the one or more nucleic acids.

7. The nucleic acid cancer vaccine of claim 1, wherein the length of each peptide epitope is determined such that the anti-cancer efficacy of the nucleic acid cancer vaccine has a maximal survival value based on the length of the one or more nucleic acids.

8. The nucleic acid cancer vaccine of any one of claims 1-7, wherein the minimum length of any peptide epitope is 8-13 amino acids.

9. The nucleic acid cancer vaccine of any one of claims 1-8, wherein the maximum length of any peptide epitope is 31-35 amino acids.

10. The nucleic acid cancer vaccine of any one of claims 1-9, wherein the cancer vaccine is a DNA cancer vaccine.

11. The nucleic acid cancer vaccine of any one of claims 1-9, wherein the cancer vaccine is an RNA cancer vaccine.

12. The nucleic acid cancer vaccine of claim 11, wherein the cancer vaccine is an mRNA cancer vaccine, and wherein the one or more nucleic acids are mRNA.

13. The nucleic acid cancer vaccine of claim 12, wherein the one or more mRNA each comprise a 5′ UTR and/or a 3′ UTR.

14. The nucleic acid cancer vaccine of claim 12 or claim 13, wherein the one or more mRNA each comprise a poly-A tail.

15. The nucleic acid cancer vaccine of claim 14, wherein the poly-A tail comprises about 100 nucleotides.

16. The nucleic acid cancer vaccine of any one of claims 12-15, wherein the one or more mRNA each comprise a cap structure or a modified cap structure.

17. The nucleic acid cancer vaccine of claim 16, wherein the cap structure or the modified cap structure is a 5′ cap structure, a 5′ cap-0 structure, a 5′ cap-1 structure, or a 5′ cap-2 structure.

18. The nucleic acid cancer vaccine of any one of claims 12-17, wherein the one or more mRNA comprise at least one chemical modification.

19. The nucleic acid cancer vaccine of claim 18, wherein the chemical modification is selected from the group consisting of pseudouridine, N1-methylpseudouridine, N1-ethylpseudouridine, 2-thiouridine, 4′-thiouridine, 5-methylcytosine, 2-thio-1-methyl-1-deaza-pseudouridine, 2-thio-1-methyl-pseudouridine, 2-thio-5-aza-uridine, 2-thio-dihydropseudouridine, 2-thio-dihydrouridine, 2-thio-pseudouridine, 4-methoxy-2-thio-pseudouridine, 4-methoxy-pseudouridine, 4-thio-1-methyl-pseudouridine, 4-thio-pseudouridine, 5-aza-uridine, dihydropseudouridine, 5-methyluridine, 5-methyluridine, 5-methoxyuridine, and 2′-O-methyl uridine.

20. The nucleic acid cancer vaccine of claim 18 or claim 19, wherein the one or more mRNA is fully modified.

21. The nucleic acid cancer vaccine of any one of claims 1-20, wherein the one or more nucleic acids encode 34 peptide epitopes, 5-10 peptide epitopes, 10-20 peptide epitopes, 20-30 peptide epitopes, 30-40 peptide epitopes, 40-50 peptide epitopes, 50-60 peptide epitopes, 60-70 peptide epitopes, 70-80 peptide epitopes, 80-90 peptide epitopes, 90-100 peptide epitopes, 100-110 peptide epitopes, 110-120 peptide epitopes, or 120-130 peptide epitopes.

22. The nucleic acid cancer vaccine of any one of claims 1-21, wherein each of the peptide epitopes is encoded by a separate open reading frame.

23. The nucleic acid cancer vaccine of any one of claims 1-22, wherein the peptide epitopes are in the form of a concatemeric cancer antigen comprised of 5-130 peptide epitopes.

24. The nucleic acid cancer vaccine of any one of claims 1-23, wherein one or more of the following conditions are met:

a) the 5-130 peptide epitopes are interspersed by cleavage sensitive sites; and/or

b) each peptide epitope is linked directly to one another without a linker; and/or

c) each peptide epitope is linked to one another with a single amino acid linker; and/or

d) each peptide epitope is linked to one another with a short peptide linker; and/or

e) each peptide epitope comprises 8-35 amino acids and includes one or more SNP mutations; and/or

f) each peptide epitope comprises 8-35 amino acids and includes a mutation causing a unique expressed peptide sequence; and/or

g) none of the peptide epitopes have a highest affinity for class II MHC molecules from a subject; and/or

h) the nucleic acid encoding the peptide epitopes is arranged such that the peptide epitopes are ordered to minimize pseudo-epitopes; and/or

i) the ratio of class I MHC molecule peptide epitopes to class II MHC molecule peptide epitopes is at least 1:1, 2:1, 3:1, 4:1, or 5:1; and/or

j) no class II MHC molecule peptide epitopes are present; and/or

k) at least 30% of the peptide epitopes have a highest affinity for class I MHC molecules and/or class II MHC class molecules from a subject; and/or

l) at least 50% of the peptide epitopes have a probability percent rank greater than 0.5% for HLA-A, HLA-B, and/or DRB1; and/or

m) wherein the open reading frames encodes 34 peptide epitopes and wherein 29 epitopes are MHC class I epitopes and 5 epitopes are MHC class II or MHC class I and II epitopes.

25. The nucleic acid cancer vaccine of any one of claims 1-24, wherein at least one of the peptide epitopes is a predicted T cell reactive epitope.

26. The nucleic acid cancer vaccine of any one of claims 1-25, wherein at least one of the peptide epitopes is a predicted B cell reactive epitope.

27. The nucleic acid cancer vaccine of any one of claims 1-26, wherein the peptide epitopes comprise a combination of predicted T cell reactive epitopes and predicted B cell reactive epitopes.

28. The nucleic acid cancer vaccine of any one of claims 1-27, wherein the peptide epitopes are predicted T cell reactive epitopes and/or predicted B cell reactive epitopes.

29. The nucleic acid cancer vaccine of any one of claims 1-26, wherein at least one of the peptide epitopes is a predicted neoepitope.

30. The nucleic acid cancer vaccine of any one of claims 1-27, wherein at least one nucleic acid has an open reading frame encoding at least a fragment of one or more traditional cancer antigens or one or more cancer/testis antigens.

31. The nucleic acid cancer vaccine of any one of claims 1-30, wherein each nucleic acid is formulated in a lipid nanoparticle.

32. The nucleic acid cancer vaccine of claim 31, wherein each nucleic acid is formulated in a different lipid nanoparticle.

33. The nucleic acid cancer vaccine of claim 31, wherein each nucleic acid is formulated in the same lipid nanoparticle.

34. The nucleic acid cancer vaccine of any one of claims 1-33, wherein the total length of the one or more nucleic acids encodes a total protein length of 50-100 amino acids, 100-200 amino acids, 200-300 amino acids, 300-400 amino acids, 400-500 amino acids, 500-600 amino acids, 600-700 amino acids, 700-800 amino acids, 800-900 amino acids, 900-1000 amino acids, 1000-1100 amino acids, or 1100-1200 amino acids.

35. The nucleic acid cancer vaccine of any one of claims 1-34, wherein the anti-cancer efficacy is calculated at least in part based on one or more factors selected from the group consisting of gene expression, RNA Seq, transcript abundance, DNA allele frequency, amino acid conservation, physiochemical similarity, oncogene, predicted binding affinity to a specific HLA allele, clonality, binding efficiency and presence in an indel.

36. The nucleic acid cancer vaccine of claim 35, wherein the one or more factors are inputted into a statistical model.

37. A nucleic acid cancer vaccine, comprising:

one or more nucleic acids each having one or more open reading frames encoding 5-130 peptide epitopes,

wherein each of the peptide epitopes are portions of personalized cancer antigens or portions of cancer hotspot antigens, and

wherein each peptide epitope has an equal length.

38. A method of making a cancer vaccine comprising:

a) identifying between 1-34 cancer hotspots;

b) identifying between 5-130 personalized cancer antigens for a patient;

c) determining the anti-tumor efficacy of at least two peptide epitopes for each of the 5-130 personalized cancer antigens; and

d) preparing a cancer vaccine in which the total anti-cancer efficacy of the cancer vaccine is maximized for a given total length of the cancer vaccine and wherein the vaccine comprises portions of 1-34 cancer hotspot neoantigens.

39. A method for treating a patient having cancer, comprising:

a) analyzing a sample derived from a patient in order to identify one or more personalized cancer antigens;

b) determining the anti-tumor efficacy of at least two peptide epitopes for each of the identified personalized cancer antigens;

c) preparing a cancer vaccine in which the total anti-cancer efficacy of the cancer vaccine is maximized for a given total length of the cancer vaccine, wherein the cancer vaccine further comprises portions of 1-34 cancer hotspot antigens; and

d) administering the cancer vaccine to the patient.

40. The method of claim 38 or claim 39, wherein the portions of 1-34 cancer hotspot neoantigens comprises at least one of the following mutations: a KRAS G12 mutation, a KRAS G13 mutation, a NRAS Q61 mutation, a BRAF V600 mutation, a PIK3CA R88 mutation, a PIK3CA E545 mutation, a PIK3CA H1047 mutation, a TP53 R175 mutation, a TP53 R282 mutation, an EGFR L858 mutation, a FGFR3 S249 mutation, an ERBB2 S310 mutation, a PTEN R130 mutation, and a BCOR N1459 mutation.

41. The method of claim 38 or claim 39, wherein the portions of 1-34 cancer hotspot neoantigens comprise a KRAS G12 mutation or a KRAS G13 mutation or both mutations.

42. The method of claim 38 or claim 39, wherein the cancer vaccine is a nucleic acid cancer vaccine comprising one or more nucleic acids each having one or more open reading frames.

43. The method of any one of claims 38-42, wherein the cancer vaccine is a DNA cancer vaccine.

44. The method of any one of claims 38-43, wherein the cancer vaccine is an RNA cancer vaccine.

45. The method of claim 44, wherein the cancer vaccine is an mRNA cancer vaccine.

46. The method of claim 38 or claim 39, wherein the cancer vaccine is a peptide cancer vaccine.

47. The method of any one of claims 39-46, wherein the cancer vaccine is administered at a dosage level sufficient to deliver between 0.02-1.0 mg of the cancer vaccine to the subject.

48. The method of claim 47, wherein the cancer vaccine is administered to the subject twice, three times, four times, or more.

49. The method of any one of claims 39-48, wherein the cancer vaccine is administered by intradermal, intramuscular, intravascular, intratumoral, and/or subcutaneous administration.

50. The method of claim 49, wherein the cancer vaccine is administered by intramuscular administration.

51. The method of any one of claims 39-50, wherein the cancer is selected from the group consisting of non-small cell lung cancer (NSCLC), small cell lung cancer, melanoma, bladder urothelial carcinoma, HPV-negative head and neck squamous cell carcinoma (HNSCC), a solid malignancy that is microsatellite high (MSI H) / mismatch repair (MMR) deficient, renal cancer, gastric cancer, and tumor mutational burden high tumors.

52. The method of claim 51, wherein the NSCLC lacks an EGFR sensitizing mutation and/or an ALK translocation.

53. The method of claim 51, wherein the solid malignancy that is microsatellite high (MSI H) / mismatch repair (MMR) deficient is selected from the group consisting of colorectal cancer, stomach adenocarcinoma, esophageal adenocarcinoma, and endometrial cancer.

54. The method of any one of claims 45-53, wherein the one or more mRNA each comprise a 5′ UTR and/or a 3′ UTR.

55. The method of any one of claims 45-54, wherein the one or more mRNA each comprise a poly-A tail.

56. The method of claim 55, wherein the poly-A tail comprises about 100 nucleotides.

57. The method of any one of claims 45-56, wherein the one or more mRNA each comprise a cap structure or a modified cap structure.

58. The nucleic acid cancer vaccine of claim 57, wherein the cap structure or the modified cap structure is a 5′ cap structure, a 5′ cap-0 structure, a 5′ cap-1 structure, or a 5′ cap-2 structure.

59. The method of any one of claims 45-58, wherein the one or more mRNA comprise at least one chemical modification.

60. The method of claim 59, wherein the chemical modification is selected from the group consisting of pseudouridine, N1-methylpseudouridine, N1-ethylpseudouridine, 2-thiouridine, 4′-thiouridine, 5-methylcytosine, 2-thio-1-methyl-1-deaza-pseudouridine, 2-thio-1-methyl-pseudouridine, 2-thio-5-aza-uridine, 2-thio-dihydropseudouridine, 2-thio-dihydrouridine, 2-thio-pseudouridine, 4-methoxy-2-thio-pseudouridine, 4-methoxy-pseudouridine, 4-thio-1-methyl-pseudouridine, 4-thio-pseudouridine, 5-aza-uridine, dihydropseudouridine, 5-methyluridine, 5-methyluridine, 5-methoxyuridine, and 2′-O-methyl uridine.

61. The method of claim 59 or claim 60, wherein the one or more mRNA is fully modified.

62. The method of any one of claims 42-45, wherein the one or more nucleic acids encode 5-10 peptide epitopes, 10-20 peptide epitopes, 20-30 peptide epitopes, 30-40 peptide epitopes, 40-50 peptide epitopes, 50-60 peptide epitopes, 60-70 peptide epitopes, 70-80 peptide epitopes, 80-90 peptide epitopes, 90-100 peptide epitopes, 100-110 peptide epitopes, 110-120 peptide epitopes, or 120-130 peptide epitopes.

63. The method of any one of claims 38-62, wherein each of the peptide epitopes is encoded by a separate open reading frame.

64. The method of any one of claims 38-63, wherein the peptide epitopes are in the form of a concatemeric cancer antigen comprised of 5-130 peptide epitopes.

65. The method of any one of claims 38-64, wherein one or more of the following conditions are met:

a) the 5-130 peptide epitopes are interspersed by cleavage sensitive sites; and/or

b) each peptide epitope is linked directly to one another without a linker; and/or

c) each peptide epitope is linked to one or another with a single amino acid linker; and/or

d) each peptide epitope is linked to one another with a short linker; and/or

e) each peptide epitope comprises 8-35 amino acids and includes one or more SNP mutations; and/or

f) each peptide epitope comprises 8-35 amino acids and includes a mutation causing a unique expressed peptide sequence; and/or

g) none of the peptide epitopes have a highest affinity for class II MHC molecules from a subject; and/or

h) the nucleic acid encoding the peptide epitopes is arranged such that the peptide epitopes are ordered to minimize pseudo-epitopes; and/or

i) the ratio of class I MHC molecule peptide epitopes to class II MHC molecule peptide epitopes is at least 1:1, 2:1, 3:1, 4:1, or 5:1; and/or

j) no class II MHC molecule peptide epitopes are present; and/or

k) at least 30% of the peptide epitopes have a highest affinity for class I MHC molecules and/or class II MHC class molecules from a subject; and/or

l) at least 50% of the peptide epitopes have a probability percent rank greater than 0.5% for HLA-A, HLA-B, and/or DRB1, and/or

m) wherein the open reading frames encodes 34 peptide epitopes and wherein 29 epitopes are MHC class I epitopes and 5 epitopes are MHC class II or MHC class I and II epitopes.

66. The method of any one of claims 38-65, wherein at least one of the peptide epitopes is a predicted T cell reactive epitope.

67. The method of any one of claims 38-66, wherein at least one of the peptide epitopes is a predicted B cell reactive epitope.

68. The method of any one of claims 38-67, wherein the peptide epitopes comprise a combination of predicted T cell reactive epitopes and predicted B cell reactive epitopes.

69. The method of any one of claims 38-67, wherein the peptide epitopes are predicted T cell reactive epitopes and/or predicted B cell reactive epitopes.

70. The method of any one of claims 38-69, wherein at least one of the peptide epitopes is a predicted neoepitope.

71. The method of any one of claims 42-45 or 62-69, wherein at least one nucleic acid has an open reading frame encoding at least a fragment of one or more traditional cancer antigens or one or more cancer/testis antigens.

72. The method of any one of claims 42-45 or 62-71, wherein each nucleic acid is formulated in a lipid nanoparticle.

73. The method of claim 72, wherein each nucleic acid is formulated in a different lipid nanoparticle.

74. The method of claim 72, wherein each nucleic acid is formulated in the same lipid nanoparticle.

75. The method of any one of claims 42-45 or 62-74, wherein the total length of the one or more nucleic acids encodes a total protein length of 50-100 amino acids, 100-200 amino acids, 200-300 amino acids, 300-400 amino acids, 400-500 amino acids, 500-600 amino acids, 600-700 amino acids, 700-800 amino acids, 800-900 amino acids, 900-1000 amino acids, 1000-1100 amino acids, or 1100-1200 amino acids.

76. The method of any one of claims 38-75, wherein the anti-cancer efficacy is calculated at least in part based on one or more factors selected from the group consisting of gene expression, RNA Seq, transcript abundance, DNA allele frequency, amino acid conservation, physiochemical similarity, oncogene, predicted binding affinity to a specific HLA allele, clonality, binding efficiency and presence in an indel.

77. The method of claim 76, wherein the one or more factors are inputted into a statistical model.

78. A computerized system for selecting nucleic acids to include in a nucleic acid cancer vaccine having a maximum length, the system comprising:

a communication interface configured to receive a plurality of sequences of nucleic acids encoding a plurality of peptide epitopes, wherein each of the peptide epitopes are portions of personalized cancer antigens; and

at least one computer processor programmed to:

for each of the plurality of peptide epitopes, calculate a score for each of a plurality of nucleic acids in the peptide, each of which includes at least one of the one or more peptide epitopes, wherein at least two of the nucleic acid sequences have different lengths; and

ranking based on the calculated scores, the plurality of nucleic acid sequences in the plurality of peptides; and

selecting based on the ranking and the maximum length of the vaccine, nucleic acid sequences for inclusion in the vaccine.

79. The computerized system of claim 78, wherein the minimum length of any peptide epitope is 8 amino acids.

80. The computerized system of claim 78 or claim 79, wherein the maximum length of any peptide epitope is 31 amino acids.

81. The computerized system of any one of claims 78-80, wherein the plurality of nucleic acids encode 5-10 peptide epitopes, 10-20 peptide epitopes, 20-30 peptide epitopes, 30-40 peptide epitopes, 34 epitopes, 40-50 peptide epitopes, 50-60 peptide epitopes, 60-70 peptide epitopes, 70-80 peptide epitopes, 80-90 peptide epitopes, 90-100 peptide epitopes, 100-110 peptide epitopes, 110-120 peptide epitopes, or 120-130 peptide epitopes.

82. The computerized system of any one of claims 78-81, wherein one or more of the following conditions are met:

a) each peptide epitope comprises 8-31 amino acids and includes one or more SNP mutations; and/or

b) each peptide epitope comprises 8-31 amino acids and includes a mutation causing a unique expressed peptide sequence; and/or

c) none of the peptide epitopes have a highest affinity for class II MHC molecules from a subject; and/or

d) the ratio of class I MHC molecule peptide epitopes to class II MHC molecule peptide epitopes is at least 1:1, 2:1, 3:1, 4:1, or 5:1; and/or

e) no class II MHC molecule peptide epitopes are present

f at least 30% of the peptide epitopes have a highest affinity for class I MHC molecules and/or class II MHC class molecules from a subject; and/or

g) at least 50% of the peptide epitopes have a probability percent rank greater than 0.5% for HLA-A, HLA-B, and/or DRB1.

83. The computerized system of any one of claims 78-82, wherein at least one of the peptide epitopes is a predicted T cell reactive epitope.

84. The computerized system of any one of claims 78-83, wherein at least one of the peptide epitopes is a predicted B cell reactive epitope.

85. The computerized system of any one of claims 78-84, wherein the peptide epitopes comprise a combination of predicted T cell reactive epitopes and predicted B cell reactive epitopes.

86. The computerized system of any one of claims 78-85, wherein the peptide epitopes are predicted T cell reactive epitopes and/or predicted B cell reactive epitopes.

87. The computerized system of any one of claims 78-86, wherein at least one of the peptide epitopes is a predicted neoepitope.

88. The computerized system of any one of claims 78-87, wherein at least one nucleic acid has an open reading frame encoding at least a fragment of one or more traditional cancer antigens or one or more cancer/testis antigens.

89. The computerized system of any one of claims 78-88, wherein the total length of the vaccine encodes a total protein length of 50-100 amino acids, 100-200 amino acids, 200-300 amino acids, 300-400 amino acids, 400-500 amino acids, 500-600 amino acids, 600-700 amino acids, 700-800 amino acids, 800-900 amino acids, 900-1000 amino acids, 1000-1100 amino acids, or 1100-1200 amino acids.

90. The computerized system of any one of claims 78-89, wherein the score is calculated at least in part based on one or more factors selected from the group consisting of gene expression, RNA Seq, transcript abundance, DNA allele frequency, amino acid conservation, physiochemical similarity, oncogene, predicted binding affinity to a specific HLA allele, clonality, binding efficiency and presence in an indel.

91. The computerized system of claim 90, wherein the one or more factors are input into a statistical model.