Description

Title of Invention: ALPHAVIRUS REPLICON PARTICLE

Technical Field

[0001] The present invention relates to an alphavirus replicon particle that can be used as a gene delivery system.

Background Art

[0002] Gene therapy is designed to introduce genetic material into cells to compensate for abnormal genes or to make a beneficial protein. If a mutated gene causes a necessary protein to be faulty or missing, gene therapy may be able to introduce a normal copy of the gene to restore the function of the protein.

[0003] Gene therapy is an emerging field in medical and pharmaceutical sciences because of its potential in treating chronic diseases like cancer, viral infections, myocardial infarctions, and genetic disorders, etc.

[0004] A gene that is inserted directly into a cell usually does not function. Instead, a carrier called a vector is genetically engineered to deliver the gene. Certain viruses are often used as vectors because they can deliver the new gene by infecting the cell. The viruses are modified so they can't cause disease when used in people. Some types of virus, such as retroviruses, integrate their genetic material (including the new gene) into a chromosome in the human cell. Other viruses, such as adenoviruses, introduce their gene into the nucleus of the cell, but the gene is not integrated into the chromosome.

[0005] The vector can be injected directly into a specific tissue where it is taken up by individual cells or given intravenously (by IV)in the body. Alternately, a sample of the patient's cells can be removed and exposed to the vector in a laboratory setting. The cells containing the vector are then returned to the patient. If the treatment is successful, the new gene delivered by the vector will make a functioning protein. (https://ghr.nlm.nih.gov/primer/therapy/procedures, Int J Pharm Investig. 2013 Jan-Mar; 3(1): 1-7.)

[0006] Alphaviruses comprise a set of genetically, structurally, and serologically related mosquito-borne viruses of the Togaviridae family. The alphaviruses include Eastern Equine Encephalitis Virus (EEEV), Venezuelan Equine Encephalitis Virus (VEEV), Everglades Virus, Mucambo Virus, Pixuna Virus, Western Equine Encephalitis Virus (WEEV), Sindbis Virus, Semliki Forest Virus, Middleburg Virus, Chikungunya Virus (CHIKV), O'nyong-nyong Virus, Ross River Virus, Barmah Forest Virus, Getah Virus, Sagiyama Virus, Bebaru Virus, Mayaro Virus, Una Virus, Aura Virus, Whataroa Virus, Babanki Virus, Kyzylagach Virus, Highlands J virus, Fort Morgan Virus, Ndumu Virus, and Buggy Creek Virus. Structural subunits containing a single viral

protein, capsid, associate with the RNA genome in an icosahedral nucleocapsid. In the virion, the capsid is surrounded by a lipid envelope covered with a regular array of transmembrane protein spikes, each of which consists of a heterodimeric complex of two glycoproteins, E1 and E2.

[0007] An alphavirus replicon particle (ARP) is produced in cells or cultures and incorporates a "replicon" that can express non-alphavirus genes within the virion shell comprising alphavirus structural proteins and membrane lipid.

[0008] Alphavirus replicon particles are described in U.S. Pat. No. 7,045,335, WO 2004/085660 and Virology 239, 389-401, 1997. Processes for their manufacture are described in U.S. Pat. No. 7,078,218, the contents of the documents cited in this paragraph are incorporated by reference.

[0009] Clinical application of gene therapy is still limited because of lack of suitable methods for proper introduction of genes into cells and therefore, this is an area of interest for many researchers. To achieve successful gene therapy, development of proper gene delivery systems could be one of the most important factors (Int. J Pharm Investig. 2013 Jan-Mar; 3(1): 1-7).

Citation List

Patent Literature

[0010] PTL 1: US Patent No. 7,045,335
PTL 2: WO2004/085660
PTL 3: US Patent No. 7,078,218

Non Patent Literature

[0011] NPL 1: Int J Pharm Investig. 2013 Jan-Mar; 3(1): 1-7
NPL 2: Virology 239, 389-401, 1997

Summary of Invention

[0012] The present invention relates to a gene delivery system comprising an improved alphavirus replicon particle.

[0013] In one aspect, an alphavirus replicon particle (ARP), which comprises

(i) alphavirus structural proteins comprising capsid and/or envelope, and

(ii) an alphavirus replicon comprising a polynucleotide encoding alphavirus non-structural proteins nsp1, nsp2, nsp3 and nsp4 and at least one gene of interest

wherein at least one of capsid, E3 and E2 in the envelope comprise one or more amino acid alteration but E1 in the envelope comprises no amino acid alteration.

[0014] In one aspect, the invention provides an ARP, wherein the virus structural protein has one or more alterations in the alphavirus capsid protein Nuclear Localization Signal (NLS).

[0015] In one aspect, the alphavirus capsid protein is an EEEV, WEEV, VEEV, CHIKV,

Ross River virus, or Barmah Forest virus capsid protein. In various embodiments of the above aspects or any other aspect of the invention described herein, the one or more alterations is in an NLS at amino acids 67-70 of an EEEV capsid protein; at amino acids 67-70 of an WEEV capsid protein; at amino acids 64-68 of an VEEV capsid protein; at amino acids 62-69 of a CHIKV capsid protein; at amino acids 71-74 of a Ross River virus capsid protein; or at amino acids 64-68 of a Barmah Forest virus capsid protein.

[0016] In various embodiments of the above aspects or any other aspect of the invention described herein, the alteration is a substitution in a charged amino acid of the NLS or basic charged amino acid of the NLS. In some embodiments, the charged amino acid or basic charged amino acid is lysine or arginine. In certain embodiments, the lysine or arginine is substituted with a non-lysine or non-arginine amino acids. In specific embodiments, the lysine or arginine is substituted with asparagine or alanine.

[0017] In various embodiments of the above aspects or any other aspect of the invention described herein, the EEEV virus capsid protein NLS is altered at amino acid 67. In particular embodiments, the EEEV virus capsid protein NLS has a substitution K67N.

[0018] In various embodiments of the above aspects or any other aspect of the invention described herein, the WEEV virus capsid protein NLS is altered at one or more of amino acids 67, 68, and 69. In particular embodiments, the WEEV capsid protein NLS comprises K67N, K68N, and/or K69N.

[0019] In various embodiments of the above aspects or any other aspect of the invention described herein, the VEEV capsid protein NLS is altered at one or more of amino acids 64, 65, and 67. In particular embodiments, the VEEV virus capsid protein NLS comprises K64N, K65A or K65N, and/or K67A or K67N.

[0020] In various embodiments of the above aspects or any other aspect of the invention described herein, the Ross River virus capsid protein NLS is altered at one or more of amino acids 71, 72, 73, and 74. In particular embodiments, the Ross River virus capsid protein NLS comprises R71N, R72N, R73N, and/or R74N.

[0021] In various embodiments of the above aspects or any other aspect of the invention described herein, the Barmah Forest virus capsid protein NLS is altered at one or more of amino acids 64, 65, 67, and 68. In particular embodiments, the Barmah Forest virus capsid protein NLS comprises K64A, K65A or K65N, K67A, K67N, K68A and/or K68N.

[0022] Alteration in the capsid NLS is described in detail in US Patent Publication Nos. 2014-170186 or 2017-073377. The contents of these publications are herein incorporated by reference.

[0023] In one aspect, alphavirus E2 protein may have a non-lysine residue (e.g., asparagine) at the amino acid position corresponding to amino acid 234 in the CHIKV E2 protein

and/or a modification at the amino acid position corresponding to amino acid 251 in the CHIKV E2 protein that destabilizes the E2 protein during viral budding.

[0024] In one aspect, the alphavirus E3 protein may comprise an alteration/mutation in the amino acid sequence at the furin site (Arg-X-X-Arg).

[0025] The term "Arg-X-X-Arg" indicates the minimal cleavage site of furin and "X-X" includes any combination of two amino acids. Example of the alteration to the amino acid sequence at furin site includes the alteration to Ile-Glu/Asp-Gly-Arg, Asp-Asp-Asp-Asp-Lys or Ser-Gly-Gly-Gly-Ser. Details regarding furin site alteration are described in US Patent Publication Nos. 2016-0040134 and 2016-0200775 (the cited documents are herein incorporated by reference).

[0026] For example, VEEV CT83 strain has a furin site of RKRR at the end of its E3 region and RKRR may be replaced with SGGGS.

[0027] According to the present invention, the alphavirus replicon comprises nucleotide encoding alphavirus nonstructural proteins nsp1, nsp2, nsp3 and nsp4, and at least one gene of interest. The alphavirus nonstructural proteins may be those derived from the same alphavirus as the alphavirus from which the structural proteins are derived. The alphavirus nonstructural proteins may be those derived from an alphavirus different from the alphavirus from which the structural proteins are derived (chimeric alphavirus replicon particle).

For example, ARPs comprising the alphavirus structural proteins derived from CHIKV and alphavirus replicon comprising nucleotides encoding VEEV nsp1, nsp2, nsp3 and nsp4 and a gene of interest may be provided according to the present invention.

[0028] In one aspect, the present invention provides an alphavirus replicon particle (ARP), which comprises

(i) CHIKV structural proteins comprising capsid and/or envelope, and

(ii) a VEEV replicon comprising a polynucleotide encoding VEEV non-structural proteins nsp1, nsp2, nsp3 and nsp4 and at least one gene of interest.

[0029] The gene of interest may be chosen from a wide variety of sequences derived from any desired source, e.g., viruses, prokaryotes, eukaryotes, archaea. Examples of categories of gene of interest include, for example, immunogens, including antigenic proteins, cytokines, toxins, therapeutic proteins, enzymes, antisense sequences, and immune response modulators.

[0030] In another aspect, the invention provides a method for preparing alphavirus replicon particles, comprising the steps of

co-transfecting cells with

i) a vector comprising a polynucleotide encoding alphavirus non-structural protein nsp1, nsp2, nsp3 and nsp4, and at least one gene of interest,

ii) a vector comprising a polynucleotide encoding an alphavirus capsid protein, and
iii) a vector comprising a polynucleotide encoding an alphavirus E3-E2-6K-E1,
wherein at least one of the capsid, E3 and E2 comprises one or more amino acid alteration but E1 comprises no amino acid alteration,
culturing the transfected cells, and
purifying the ARPs from the cell culture.

[0031] In general, nucleotides encoding alphavirus structural proteins comprise those encoding E1, E2, 6k and E3. Upon expression of the wild type virus structural proteins, 6K and E3 are naturally cleaved during the process of assemble and removed from the ARPs. The mature wild type ARPs may comprise capsid, E1 and E2 proteins. When one or more alterations of the amino acid sequences are introduced in, for example, the furin site of E3 protein, E3 may not be cleaved and contained in the ARPs. In the present specification and claims, "viral structural proteins" refers not only those having 6k and/or E3 but also those not having 6K and/or E3.

[0032] Representative ARPs wherein the alphavirus is CHIKV or VEEV are exemplified by Figure 1.

[0033] In one aspect, the present invention provides a chimeric alphavirus replicon particle,
(i) CHIKV structural proteins comprising capsid and/or envelope, and
(ii) a VEEV replicon comprising a polynucleotide encoding VEEV non-structural proteins nsp1, nsp2, nsp3 and nsp4 and at least one gene of interest.

Brief Description of Drawings

[0034] [fig.1]Figure 1 shows a schematic protocol for producing ARPs.
[fig.2]Figure 2 shows the construction of the VEEV replicon particle.
[fig.3]Figure 3 shows VEEV_pBR322_NLuc vector.
[fig.4]Figure 4 shows TC83_CMV_CAwt_helper 1p vector.
[fig.5]Figure 5 shows TC83_CMV_CAmut_helper 1P vector.
[fig.6]Figure 6 shows TC83_CMV_WTgp_helper 2P vector
[fig.7]Figure 7 shows TC83_CMV_E3gp_helper 2P vector
[fig.8]Figure 8 shows schematic protocol for determining the packaging ability of VEEV replicon into VEE VRPs.
[fig.9]Figure 9 shows results of the test shown in Figure 8.
[fig.10]Figure 10 shows incorporation of multiple cloning site in the construction of the VEEV replicon particle.
[fig.11]Figure 11 shows VEEV_pBR322_MCS_NLuc_Putative vector.
[fig.12]Figure 12 shows result of western blotting of purified VEE virus replicon particles.
[fig.13]Figure 13 shows western blotting of the cells infected by VRPs comprising a

nucleotide encoding IKK. Lane 1, cells infected by VRPs, Lane 2, cells transfected by IKK plasmid vector (positive control).

[fig.14]Figure 14 shows western blotting of the cells infected by VRPs comprising a nucleotide encoding JNK2.

[fig.15]Figure 15 shows the results of luciferase assay of the cells infected with VRPs comprising a gene encoding luciferase.

[fig.16]Figure 16 shows the expression of GFP in the cells infected with VRPs comprising a gene encoding GFP.

[fig.17]Figure 17 shows the GFP expression in M2 polarized macrophage cells infected with VRPs comprising a gene encoding GFP.

[fig.18]Figure 18 shows schematic protocol of Example 9.

[fig.19]Figure 19 shows the results of Example 9.

[fig.20]Figure 20 shows western blotting of purified chimeric ARPs obtained from CHIKV structural proteins and VEEV replicon.

[fig.21]Figure 21 shows FACS analysis of the cells infected by the ARPs of Figure 20.

Description of Embodiments

[0035] Definitions

As used herein "alphavirus" is meant to refer to RNA-containing viruses that belong to the Togaviridae family of viruses. Exemplary Togaviridae viruses include but are not limited to Eastern Equine Encephalitis Virus (EEEV), Venezuelan Equine Encephalitis Virus (VEEV), Everglades Virus, Mucambo Virus, Pixuna Virus, Western Equine Encephalitis Virus (WEEV), Sindbis Virus, Semliki Forest Virus, Middleburg Virus, Chikungunya Virus (CHIKV), O'nyong-nyong Virus, Ross River Virus, Barmah Forest Virus, Getah Virus, Sagiyama Virus, Bebaru Virus, Mayaro Virus, Una Virus, Aura Virus, Whataroa Virus, Babanki Virus, Kyzylagach Virus, Highlands J virus, Fort Morgan Virus, Ndumu Virus, Buggy Creek Virus and Ockelbo virus.

[0036] By "alphavirus structural protein" is meant a polypeptide or fragment thereof having at least about 80% amino acid sequence identity to a naturally occurring viral capsid or envelope protein. In one embodiment, the alphavirus structural protein has at least about 85%, 90%, 95% or greater amino acid sequence identity with Eastern Equine Encephalitis Virus (EEEV), Venezuelan Equine Encephalitis Virus (VEEV), Everglades Virus, Mucambo Virus, Pixuna Virus, Western Equine Encephalitis Virus (WEEV), Sindbis Virus, Semliki Forest Virus, Middleburg Virus, Chikungunya Virus (CHIKV), O'nyong-nyong Virus, Ross River Virus, Barmah Forest Virus, Getah Virus, Sagiyama Virus, Bebaru Virus, Mayaro Virus, Una Virus, Aura Virus, Whataroa Virus, Babanki Virus, Kyzylagach Virus, Highlands J virus, Fort Morgan Virus, Ndumu Virus, and Buggy Creek Virus. Wild type amino acid sequences of alphavirus

structural proteins can be obtained from GenBank.

[0037] In specific embodiments, the alphavirus is a CHIKV, for example CHIKV strain 37997 or LR2006 OPY-1. In other embodiments, the alphavirus is a VEEV, for example VEEV strain TC-83.

[0038] By "an alphavirus replicon" is meant an RNA molecule which can direct its own amplification in vivo in a target cell. The replicon encodes the polymerase(s) which catalyze RNA amplification (nspl, nsp2, nsp3, nsp4) and contains cis RNA sequences required for replication which are recognized and utilized by the encoded polymerase(s). An alphavirus replicon typically contains the following ordered elements: 5' UTR, sequences which encode alphavirus nonstructural proteins (nspl, nsp2, nsp3, nsp4), 3' UTR, and a poly A signal. An alphavirus replicon also contains one or more viral sub-genomic promoters directing the expression of the gene of interest. Those sequences may have one or more mutations taught in a prior art.

[0039] By "alphavirus replicon particle" (ARP) is meant an alphavirus replicon packaged with alphavirus structural proteins. ARP does not contain polynucleotide encoding any of the alphavirus structural proteins.

[0040] By "agent" is meant any small molecule chemical compound, antibody, nucleic acid molecule, or polypeptide, or fragments thereof.

[0041] As used herein, the term "adjuvant" is meant to refer to a compound that, when used in combination with a specific immunogen in a formulation, will augment, alter or modify the resultant immune response. In certain embodiments, the adjuvant is used in combination with a ARP. Modification of the immune response includes intensification or broadening the specificity of either or both antibody and cellular immune responses. Modification of the immune response can also mean decreasing or suppressing certain antigen- specific immune responses. In one embodiment, the adjuvant is Ribi adjuvant.

[0042] By "ameliorate" is meant decrease, suppress, attenuate, diminish, arrest, or stabilize the development or progression of a disease or a symptom thereof.

[0043] By "alteration" is meant a change in an amino acid or nucleotide at a specified position with reference to a polypeptide sequence or polynucleotide sequence. As used herein, an alteration includes a substitution, deletion, or insertion of an amino acid or nucleotide at a specified position of a polypeptide or polynucleotide. In some embodiments, an alteration in an alphavirus capsid protein nuclear localization signal includes substitution of a charged amino acid (e.g., lysine or arginine) with an uncharged amino acid (e.g., alanine or asparagine, or any amino acid except a basic charged amino acid such as lysine or arginine).

[0044] By "alteration" is meant a change (increase or decrease) with reference to the expression levels or activity of a gene or polypeptide as detected by standard art known

methods, such as those described herein. As used herein, an alteration includes a 10%, 25%, 50%, 75%, 100% or greater change in expression levels. An alteration includes a 10-, 20-, 50-, 70-, 80-, 90-, 100-, 200-, 500-, 1000-fold or greater change in expression levels.

[0045] By "analog" is meant a molecule that is not identical, but has analogous functional or structural features. For example, a polypeptide analog retains the biological activity of a corresponding naturally-occurring polypeptide, while having certain biochemical modifications that enhance the analog's function relative to a naturally occurring polypeptide. Such biochemical modifications could increase the analog's protease resistance, membrane permeability, or half-life, without altering, for example, ligand binding. An analog may include an unnatural amino acid.

[0046] In this disclosure, "comprises," "comprising," "containing" and "having" and the like can have the meaning ascribed to them in U.S. Patent law and can mean " includes," "including," and the like; "consisting essentially of or "consists essentially" likewise has the meaning ascribed in U.S. Patent law and the term is open-ended, allowing for the presence of more than that which is recited so long as basic or novel characteristics of that which is recited is not changed by the presence of more than that which is recited, but excludes prior art embodiments.

[0047] "Detect" refers to identifying the presence, absence or amount of the analyte to be detected.

[0048] By "disease" is meant any condition or disorder that damages or interferes with the normal function of a cell, tissue, or organ.

[0049] By "effective amount" is meant the amount of an agent required to ameliorate the symptoms of a disease relative to an untreated patient. The effective amount of active compound(s) used to practice the present invention for prevention or treatment of a disease varies depending upon the manner of administration, the age, body weight, and general health of the subject. Ultimately, the attending physician or veterinarian will decide the appropriate amount and dosage regimen. Such amount is referred to as an "effective" amount.

[0050] By "fragment" is meant a portion of a polypeptide or nucleic acid molecule. This portion contains, preferably, at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, or 90% of the entire length of the reference nucleic acid molecule or polypeptide. A fragment may contain 10, 20, 30, 40, 50, 60, 70, 80, 90, or 100, 200, 300, 400, 500, 600, 700, 800, 900, or 1000 nucleotides or amino acids.

[0051] By "marker" is meant any protein or polynucleotide having an alteration in expression level or activity that is associated with a disease or disorder.

[0052] As used herein, "nuclear localization signal" or "NLS" is an amino acid sequence that, when present on the surface of a polypeptide, targets the polypeptide to the

nucleus of the cell. NLS sequences are known in the art. See, for example, Goldfarb, D., and N. Michaud (1991) Trends Cell Biol. 1, 20-24; Gorlich, D., and I. W. Mattaj (1996) Science 271, 1513-1518). In one embodiment, an NLS includes one or more short sequences of positively charged amino acids, such as lysines or arginines. Consensus sequences for NLS include K-K/R-X-K/R (Schneider, J. et al. (1988) Cell 54,117-125) and two clusters of basic amino acids, separated by a spacer of about 10 amino acids, e.g., KR[PAATKKAGQA] KKKK (Dingwall et al., / Cell Biol. 107 (3): 841-9). With reference to the alphavirus amino acid sequences of the invention, NLS are present at amino acids 67-70 of an EEEV capsid protein (KRKK); at amino acids 67-70 of an WEEV capsid protein (KKKK); at amino acids 64-68 of a VEEV capsid protein (KKPKK); at amino acids 62-69 of a CHIKV capsid protein (RRNRKNKK); at amino acids 71-74 of a Ross River virus capsid protein (RKKK); and at amino acids 64-68 of a Barmah Forest virus capsid protein (KKPKK). For example, K64N of VEEV TC83 capsid protein may be employed.

[0053] As used herein, "obtaining" as in "obtaining an agent" includes synthesizing, purchasing, or otherwise acquiring the agent.

[0054] By "reduces" is meant a negative alteration of at least 10%, 25%, 50%, 75%, or 100%.

[0055] By "reference" is meant a standard or control condition.

[0056] A "reference sequence" is a defined sequence used as a basis for sequence comparison. A reference sequence may be a subset of or the entirety of a specified sequence; for example, a segment of a full-length cDNA or gene sequence, or the complete cDNA or gene sequence. For polypeptides, the length of the reference polypeptide sequence will generally be at least about 16 amino acids, preferably at least about 20 amino acids, more preferably at least about 25 amino acids, and even more preferably about 35 amino acids, about 50 amino acids, or about 100 amino acids. For nucleic acids, the length of the reference nucleic acid sequence will generally be at least about 50 nucleotides, preferably at least about 60 nucleotides, more preferably at least about 75 nucleotides, and even more preferably about 100 nucleotides or about 300 nucleotides or any integer thereabout or there between.

[0057] Sequence identity is typically measured using sequence analysis software (for example, Sequence Analysis Software Package of the Genetics Computer Group, University of Wisconsin Biotechnology Center, 1710 University Avenue, Madison, Wis. 53705, BLAST, BESTFIT, GAP, or PILEUP/PRETTYBOX programs). Such software matches identical or similar sequences by assigning degrees of homology to various substitutions, deletions, and/or other modifications. Conservative substitutions typically include substitutions within the following groups: glycine, alanine; valine, isoleucine, leucine; aspartic acid, glutamic acid, asparagine, glutamine; serine,

threonine; lysine, arginine; and phenylalanine, tyrosine. In an exemplary approach to determining the degree of identity, a BLAST program may be used, with a probability score between e<"3> and e<"100> indicating a closely related sequence.

[0058] By "structural polyprotein" is meant a composite amino acid molecule comprising at least two separable polypeptides that contribute to a viral capsid or envelope. In one embodiment, the polypeptides are susceptible to cleavage with a viral enzyme (e.g., capsid autoproteinase and signalases).

[0059] By "subject" is meant a mammal, including, but not limited to, a human or non-human mammal, such as a bovine, equine, canine, ovine, or feline.

[0060] Ranges provided herein are understood to be shorthand for all of the values within the range. For example, a range of 1 to 50 is understood to include any number, combination of numbers, or sub-range from the group consisting 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50.

[0061] As used herein, the terms "treat," treating," "treatment," and the like refer to reducing or ameliorating a disorder and/or symptoms associated therewith. It will be appreciated that, although not precluded, treating a disorder or condition does not require that the disorder, condition or symptoms associated therewith be completely eliminated.

[0062] Unless specifically stated or obvious from context, as used herein, the term "or" is understood to be inclusive.

[0063] Unless specifically stated or obvious from context, as used herein, the terms "a", "an", and "the" are understood to be singular or plural.

[0064] Unless specifically stated or obvious from context, as used herein, the term "about" is understood as within a range of normal tolerance in the art, for example within 2 standard deviations of the mean. About can be understood as within 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, 1%, 0.5%, 0.1%, 0.05%, or 0.01% of the stated value. Unless otherwise clear from context, all numerical values provided herein are modified by the term about.

[0065] Any compositions or methods provided herein can be combined with one or more of any of the other compositions and methods provided herein.

[0066] Alphavirus replicon

The alphavirus replicon can, when delivered to an eukaryote cell, lead to the production of multiple daughter RNAs by transcription from itself (via an antisense copy which it generates from itself). The alphavirus replicon can be directly translated after delivery to a cell, and this translation provides a RNA-dependent RNA polymerase which then produces both antisense and sense transcripts from the delivered RNA. Thus the delivered RNA leads to the production of multiple daughter RNAs. These daughter RNAs, as well as collinear subgenomic transcripts, may be

translated themselves to provide in situ expression of an encoded protein, or may be transcribed to provide further transcripts with the same sense as the delivered RNA which are translated to provide in situ expression of the protein. The overall result of this sequence of transcriptions is a huge amplification in the number of the introduced replicon RNAs and so the encoded protein becomes a major polypeptide product of the cells.

[0067] According to the present invention, the alphavirus replicon comprises polynucleotides that encode non-structural proteins n1, n2, n3 and n4, and at least one gene of interest. Alphavirus replicon does not encode any of the alphavirus structural proteins.

[0068] The alphavirus nonstructural proteins may be wild type proteins derived from one of the above discussed alphaviruses or may have one or more alterations in the wild type amino acid sequences. Alterations of alphavirus nonstructural proteins are disclosed in various prior art references and the art can choose a suitable alphavirus nonstructural protein based on those publicly known information.

[0069] Alphavirus replicons are well known in the art and may be employed any of those previously disclosed replicons (for example, Virology. 1997 Dec 22;239(2):389-401., WO2009/131604, WO2011/005799, WO2012/031043, WO2014/1270493 and WO2015/095167, the contents of the cited documents are herein incorporated by reference).

[0070] Alphavirus Structural Proteins

The ARP has alphavirus structural proteins of capsid and envelope proteins. Preferably, the alphavirus structural proteins comprise the capsid protein and E2 and E1 proteins of the envelope, and may also have E3 protein of the envelope. According to the present invention, at least one of capsid and envelope has at least one alteration that enhances ARP expression in mammalian cells.

[0071] In one embodiment, the alphavirus structural proteins includes at least an alphavirus capsid protein having a non-lysine residue (e.g., alanine or asparagine) at an amino acid position corresponding to a lysine residue in the alphavirus capsid protein NLS and/or a non-arginine residue (e.g., alanine or asparagine) at an amino acid position corresponding to an arginine residue in the alphavirus capsid protein NLS. In specific embodiments, the alphavirus capsid protein is a WEEV CBA87 strain capsid protein having one or more of the alterations K67N, K68N, and K69N. In certain embodiments, the alphavirus capsid protein is a VEEV TC83 strain capsid protein having one or more of the alterations K64N, K65A, K65N, K67A, and K67N. In some embodiments, the alphavirus capsid protein is a EEEV PE-6 strain capsid protein having an alteration K67N. In particular embodiments, the alphavirus capsid protein is CHIKV Strain 37997 capsid protein having one or more of the alterations R62A, R63A, R65A, K66A, K68A, and K69A; the alphavirus capsid protein is a Ross River

Virus capsid protein having one or more of the alterations R71N, K72N, K73N, and K74N; the alphavirus capsid protein is a Barmah Forest Virus capsid protein having one or more of the alterations K64A, K64N, K65A, K65N, K67A, K67N, K68A and K68N. The wild type capsid protein amino acid sequences of the above-discussed alphaviruses are available at GenBank.

[0072] In one embodiment, the alphavirus E2 protein has a non-lysine residue (e.g., asparagine) at the amino acid position corresponding to amino acid 234 in the CHIKV E2 protein and/or a modification at the amino acid position corresponding to amino acid 251 in the CHIKV E2 protein that destabilizes the E2 protein during viral budding.

[0073] In one embodiment, the polynucleotide encoding alphavirus E3 may be modified to comprise an alteration/mutation to the amino acid sequence at the furin site (Arg-X-X-Arg).

[0074] The term "Arg-X-X-Arg" indicates the minimal cleavage site of furin and "X-X" includes any combination of two amino acids. Example of the alteration to the amino acid sequence at furin site includes the alteration to Ile-Glu/Asp-Gly-Arg, Asp-Asp-Asp-Asp-Lys or Ser-Gly-Gly-Gly-Ser. Detailed descriptions described in US Patent Publication Nos. 2016-0040134 and 2016-0200775 (the cited documents are herein incorporated by reference).

[0075] For example, VEEV CT83 strain has a furin site include RKRR at the end of its E3 region and the polynucleotide sequence encoding RKRR may be replaced with that encoding SGGGS.

[0076] In some embodiments of the invention, proteins may comprise mutations containing alterations which produce silent substitutions, additions, or deletions, but do not alter the properties or activities of the encoded protein or how the proteins are made.

[0077] Method of preparing ARPs

ARPs may be prepared by procedures that are known to the art. Exemplified procedure for producing ARPs is disclosed in Virology 239, 389-401, 1997, the contents of the cited document is herein incorporated by reference.

[0078] In general, ARPs may be produced by co-transfection of suitable host cells with a vector encoding an alphavirus replicon, i.e. a vector comprising a polynucleotide encoding nsp1, nsp2, nsp3 and nsp4, and a gene of interest; and at least one helper vector encoding the alphavirus virus structural proteins. Preferably, cells are co-transfected with a vector encoding an alphavirus replicon, a vector encoding a capsid protein and a vector encoding envelope proteins.

[0079] In particular, the invention provides a method for preparing alphavirus replicon particles, comprising the steps of

co-transfecting cells with

i) a vector comprising a polynucleotide encoding alphavirus non-structural protein nsp1, nsp2, nsp3 and nsp4, and at least one gene of interest,
ii) a vector comprising a polynucleotide encoding an alphavirus capsid protein, and
iii) a vector comprising a polynucleotide encoding an alphavirus E3-E2-6K-E1,
wherein at least one of the capsid, E3 and E2 comprises one or more amino acid alteration but E1 comprises no amino acid alteration,
culturing the transfected cells, and
purifying the ARPs from the cell culture.

[0080] Those skilled in the field of molecular biology will understand that any of a wide variety of expression systems may be used to produce ARPs. The precise cell to be co-transfected (host cells) is not critical to the invention. ARP may be produced in a prokaryotic host (e.g., E. coli) or in a eukaryotic host (e.g., Saccharomyces cerevisiae, insect cells, e.g., Sf21 cells, or mammalian cells, e.g., NIH 3T3, HeLa, COS cells). Such cells are available from a wide range of sources (e.g., the American Type Culture Collection, Rockland, Md.; also, see, e.g., Ausubel et al., supra). Non limiting examples of insect cells are, Spodoptera frugiperda (Sf) cells, e.g., Sf9, Sf21, Trichoplusia ni cells, e.g., High Five cells, and Drosophila S2 cells. Examples of fungi (including yeast) host cells are S. cerevisiae, Kluyveromyces lactis (K lactis), species of Candida including C. albicans and C. glabrata, Aspergillus nidulans, Schizosaccharomyces pombe (S. pombe), Pichia pastoris, and Yarrowia lipolytica. Examples of mammalian cells are COS cells, baby hamster kidney cells, mouse L cells, LNCaP cells, Chinese hamster ovary (CHO) cells, human embryonic kidney (HEK) cells, African green monkey cells, CV1 cells, HeLa cells, MDCK cells, Vero and Hep-2 cells. Xenopus laevis oocytes, or other cells of amphibian origin, may also be used. Prokaryotic host cells include bacterial cells, for example, E. coli, B. subtilis, and mycobacteria.

[0081] Methods of obtaining polynucleotides encoding said proteins are known in the art. For example, the gene encoding a specific alphavirus protein, e.g., a CHIKV, WEEV, EEEV, VEEV, Ross River virus, or Barmah Forest virus structural protein can be isolated by RT-PCR from polyadenylated mRNA extracted from cells which had been infected with said virus. The resulting product gene can be cloned as a polynucleotide insert into a vector.

[0082] The term "vector" refers to the means by which a nucleic acid sequence can be propagated and/or transferred between organisms, cells, or cellular components. Vectors include plasmids, viruses, bacteriophages, pro-viruses, phagemids, transposons, artificial chromosomes, and the like, that replicate autonomously or can integrate into a chromosome of a host cell. A vector can also be a naked RNA polynucleotide, a naked DNA polynucleotide, a polynucleotide composed of both DNA and

RNA within the same strand, a poly-lysine-conjugated DNA or RNA, a peptide-conjugated DNA or RNA, a liposome-conjugated DNA, or the like, that is not autonomously replicating. In many, but not all, common embodiments, the vectors of the present invention are plasmids or bacmids.

[0083] Typically, the nucleic acid molecule to be expressed is "operably linked" to a promoter and/or enhancer, and is subject to transcription regulatory control by the promoter and/or enhancer.

[0084] The method of transfection and the choice of expression vehicle will depend on the host system selected. Transfection methods are described, e.g., in Ausubel et al. (supra); expression vehicles may be chosen from those provided, e.g., in Cloning Vectors: A Laboratory Manual (P. H. Pouwels et al., 1985, Supp. 1987) The references cited in this paragraph are herein incorporated by reference.

[0085] A variety of expression systems exist for the production of the ARPs of the invention. Expression vectors useful for producing such ARPs include, without limitation, chromosomal, episomal, and virus-derived vectors, e.g., vectors derived from bacterial plasmids, from bacteriophage, from transposons, from yeast episomes, from insertion elements, from yeast chromosomal elements, from viruses such as baculoviruses, papova viruses, such as SV40, vaccinia viruses, adenoviruses, fowl pox viruses, pseudorabies viruses and retroviruses, and vectors derived from combinations thereof.

[0086] Constructs and/or vectors used herein comprise alphavirus polynucleotides that encode structural proteins, including envelope proteins or capsid protein as described herein. Also, constructs and/or vectors used herein comprise alphavirus polynucleotides that encode nonstructural proteins nsp1, nsp2, nsp3 and nsp4 and a gene of interest.

[0087] The vector may be, for example, a phage, plasmid, viral, or retroviral vector. The constructs and/or vectors that comprise the nucleotides should be operatively linked to an appropriate promoter, such as the CMV promoter, phage lambda PL promoter, the E. coli lac, phoA and tac promoters, the SV40 early and late promoters, and promoters of retroviral LTRs are non-limiting examples. Other suitable promoters will be known to the skilled artisan depending on the host cell and/or the rate of expression desired. The expression constructs will further contain sites for transcription initiation, termination, and, in the transcribed region, a ribosome-binding site for translation. The coding portion of the transcripts expressed by the constructs will preferably include a translation initiating codon at the beginning and a termination codon appropriately positioned at the end of the polypeptide to be translated.

[0088] Vectors will preferably include at least one selectable marker. Such markers include dihydrofolate reductase, G418 or neomycin resistance for eukaryotic cell culture and

tetracycline, kanamycin or ampicillin resistance genes for culturing in E. coli and other bacteria. Among vectors preferred are virus vectors, such as baculovirus, poxvirus (e.g., vaccinia virus, avipox virus, canarypox virus, fowlpox virus, raccoonpox virus, swinepox virus, etc.), adenovirus (e.g., canine adenovirus), herpesvirus, and retrovirus. Other vectors that can be used with the invention comprise vectors for use in bacteria, which comprise pQE70, pQE60 and pQE-9, pBluescript vectors, Phagescript vectors, pNH8A, pNH16a, pNH18A, pNH46A, ptrc99a, pKK223-3, pKK233-3, pDR540, pRIT5. Among preferred eukaryotic vectors are pFastBacl pWINEO, pSV2CAT, pOG44, pXTl and pSG, pSVK3, pBPV, pMSG, and pSVL. Other suitable vectors will be readily apparent to the skilled artisan.

[0089] Recombinant constructs can be prepared and used to transfect, can express viral proteins, including those described herein, into eukaryotic cells and/or prokaryotic cells. Thus, the invention provides for host cells which comprise a vector (or vectors) that contain nucleic acids which encode alphavirus structural proteins, including capsid, E3, E2, 6K, and El or portions thereof, and a vector that comprises nucleic acids which encode alphavirus nsp1, nsp2, nsp3 and nsp4, and at least one of gene of interest under conditions which allow the formation of ARPs.

[0090] In one embodiment, said vector is a recombinant baculovirus. In another embodiment, said recombinant baculovirus is transfected into an insect cell. In a preferred embodiment, said cell is an insect cell. In another embodiment, said insect cell is a Sf9 cell.

[0091] One particular bacterial expression system for polypeptide production is the E. coli pET expression system (Novagen, Inc., Madison, Wis). According to this expression system, DNA encoding a polypeptide is inserted into a pET vector in an orientation designed to allow expression. Since the gene encoding such a polypeptide is under the control of the T7 regulatory signals, expression of the polypeptide is achieved by inducing the expression of T7 RNA polymerase in the host cell. This is typically achieved using host strains that express T7 RNA polymerase in response to IPTG induction. Once produced, a recombinant polypeptide is then isolated according to standard methods known in the art, for example, those described herein.

[0092] Another bacterial expression system for polypeptide production is the pGEX expression system (Pharmacia). This system employs a GST gene fusion system that is designed for high-level expression of genes or gene fragments as fusion proteins with rapid purification and recovery of functional gene products. The protein of interest is fused to the carboxyl terminus of the glutathione S-transferase protein from Schistosoma japonicum and is readily purified from bacterial lysates by affinity chromatography using Glutathione Sepharose 4B. Fusion proteins can be recovered under mild conditions by elution with glutathione. Cleavage of the glutathione S-transferase

domain from the fusion protein is facilitated by the presence of recognition sites for site-specific proteases upstream of this domain. For example, proteins expressed in pGEX-2T plasmids may be cleaved with thrombin; those expressed in pGEX-3X may be cleaved with factor Xa.

[0093] Depending on the vectors and host cells selected, the ARPs are produced by growing host cells transfected by the vectors under conditions whereby the recombinant proteins are expressed and the alphavirus replicon is generated, and ARPs containing alphavirus replicon being packaged with the particle of alphavirus structural proteins are formed. In one embodiment, the invention comprises a method of producing an ARP, that involves co-transfecting a vector comprising a polynucleotide encoding alphavirus non-structural protein nsp1, nsp2, nsp3 and nsp4, and at least one gene of interest, at least one vectors each encoding at least one alphavirus protein into suitable host cells and expressing said alphavirus protein under conditions that allow ARP formation. In another embodiment, the eukaryotic cell is selected from the group consisting of, yeast, insect, amphibian, avian or mammalian cells. The selection of the appropriate growth conditions is within the skill or a person with skill of one of ordinary skill in the art.

[0094] Methods to grow cells that produce ARPs of the invention include, but are not limited to, batch, batch-fed, continuous and perfusion cell culture techniques. In one embodiment, cells co-transfected with a vector encoding an alphavirus replicon and a vector comprising a polypeptide encoding capsid, and a vector comprising a polynucleotide encoding envelope proteins, such as those derived from a CHIKV or VEEV are grown in a bioreactor or fermentation chamber where cells propagate and express protein (e.g., recombinant proteins) for purification and isolation. Typically, cell culture is performed under sterile, controlled temperature and atmospheric conditions. A bioreactor is a chamber used to culture cells in which environmental conditions such as temperature, atmosphere, agitation and/or pH can be monitored. In one embodiment, the bioreactor is a stainless steel chamber. In another embodiment, said bioreactor is a pre-sterilized plastic bag (e.g., Cellbag.RTM., Wave Biotech, Bridgewater, N.J.). In other embodiment, said pre-sterilized plastic bags are about 50 L to 1000 L bags.

[0095] The ARPs are isolated using methods that preserve the integrity thereof, such as by gradient centrifugation, e.g., cesium chloride, sucrose and iodixanol, as well as standard purification techniques including, e.g., ion exchange and gel filtration chromatography.

[0096] The following is an example of how ARPs of the invention can be made, isolated and purified. A person of skill in the art appreciates that there are additional methods that can be used to make and purify ARPs. Accordingly, the invention is not limited to the methods described herein.

[0097] In general, production of ARPs of the invention is accomplished by seeding a mammalian cell (e.g., human embryonic kidney (293T) cells) or Sf9 cells (non-infected) into shaker flasks, allowing the cells to expand and scaling up as the cells grow and multiply (for example from a 125-ml flask to a 50 L Wave bag). The medium used to grow the cells is formulated for the appropriate cell line (preferably serum free media, e.g., insect medium ExCell-420, JRH). Next, the cells are transfected or infected with an appropriate vector (e.g., mammalian expression vector or for SF(cells recombinant baculovirus at the most efficient multiplicity of infection (e.g., from about 1 to about 3 plaque forming units per cell). The polynucleotides, or portions thereof, are expressed in the cells where they self-assemble into ARPs and are secreted from the cells approximately 24 to 72 hours post infection (hpi). Usually, transfection or infection is most efficient when the cells are in mid-log phase of growth (4- 8.xl0<6> cells/ml) and are at least about 90% viable. Additionally, the transfected cells may be exposed to high pH conditions in cell culture (pH > 7.2, e.g., pH 7.2, 7.3, 7.4, 7.5, 7.6, 7.7, 7.8, 7.9, 8.0, 8.1, 8.2, 8.3, 8.4, 8.5, 8.6, 8.7, 8.8, or higher) to increase ARP production.

[0098] ARPs of the invention are harvested approximately 48 to 120 hours post infection, when the levels of ARPs in the cell culture medium are near the maximum but before extensive cell lysis. The cell density and viability at the time of harvest can be about $0.5xl0^6$ cells/ml to about $1.5xl0^6$ cells/ml with at least 20% viability, as shown by dye exclusion assay. Next, the medium is removed and clarified. NaCl can be added to the medium to a concentration of about 0.4 to about 1.0 M, preferably to about 0.5 M, to avoid ARP aggregation. The removal of cell and cellular debris from the cell culture medium containing ARPs of the invention can be accomplished by tangential flow filtration (TFF) with a single use, pre- sterilized hollow fiber 0.5 or 1.00 μπι filter cartridge or a similar device.

[0099] Additionally, the ARPs may be exposed to high pH conditions during purification (pH > 7.2, e.g., pH 7.2, 7.3, 7.4, 7.5, 7.6, 7.7, 7.8, 7.9, 8.0, 8.1, 8.2, 8.3, 8.4, 8.5, 8.6, 8.7, 8.8, or higher) to increase ARP production.

[0100] Next, ARPs in the clarified culture medium are concentrated by ultrafiltration using a disposable, pre-sterilized 500,000 molecular weight cut off hollow fiber cartridge. The concentrated ARPs can be diafiltrated against 10 volumes pH 7.0 to 8.0 phosphate-buffered saline (PBS) containing 0.5 M NaCl to remove residual medium components.

[0101] The concentrated, diafiltered ARPs can be furthered purified on a 20% to 60% dis-continuous sucrose gradient in pH 7.2 PBS buffer with 0.5 M NaCl by centrifugation at 6,500 x g for 18 hours at about 4 C to about 10 C. Usually ARPs will form a distinctive visible band between about 30% to about 40% sucrose or at the interface (in a 20% and 60% step gradient) that can be collected from the gradient and stored. This product can

be diluted to comprise 200 mM of NaCl in preparation for the next step in the purification process. This product contains ARPs and may contain intact baculo virus particles.

[0102] Further purification of ARPs can be achieved by anion exchange chromatography, or 44% isopycnic sucrose cushion centrifugation. In anion exchange chromatography, the sample from the sucrose gradient (see above) is loaded into column containing a medium with an anion (e.g., Matrix Fractogel EMD TMAE) and eluded via a salt gradient (from about 0.2 M to about 1.0 M of NaCl) that can separate the ARP from other contaminates (e.g., baculovirus and DNA/RNA). In the sucrose cushion method, the sample comprising the ARPs is added to a 44% sucrose cushion and centrifuged for about 18 hours at 30,000 g. ARPs form a band at the top of 44% sucrose, while baculovirus precipitates at the bottom and other contaminating proteins stay in the 0% sucrose layer at the top. The ARP peak or band is collected.

[0103] The intact baculovirus can be inactivated, if desired. Inactivation can be accomplished by chemical methods, for example, formalin or .beta.-propiolactone (BPL). Removal and/or inactivation of intact baculovirus can also be largely accomplished by using selective precipitation and chromatographic methods known in the art, as exemplified above. Methods of inactivation comprise incubating the sample containing the ARPs in 0.2% of BPL for 3 hours at about 25° C to about 27° C. The baculovirus can also be inactivated by incubating the sample containing the ARPs at 0.05% BPL at 4° C for 3 days, then at 37° C for one hour.

[0104] After the inactivation/removal step, the product comprising ARPs can be run through another diafiltration step to remove any reagent from the inactivation step and/or any residual sucrose, and to place the ARPs into the desired buffer (e.g., PBS). The solution comprising ARPs can be sterilized by methods known in the art (e.g., sterile filtration) and stored in the refrigerator or freezer.

[0105] The above techniques can be practiced across a variety of scales. For example, T-flasks, shake-flasks, spinner bottles, up to industrial sized bioreactors. The bioreactors can comprise either a stainless steel tank or a pre- sterilized plastic bag (for example, the system sold by Wave Biotech, Bridgewater, N.J.). A person with skill in the art will know what is most desirable for their purposes.

[0106] As described herein, upon administration to a desired host, the ARPs of the present invention are taken up by cells normally infected by the alphavirus from which the structural proteins are derived. The gene of interest contained in the replicon is internalized into the cell upon ARP entry. This property facilitates the use of the ARPs described herein as delivery vehicles of the gene because they enable the delivery of the gene of interest into a desired cell.

[0107] Whereas natural alphavirus genomes encode virus structural proteins in addition to

the non-structural replicase polyprotein, the alphavirus replicon does not encode alphavirus structural proteins. Thus the alphavirus replicon can lead to the production of genomic RNA copies of itself in a cell, but not to the production of RNA-containing virions. The inability to produce these virions means that, unlike a wild-type alphavirus, the replicon cannot perpetuate itself in infectious form. The alphavirus structural proteins which are necessary for perpetuation in wild-type viruses are absent from the replicon and their place is taken by at least one gene of interest, such that the subgenomic transcript encodes the protein of interest rather than the structural alphavirus structural proteins.

[0108] Thus, in certain embodiments, the ARP comprises a gene of interest that may be, or may encode, such as a therapeutic or diagnostic agent(s) that needs to be delivered to a subject, e.g., imaging agent, nucleic acid sequence (including siRNA and microRNA), radionuclide, hormone, peptide, antiviral agent, antitumor/chemotherapeutic agent, cell growth modulating agent, cell growth inhibitor, cytokine, antigen, adjuvant and toxin. The replicon packaged in the particle of the virus structural proteins should not adversely affect the stability of the ARP. This may be determined by producing ARP containing a given gene of interest and assessing its effects, if any, on ARP stability.

[0109] Accordingly, the present invention provides methods for introducing a gene of interest into a cell. According to the present invention, the gene of interest is contained in the alphavirus replicon and alphavirus replicon is packaged with the particle of alphavirus structural proteins. In related embodiments, the ARP is contacted with a cell. In related embodiments, the ARP is allowed to enter the cell, thereby resulting in delivery of the gene of interest into the cell.

[0110] As used herein, the terms "treat," treating," "treatment," and the like refer to reducing or ameliorating a disorder and/or symptoms associated therewith. It will be appreciated that, although not precluded, treating a disorder or condition does not require that the disorder, condition or symptoms associated therewith be completely eliminated.

[0111] As used herein, the terms "prevent," "preventing," "prevention," "prophylactic treatment" and the like refer to reducing the probability of developing a disorder or condition in a subject, who does not have, but is at risk of or susceptible to developing a disorder or condition.

[0112] The gene of interest may be a gene that encodes an antigen. ARPs of the present invention may be prepared in an injectable form, either as a liquid solution or as a suspension. Solid forms suitable for injection may also be prepared as emulsions, or with the ARPs encapsulated in liposomes. Vaccine antigens are usually combined with a pharmaceutically acceptable carrier, which includes any carrier that does not induce the production of antibodies harmful to the subject receiving the carrier. Suitable carriers typically comprise large macromolecules that are slowly metabolized, such as

proteins, polysaccharides, polylactic acids, polyglycolic acids, polymeric amino acids, amino acid copolymers, lipid aggregates, and inactive virus particles. Such carriers are well known to those skilled in the art. These carriers may also function as adjuvants.

[0113] The ARPs described herein may be administered in combination with an adjuvant (e.g., Ribi). Adjuvants are immunostimulating agents that enhance vaccine effectiveness. If desired, the ARP comprising one or more alphavirus polypeptides or fragments or variants thereof are administered in combination with an adjuvant that enhances the effectiveness of the immune response generated against the antigen of interest. Effective adjuvants include, but are not limited to, aluminum salts such as aluminum hydroxide and aluminum phosphate, muramyl peptides, bacterial cell wall components, saponin adjuvants, and other substances that act as immunostimulating agents to enhance the effectiveness of the composition.

[0114] Immunogenic compositions, i.e., the ARPs described herein, pharmaceutically acceptable carrier and adjuvant, also typically contain diluents, such as water, saline, glycerol, ethanol. Auxiliary substances may also be present, such as wetting or emulsifying agents, pH buffering substances, and the like. Proteins may be formulated into the vaccine as neutral or salt forms. The immunogenic compositions are typically administered parenterally, by injection; such injection may be either subcutaneously or intramuscularly. Additional formulations are suitable for other forms of administration, such as by suppository or orally. Oral compositions may be administered as a solution, suspension, tablet, pill, capsule, or sustained release formulation.

[0115] Immunogenic compositions are administered in a manner compatible with the dose formulation. The immunogenic composition comprises an immunologically effective amount of the ARP described herein and other previously mentioned components. By an immunologically effective amount is meant a single dose, or a composition administered in a multiple dose schedule, that is effective for the treatment or prevention of an infection. The dose administered will vary, depending on the subject to be treated, the subject's health and physical condition, the capacity of the subject's immune system to produce antibodies, the degree of protection desired, and other relevant factors. Precise amounts of the active ingredient required will depend on the judgement of the practitioner, but typically range between 5μg to 250μg of antigen per dose.

[0116] Pharmaceutical Compositions and Administration

The invention features pharmaceutical compositions that comprise ARPs as described herein. The pharmaceutical compositions useful herein contain a pharmaceutically acceptable carrier, including any suitable diluent or excipient, which includes any pharmaceutical agent that does not itself induce the production of an immune response harmful to the vertebrate receiving the composition, and which may

be administered without undue toxicity and a ARP of the invention. As used herein, the term "pharmaceutically acceptable" means being approved by a regulatory agency of the Federal or a state government or listed in the U.S. Pharmacopia, European Pharmacopia or other generally recognized pharmacopia for use in mammals, and more particularly in humans. These compositions can be useful as a vaccine and/or antigenic compositions for inducing a protective immune response in a vertebrate.

[0117] Pharmaceutically acceptable carriers include but are not limited to saline, buffered saline, dextrose, water, glycerol, sterile isotonic aqueous buffer, and combinations thereof. A thorough discussion of pharmaceutically acceptable carriers, diluents, and other excipients is presented in Remington's Pharmaceutical Sciences (Mack Pub. Co. N.J. current edition). The formulation should suit the mode of administration. In a preferred embodiment, the formulation is suitable for administration to humans, preferably is sterile, non-particulate and/or non-pyrogenic.

[0118] The composition, if desired, can also contain minor amounts of wetting or emulsifying agents, or pH buffering agents. The composition can be a solid form, such as a lyophilized powder suitable for reconstitution, a liquid solution, suspension, emulsion, tablet, pill, capsule, sustained release formulation, or powder. Oral formulation can include standard carriers such as pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate, etc.

[0119] In certain embodiments, the ARP composition is supplied in liquid form, for example in a sealed container indicating the quantity and concentration of the ARP composition.

[0120] Preferably, the liquid form of the ARP composition is supplied in a hermetically sealed container at least about 50 μg/ml, more preferably at least about 100 μg/ml, at least about 200 μg/ml, at least 500 μg/ml, or at least 1 mg/ml.

[0121] Alternatively, the vaccine formulation is administered intranasally, either by drops, large particle aerosol (greater than about 10 microns), or spray into the upper respiratory tract or small particle aerosol (less than 10 microns) or spray into the lower respiratory tract. While any of the above routes of delivery results in an immune response, intranasal administration confers the added benefit of eliciting mucosal immunity at the site of entry of many viruses, including alphaviruses, for example CHIKV or VEEV.

[0122] Thus, the invention also comprises a method of formulating a vaccine or antigenic composition that induces immunity to an infection or at least one symptom thereof to a mammal, comprising adding to said formulation an effective dose of ARPs, e.g., alphavirus (e.g., CHIKV or VEEV).

[0123] In certain cases, stimulation of immunity with a single dose is preferred, however ad-

ditional dosages can be also be administered, by the same or different route, to achieve the desired effect. In neonates and infants, for example, multiple administrations may be required to elicit sufficient levels of immunity. Administration can continue at intervals throughout childhood, as necessary to maintain sufficient levels or protection.

[0124] Similarly, adults who are particularly susceptible to repeated or serious infections, such as, for example, health care workers, day care workers, family members of young children, the elderly, and individuals with compromised cardiopulmonary function or immune systems may require multiple immunizations to establish and/or maintain protective immune responses. Levels of induced immunity can be monitored, for example, by measuring amounts of neutralizing secretory and serum antibodies, and dosages adjusted or vaccinations repeated as necessary to elicit and maintain desired levels of protection.

[0125] The dosage of the pharmaceutical formulation can be determined readily by the skilled artisan, for example, by first identifying doses effective to elicit a prophylactic or therapeutic immune response, e.g., by measuring the serum titer of virus specific immunoglobulins or by measuring the inhibitory ratio of antibodies in serum samples, or urine samples, or mucosal secretions. Said dosages can be determined from animal studies. A non-limiting list of animals used to study the efficacy of vaccines include the guinea pig, hamster, ferrets, chinchilla, mouse and cotton rat, and non-human primates. Most animals are not natural hosts to infectious agents but can still serve in studies of various aspects of the disease. For example, any of the above animals can be dosed with a vaccine candidate, e.g., ARPs of the invention, to partially characterize the immune response induced, and/or to determine if any neutralizing antibodies have been produced. For example, many studies have been conducted in the mouse model because mice are small size and their low cost allows researchers to conduct studies on a larger scale.

[0126] In addition, human clinical studies can be performed to determine the preferred effective dose for humans by a skilled artisan. Such clinical studies are routine and well known in the art. The precise dose to be employed will also depend on the route of administration. Effective doses may be extrapolated from dose-response curves derived from in vitro or animal test systems.

[0127] As also well known in the art, the immunogenicity of a particular composition can be enhanced by the use of non-specific stimulators of the immune response, known as adjuvants. Adjuvants have been used experimentally to promote a generalized increase in immunity against unknown antigens. Immunization protocols have used adjuvants to stimulate responses for many years, and as such, adjuvants are well known to one of ordinary skill in the art. Some adjuvants affect the way in which antigens are presented. For example, the immune response is increased when protein antigens are

precipitated by alum. Emulsification of antigens also prolongs the duration of antigen presentation. The inclusion of any adjuvant described in Vogel et al., "A Compendium of Vaccine Adjuvants and Excipients (2nd Edition)," herein incorporated by reference in its entirety for all purposes, is envisioned within the scope of this invention.

[0128] Exemplary adjuvants include complete Freund's adjuvant (a non-specific stimulator of the immune response containing killed Mycobacterium tuberculosis), incomplete Freund's adjuvants and aluminum hydroxide adjuvant. Other adjuvants comprise GMCSP, BCG, aluminum hydroxide, MDP compounds, such as thur-MDP and nor-MDP, CGP (MTP-PE), lipid A, and monophosphoryl lipid A (MPL). RIBI, which contains three components extracted from bacteria, MPL, trehalose dimycolate (TDM) and cell wall skeleton (CWS) in a 2% squalene/Tween-80 emulsion also is contemplated. MF-59, Novasomes.RTM., MHC antigens may also be used.

[0129] The ARPs of the invention can also be formulated with "immune stimulators." These are the body's own chemical messengers (cytokines) to increase the immune system's response. Immune stimulators include, but not limited to, various cytokines, lymphokines and chemokines with immunostimulatory, immunopotentiating, and pro-inflammatory activities, such as interleukins (e.g., IL-1, IL-2, IL-3, IL-4, IL-12, IL-13); growth factors (e.g., granulocyte-macrophage (GM)-colony stimulating factor (CSF)); and other immunostimulatory molecules, such as macrophage inflammatory factor, Flt3 ligand, B7.1; B7.2, etc. The immunostimulatory molecules can be administered in the same formulation as the ARPs, or can be administered separately. Either the protein or an expression vector encoding the protein can be administered to produce an immunostimulatory effect. Thus in one embodiment, the invention comprises antigenic and vaccine formulations comprising an adjuvant and/or an immune stimulator.

[0130] According to the invention, the delivery system comprising said ARP can deliver a gene of interest into the cytoplasm of eukaryotic cells, thereby can treat cancers, viral infections, neurological disorders, autoimmune diseases, graft rejection and monogenic or polygenic hereditary diseases.

[0131] The invention will be described in detail with reference to the following examples, which, however, are not intended to limit the scope of the present application.

Example 1

[0132] Construction of VEEV Replicon Particles (VRPs)

Schematic procedure is shown in Figure 2.

Establishment of VEEV Replicon plasmid expressing luciferase

1) Full-length VEEV TC83 non-structural protein(nsp)1, nsp2, nsp3 and nsp4 fragment was synthesized (ThermoFisher).

Similarly, gblocks corresponding to various fragments of the Replicon construct

were synthesized (IDT) as follows -

[0133] 2) VEEV gblock1 - ClaI-RSVp-5'UTR-nsp1(bp1-470)-RsrII-ApaI-26Sp-sgRNA up to ATG of the VEEV CA gene. This fragment had a 36 bp overlap with the pBR322 backbone plasmid at the 5' end. This is fragment #1.

[0134] 3) VEEV gblock2 - This fragment had a 64bp overlap with VEEV gblock1 starting at the ApaI site up to the ATG start codon. This was followed by the Nano Luciferase ORF (Promega) and the first 72 bases of the VEEV 3' UTR.

[0135] 4) Cloning of fragment #2 - VEEV gblock2, full-length VEEV 3'UTR and the VEEV PolyA signal ($A_{(n=55)}$) were first assembled by overlap extension PCR using the oligomers shown in the table below:

[0136] [Table 1]

Primer Name	Sequence 5′ → 3′
VEEV_gblock2_fwd	tcattcagctacctgagaggg (SEQ ID NO: 7)
VEEV_gblock2_rev	aaataaaaattttaaggcggcatgc (SEQ ID NO: 8)
VEEV_Oligo1_fwd	ttaaaatttttatttttatttttctttttctttttccgaat cggattttgtttttaatatttc (SEQ ID NO: 9)
VEEV_Oligo2_rev	CATCAATGTATCTTATCATGTCTGtcgcgaTTTTTTTT TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT TTTTTTTTTgaaatattaaaaaca (SEQ ID NO: 10)

[0137] VEEV_Oligo2 has a 15bp overlap with the pBR322 backbone plasmid.

[0138] 5) Next, the VEEV gblock1 (fragment #1), fragment #2 and pBR322 backbone (digested with ClaI and NruI-HF) were assembled using Gibson Assembly to obtain the pBR322-RSVp-RsrII-ApaI-26Sp-sgRNA-NanoLuc-A(n=55)-SV40 pA backbone construct (BB). This backbone construct lacked the full-length TC83 nsp1-4 fragment.

[0139] 6) The nsp1-4 fragment (bp 470-7461) was amplified from the synthesized Thermo plasmid obtained in 1) using primers containing the RsrII and ApaI restriction sites -

i) 5'-ccggccCGGACCGacaagtctctatcacc-3' (SEQ ID NO: 11, fwd primer) and ii)5'-ggccggGGGCCCctctcaggtagctgaatg-3' (SEQ ID NO: 12, rev primer). This PCR amplified nsp fragment was cloned into the BB backbone plasmid using the RsrII and ApaI sites to obtain the full-length VEEV TC83 Replicon construct. (Figure 3)

[0140] Helper Plasmids

Helper plasmid constructs encoding the VEEV TC83 capsid are shown in Figures 4 and 5. The constructs express wild type VEEV capsid protein (SEQ ID NO: 1) and VEEV Capsid having a mutation in the NLS (K64N, SEQ ID NO: 2), respectively.

[0141] Helper plasmid constructs expressing the VEEV TC83 glycoproteins E3-E2-6K-E1

used herein are shown in Figures 6 and 7. The constructs express wild type VEEV TC83 glycoproteins E3-E2-6K-E1 (SEQ ID NO: 3) and E3 modified E3-E2-6K-E1(furin site at the end of E3 RKRR was replaced with SGGGS, SEQ ID NO: 4), respectively.

[0142] Cell Culture and co-Transfection

293T cells were seeded in a 6-well plate in complete DMEM containing 10% FBS, Penicillin and Streptomycin. The cells were co-transfected with equal amounts of the VEEV RSVp-NLuc construct containing (VR)(SEQ ID NO: 5) or lacking (BB) the nsp1-4 fragment, along with helper plasmid encoding capsid and that encoding glycoproteins E1-6K-E2-E3 using PEI(1.7μg of each plasmid). The combination of the helper plasmids was (SEQ ID NO: 1 and 3), (SEQ ID NO: 1 and 4), (SEQ ID NO: 2 and 3) or (SEQ ID NO: 2 and 4). Cells were incubated with the transfection mixture for about 3 hours at 37 °C, following which the transfection mixture was removed, cells were washed with 1X PBS and fresh DMEM was added. The packaged Virus Replicon Particles (VRPs) were harvested either 48 hpt(hours post transfection) or 72 hpt. To harvest the VRPs, the culture supernatant of the transfected cells was obtained by centrifuging the cell culture at 1200 rpm for 5min at 4°C to pellet any cell debris. The supernatant was filtered through a 0.45um filter. The harvested VRPs were either stored at 4°C or -80°C.

Example 2

[0143] Determining the Packaging Ability of the VEEV Replicon into VEE Virus replicon particles (VRPs)

Infection and Luciferase Assay

Schematic protocol is shown in the Figure 8.

293T cells were seeded in complete DMEM in a 96-well plate at a density of approximately 10,000 cells per well. Cells were infected with undiluted or 2-fold serial dilutions of the harvested VRPs at 37°C. At 14 hours post infection (hpi), the VRPs were removed; the cells were washed with PBS and fresh DMEM was added to the wells. Cells were further incubated and harvested for Luciferase assay at 24, 48, and 72 hpi. Luciferase assay was performed by adding equal amounts of infected cells and Nano-Glo Luciferase Assay System (Promega) to a white bottom opaque 96-well plate (Costar). Luciferase activity was measured immediately using the Bio-Tek Synergy HTX microplate reader.

[0144] Result

Results are shown in Figure 9. Luciferase expression was confirmed in the cells infected with the VRPs (VR) as early as 24 hpi. Cells infected with the backbone construct lacking nsp 1-4 (BB) expressed almost no luciferase.

Example 3

[0145] New VEEV Replicon Constructs

New VEEV TC83 replicon plasmid constructs was prepared by introducing multiple cloning site (MCS) to enable introducing various "gene of interest". The schematic protocol is shown in Figure 10. The construct is shown in Figure 11 and SEQ ID NO: 6. In the construct of Figure 11, a nucleotide encoding luciferase is introduced as gene of interest. Promoter in the plasmid may be selected in view of the cells to be infected.

Example 4

[0146] Preparation of VRPs with/without a mutation in Capsid and/or E3-E2-6K-E1, and having a gene of interest

In this example, VEEV replicon plasmid containing a gene of interest was co-transfected with VEEV Capsid helper plasmid and VEEV E3-E2-6K-E1 glycoprotein helper plasmid to 293T cells in the similar manner as Example 1. As the gene of interest, genes encoding luciferase, GFP, IKK or JNK2 was employed.

[0147] The following plasmids were used:

i-1) plasmid comprising a polynucleotide encoding VEEV CT83 wild type capsid protein, or

i-2) plasmid comprising a polynucleotide encoding VEEV CT83 capsid protein having a mutation in the NLS (K64N).

ii-1) plasmid comprising a polynucleotide encoding wild type VEEV CT83 E3-E2-6K-E1, or

ii-2) plasmid comprising a polynucleotide encoding VEEV CT83 E3-E2-6K-E1, having mutation in the furin site in E3

and

iii) plasmid comprising polynucleotide encoding VEEV CT83 nsp1, nsp2, nsp3 and nsp4, and a gene of interest encoding luciferase, GFP, IKK or JNK2.

[0148] Co-transfection

293T cells were co-transfected with the VEEV TC83 replicon plasmid comprising the gene of interest, a helper plasmid encoding VEEV capsid (WT or mutation) and a helper plasmid encoding E3-E2-6K-E1 (WT or mutation). Three plasmids (1μg of capsid, 1μg of E3-E2-6K-E1 and 10μg of VEEV replicon containing the gene of interest) were transfected to 293T cells (by PEI method) and cells were incubated 4-7 days. Then, VRPs were harvested from the supernatant and purified by optiprep density sedimentation.

[0149] The purified VRPs were confirmed by western blotting using anti-VEEV antibody (ATCC) as the first antibody (1:2000) and anti-mouse IgG (1:4000) as the secondary antibody. The results of VRPs obtained by using the following plasmids are shown in

Figure 12:

i) plasmid comprising a polynucleotide encoding VEEV CT83 capsid protein having a mutation in the NLS (K64N).

ii) plasmid comprising a polynucleotide encoding wild type VEEV CT83 E3-E2-6K-E1, and

iii) plasmid comprising polynucleotide encoding VEEV CT83 nsp1, nsp2, nsp3 and nsp4, and also a polynucleotide encoding GFP, IKK or JNK2.

Example 5

[0150] VRPs obtained in Example 4 using the following vectors were used:

i) plasmid comprising a polynucleotide encoding VEEV CT83 capsid protein having a mutation in the NLS (K64N).

ii) plasmid comprising a polynucleotide encoding wild type VEEV CT83 E3-E2-6K-E1, and

iii) plasmid comprising polynucleotide encoding VEEV CT83 nsp1, nsp2, nsp3 and nsp4, and also a polynucleotide encoding IKK or JNK2.

[0151] 293T Cells were infected with the VRPs that contain IKK or JNK2 gene. The infection of VRPs to 293T cells was conducted in the same manner as Example 2.

[0152] The expression of IKK in the VRP-infected 293T cells was confirmed by western blotting. As positive control, 293T cells transfected with IKK expression vector plasmid were used. Cell lysate was subjected to western blotting with Rb anti-IKK antibody (Proteintech) as the first antibody (1:500) and HRP labelled anti-RbIgG as the secondary antibody (1:4000).

[0153] Results are shown in Figure 13. In Figure 13, Lane 1 shows 293T cells infected with the VRPs. Lane 2 shows positive control, i.e. cells transfected with IKK expression vector plasmids. As shown in this figure, the 293T cells infected with the VRPs expressed IKK protein.

[0154] The expression of JNK2 from the replicon inserted JNK2 gene in the infected 293T cells was confirmed by western blotting. As the control, 293T cells without infection were used. Mouse anti-JNK2 antibody (Santa Cruz) was used as the first antibody (1:500) and HRP labelled anti-mouse IgG was used as the second antibody (1:4000). Results are shown in Figure 14. 293T cells infected by the VRPs having JNK2 as the gene of interest were confirmed to express JNK2.

Example 6

[0155] Effect of mutation in Capsid NLS

Two types of VRPs obtained in Example 4 using the following plasmids were used:

i-1) plasmid comprising a polynucleotide encoding VEEV CT83 wild type capsid protein, or

i-2) plasmid comprising a polynucleotide encoding VEEV CT83 capsid protein having a mutation in the NLS (K64N).

ii) plasmid comprising a polynucleotide encoding wild type VEEV CT83 E3-E2-6K-E1, and

iii) plasmid comprising polynucleotide encoding VEEV CT83 nsp1, nsp2, nsp3 and nsp4, and also a polynucleotide encoding luciferase.

[0156] 293T cells were infected with the VRPs in the same manner as Example 2. Luciferase assay was conducted in the same manner as Example 2. Results are shown in Figure 15.

[0157] In this Figure, control correspond background of luciferase activity. VRPs with Capsid having mutation in NLS showed much higher luciferase activity (900556) compared to the VRPs with the WT capsid (118063).

[0158] The data indicated that modification of the capsid leads to higher yield and expression compared to the alphavirus replicon particle without modification in the capsid.

Example 7

[0159] VEE Virus replicon particles (VRPs) obtained in Example 4 by co-transfection of 293T cells with the following vectors were used:

i) plasmid comprising a polynucleotide encoding VEEV CT83 capsid protein having a mutation in the NLS (K64N).

ii) plasmid comprising a polynucleotide encoding wild type VEEV CT83 E3-E2-6K-E1.

iii) plasmid comprising polynucleotide encoding VEEV CT83 nsp1, nsp2, nsp3 and nsp4, and a polynucleotide encoding GFP.

293T cells were infected with thus obtained VRPs in the same manner as Example 2. Control shows normal cells with no infection. The expression of GFP in the cells was confirmed. Results are shown in Figure 16.

Example 8

[0160] The same VEEV replicon particles as those used in Example 7 were used. Macrophage J774.A1 cells and Bone Marrow-derived macrophage (BMDM) cells were infected with the VRPs. The gene of interest encoding GFP was expressed in the transfected macrophage cells.

The both cells were treated with IL-4 for 48 hours to be polarized and analyzed for GFP expression. Results are shown in Figure 17.

Example 9

[0161] In vivo efficacy study on CT26 models

The schematic protocol of this example is shown in Figure 18. VRPs used in this

example were those prepared in Example 4 using the following plasmids:

i) plasmid comprising a polynucleotide encoding VEEV CT83 capsid protein having a mutation in the NLS (K64N),

ii) plasmid comprising a polynucleotide encoding wild type VEEV CT83 E3-E2-6K-E1, and

iii) plasmid comprising polynucleotide encoding VEEV CT83 nsp1, nsp2, nsp3 and nsp4, and a polynucleotide encoding human IκB kinases (IKK).

[0162] This study consists of 4 arms, n=8, for a total of 32 animals. Animals were received Animals was randomized on Day 0 when tumor volume reaches 60-100 mm^3 and dosing will begin on Day 0 with G1: vehicle, G2: anti-PD-1 mAb, G3: VRPs and G4: a combination of VRPs and anti-PD-1 mAb at 10 mg/kg (BIWx3 and IP). The tumor growth was monitored until 30 days after the initiation of the treatment.

[0163] For groups 1, 3, 4, mice were intra-tumoraly injected with 50 μl of vehicle or VRPs dispersed in the vehicle on day 0, 2, 4, 6, 8 and 10. The VRPs or vehicle were administered into the tumor at the right flank using a 0.3 ml insulin type syringe. The aim was to use one entry point, but distribute the material through the tumor by moving the needle in and out.

[0164] Measurements of tumor size were performed two times a week. The humane endpoint for this study is tumor burden of 3000mm^3 and/or body weight loss of 20% or greater. The results of all 8 animals in each group are shown in Figure 19.

[0165] The data indicated that VRPs comprising the gene encoding IKK and the combination of VRPs and anti-PD-1 antibody demonstrated superior anti-tumor effect over control and anti-PD-1 single immunotherapy.

Example 10

[0166] Chimeric Alphavirus replicon particles were prepared in the same manner as Example 4 using the following sets of vectors.

Chimeric ARP 1

i) plasmid comprising a polynucleotide encoding wild type CHIKV 37997 strain capsid protein,

ii) plasmid comprising a polynucleotide encoding wild type CHIKV 37997 strain E3-E2-6K-E1, and

iii) plasmid comprising polynucleotide encoding VEEV CT83 nsp1, nsp2, nsp3 and nsp4, and a polynucleotide encoding GFP.

[0167] Chimeric ARP 2

i) plasmid comprising a polynucleotide encoding wild type CHIKV OPY-1 strain capsid protein,

ii) plasmid comprising a polynucleotide encoding wild type CHIKV OPY-1 strain

E3-E2-6K-E1, and

iii) plasmid comprising polynucleotide encoding VEEV CT83 nsp1, nsp2, nsp3 and nsp4, and a polynucleotide encoding GFP.

[0168] The obtained ARPs were purified in the same manner as Example 1. The purified chimeric ARPs were confirmed by western blotting using anti-CHIKV rabbit serum(1:2000) as the first antibody and goat anti- Rabbit IgG-HRP (1:4000) as the secondary antibody. The results are shown in Figure 20. Generation of both chimeric ARPs were confirmed.

[0169] The 293T cells were infected with the chimeric ARPs encoding GFP. After the infection, cells were incubated for 48 hours and the expression of GFP was confirmed by FACS analysis. As a control, cells with no infection were used. Results are shown in Figure 21. 5.21% and 4.07% of the cells infected with the ARPS expressed GFP, suggesting that the chimeric ARPs successfully expressed GFP protein (gene of interest is a nucleotide encoding GFP) in the cells.

Claims

[Claim 1] An alphavirus replicon particle (ARP), which comprises
(i) alphavirus structural proteins comprising capsid and/or envelope, and
(ii) an alphavirus replicon comprising a polynucleotide encoding alphavirus non-structural proteins nsp1, nsp2, nsp3 and nsp4 and at least one gene of interest
wherein at least one of capsid, and E3 and E2 in the envelope comprise one or more amino acid alteration but E1 in the envelope comprises no amino acid alteration.

[Claim 2] The ARP of claims 1, wherein the alphavirus structural proteins comprise one or more alterations in the alphavirus capsid protein Nuclear Localization Signal (NLS).

[Claim 3] The ARP of claim 2, wherein one or more of lysine or arginine in the alphavirus capsid protein Nuclear Localization Signal is substituted with asparagine or alanine.

[Claim 4] The ARP of any one of claims 1-3, wherein the alphavirus structural proteins comprise one or more alterations in the alphavirus E2 protein.

[Claim 5] The ARP of any one of claim 1-5, wherein the alphavirus structural proteins comprise one or more alterations at the furin site in alphavirus E3 protein.

[Claim 6] The ARP of any one of claims 1-5, wherein the alphavirus is a CHIKV or VEEV.

[Claim 7] The ARP of claim 6, wherein the CHIKV is CHIKV strain 37997 or strain OPY-1.

[Claim 8] The ARP of claim 6, wherein the VEEV is VEEV strain TC-83.

[Claim 9] The ARP of any of claims 1-8, wherein the gene of interest encodes an antigen.

[Claim 10] A method for preparing alphavirus replicon particles, comprising the steps of
co-transfecting cells with
i) a vector comprising a polynucleotide encoding alphavirus non-structural protein nsp1, nsp2, nsp3 and nsp4, and at least one gene of interest,
ii) a vector comprising a polynucleotide encoding an alphavirus capsid protein, and
iii) a vector comprising a polynucleotide encoding an alphavirus

E3-E2-6K-E1,
wherein at least one of the capsid, E3 and E2 comprises one or more amino acid alteration but E1 comprises no amino acid alteration,
culturing the transfected cells, and
purifying the ARPs from the cell culture.

[Claim 11] The method of claim 10, wherein the alphavirus structural proteins comprise one or more alterations in the alphavirus capsid protein Nuclear Localization Signal (NLS).

[Claim 12] The method of claim 11, wherein one or more of lysine or arginine in the alphavirus capsid protein Nuclear Localization Signal is substituted with asparagine or alanine.

[Claim 13] The method of any one of claims 10-12, wherein the alphavirus structural proteins comprise one or more alternations in the alphavirus E2 protein.

[Claim 14] The method of claim 13, wherein the alphavirus structural proteins comprise one or more alterations at the furin site in alphavirus E3 protein.

[Claim 15] The method of any one of claims 10-14, wherein the alphavirus is a CHIKV or VEEV.

[Claim 16] The method of claim 15, wherein the CHIKV is CHIKV strain 37997 or strain OPY-1.

[Claim 17] The method of claim 15, wherein the VEEV is VEEV strain TC-83.

[Claim 18] The method of any of claims 10-17, wherein the gene of interest encodes an antigen.

[Claim 19] An alphavirus replicon particle (ARP), which comprises
(i) CHIKV structural proteins comprising capsid and/or envelope, and
(ii) a VEEV replicon comprising a polynucleotide encoding VEEV non-structural proteins nsp1, nsp2, nsp3 and nsp4 and at least one gene of interest.

[Claim 20] The ARP of claim 19, wherein the CHIKV is CHIKV strain 37997 or strain OPY-1, and VEEV is VEEV strain TC-83.