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(19) **United States**(12) **Patent Application Publication****Talaat et al.**(10) **Pub. No.: US 2020/0390883 A1**(43) **Pub. Date: Dec. 17, 2020**(54) **NOVEL ADJUVANT FOR ANIMAL AND HUMAN VACCINES**(71) Applicant: **Wisconsin Alumni Research Foundation, Madison, WI (US)**(72) Inventors: **Adel M. Talaat, Madison, WI (US); Shaswath Chandrasekar, Madison, WI (US)**(21) Appl. No.: **16/900,070**(22) Filed: **Jun. 12, 2020****Related U.S. Application Data**

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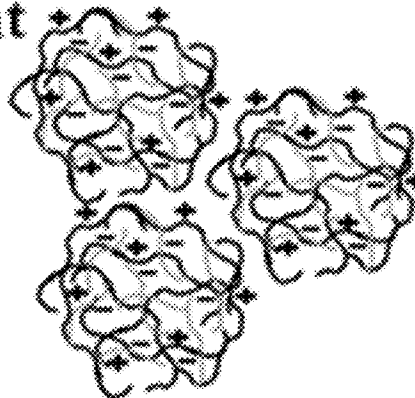
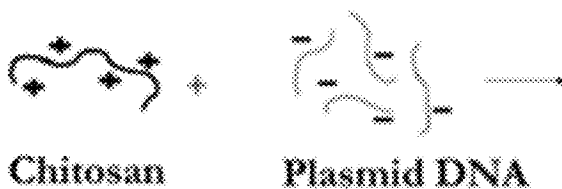
(57)

ABSTRACT

Quil-A chitosan spherical nanostructure complexes as well as methods of making and using such complexes are disclosed herein. Also provided are Quil-A chitosan spherical nanostructures loaded with one or more RNA, DNA, or protein payload molecules as well as methods of making and using such loaded complexes.

Specification includes a Sequence Listing.

Chitosan-DNA Adjuvant



**Chitosan-DNA complexes
(aggregated nanostructures)**

QAC-DNA complex

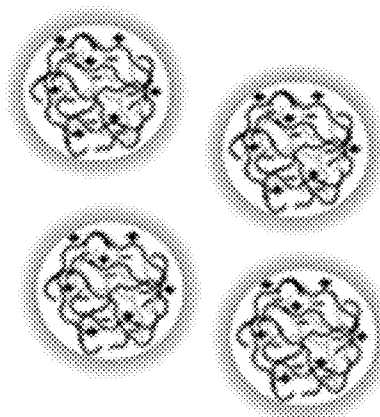
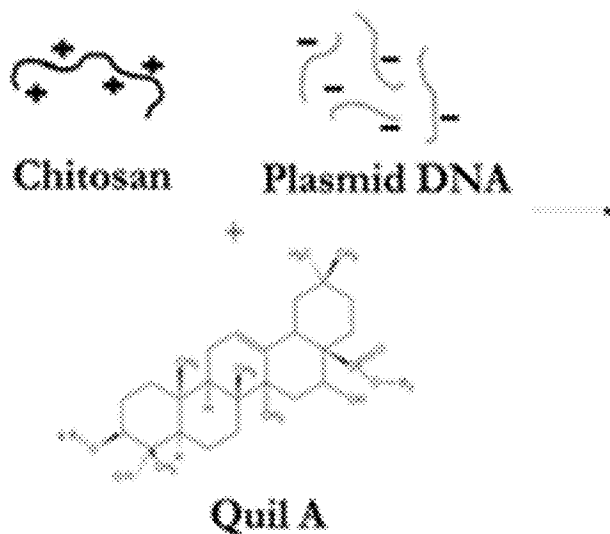


FIG. 1

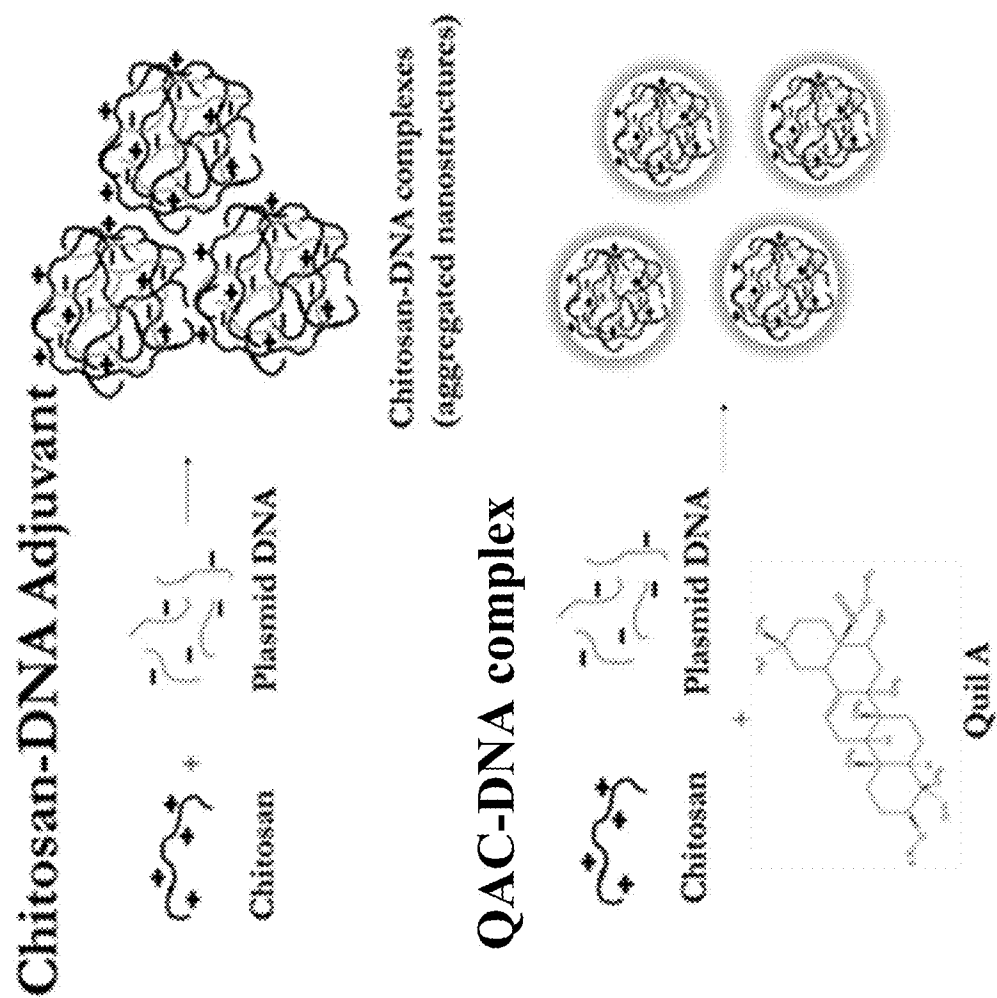


FIG. 2

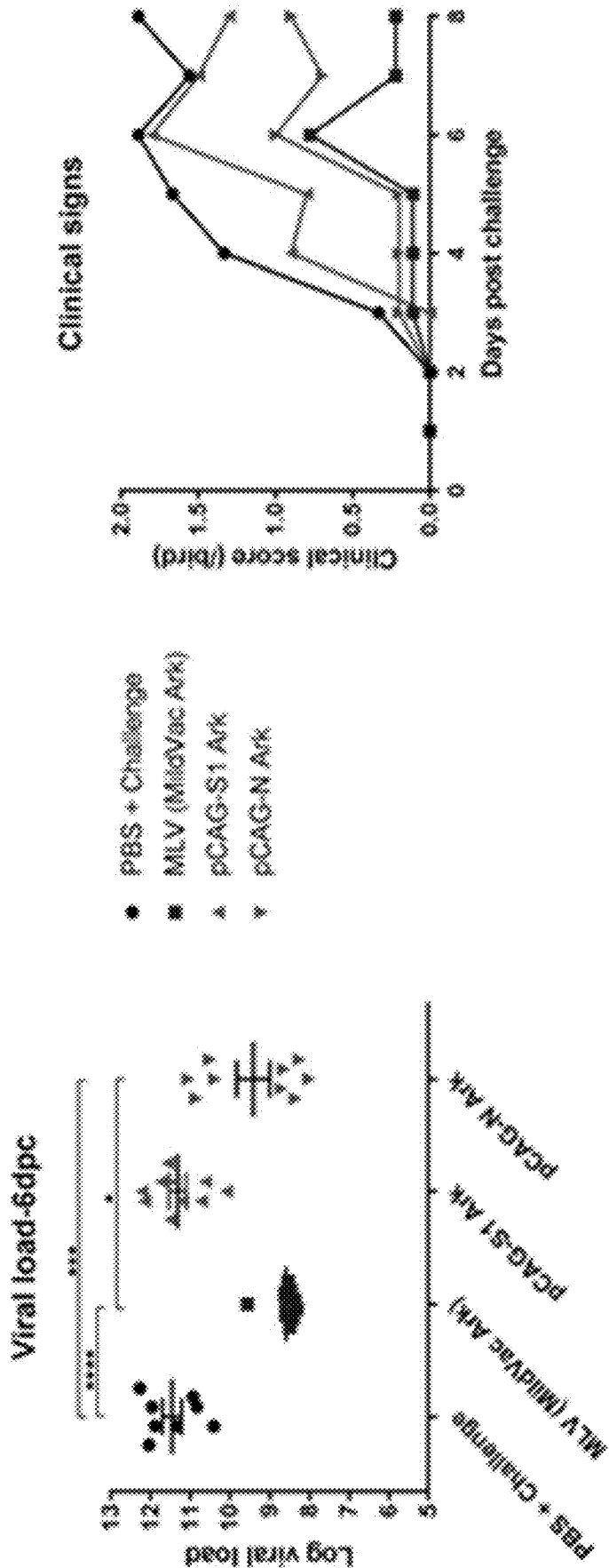


FIG. 2 CONTINUED

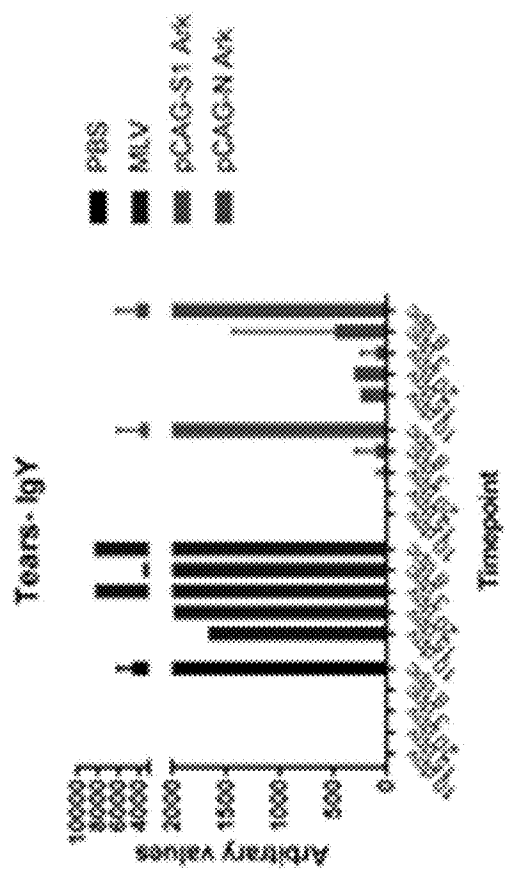
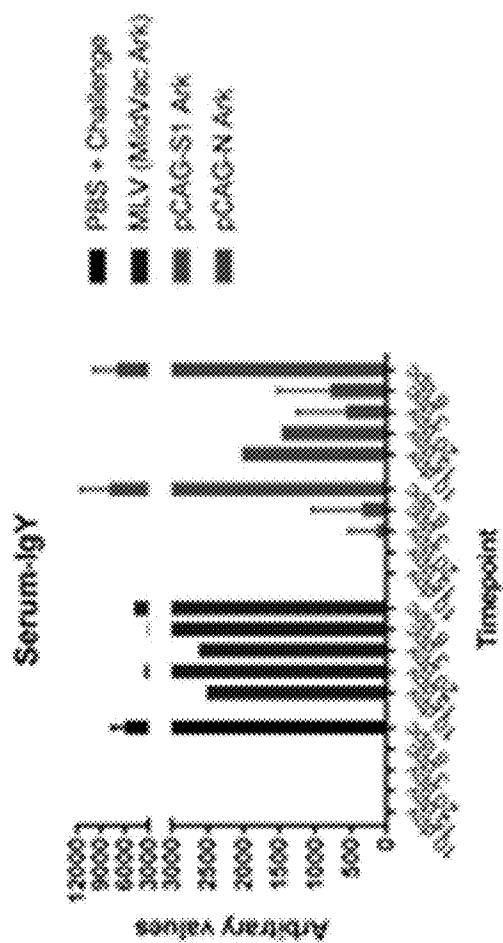


FIG. 3

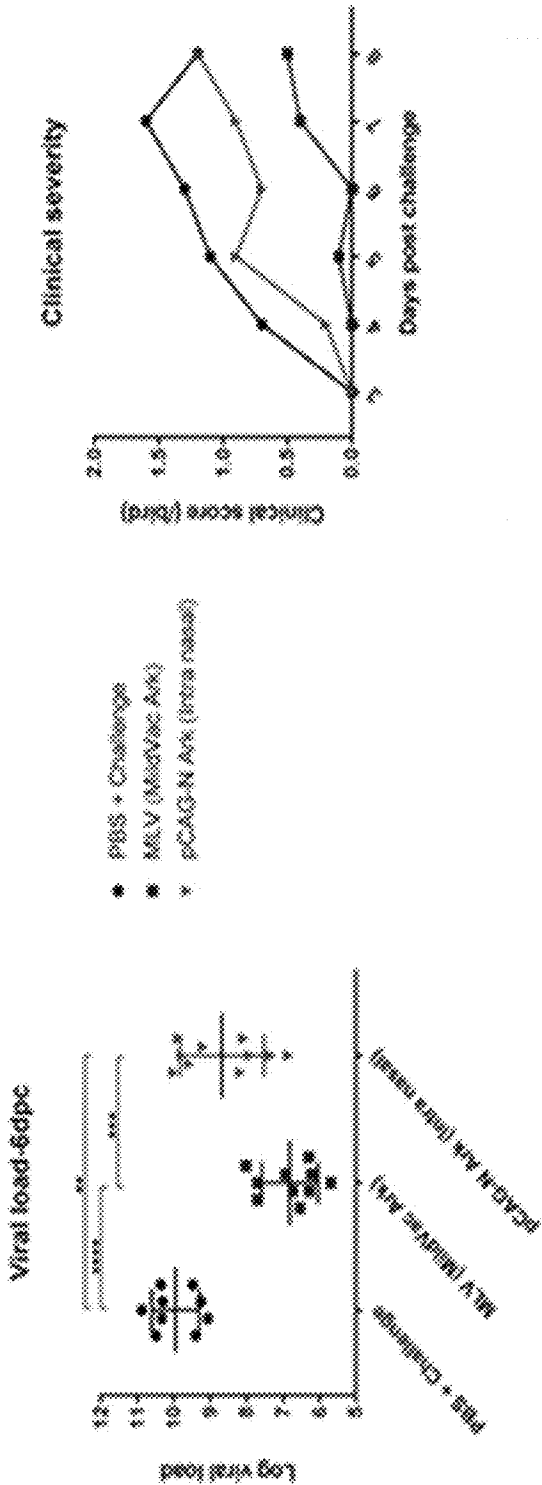


FIG. 3 CONTINUED

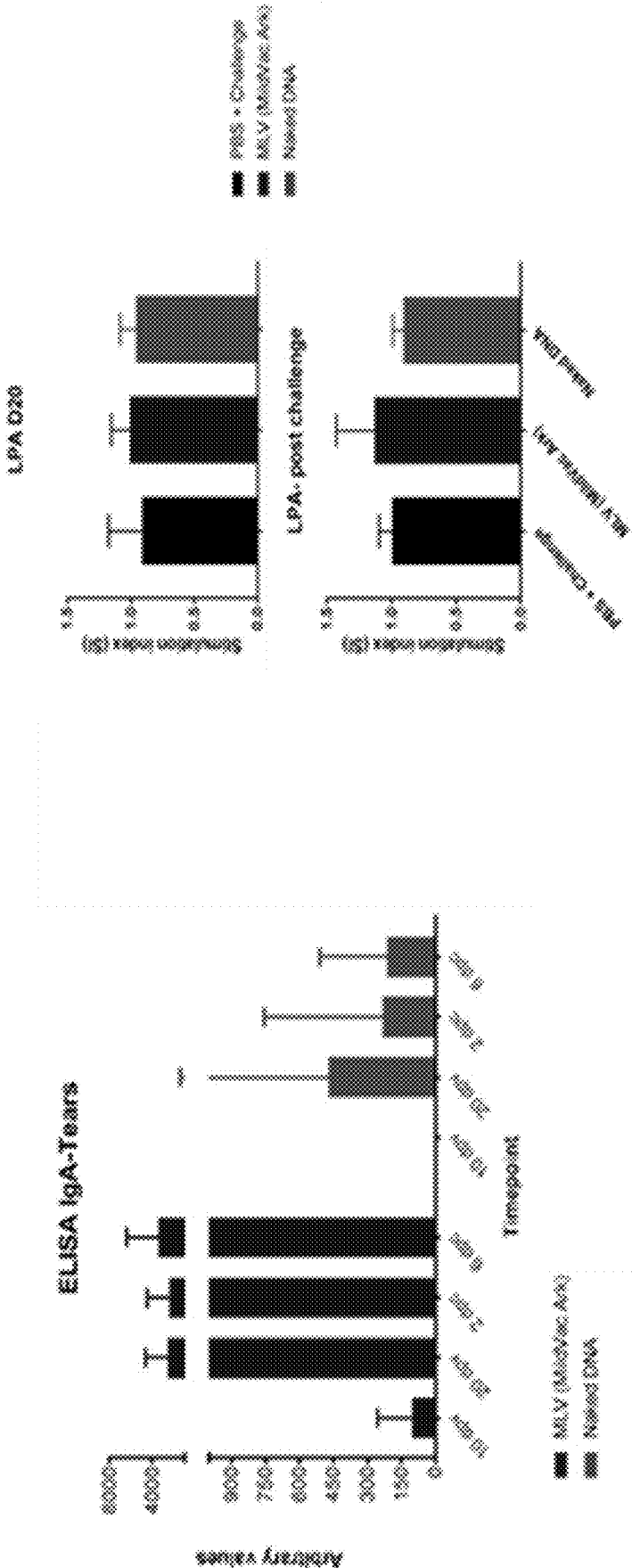


FIG. 4

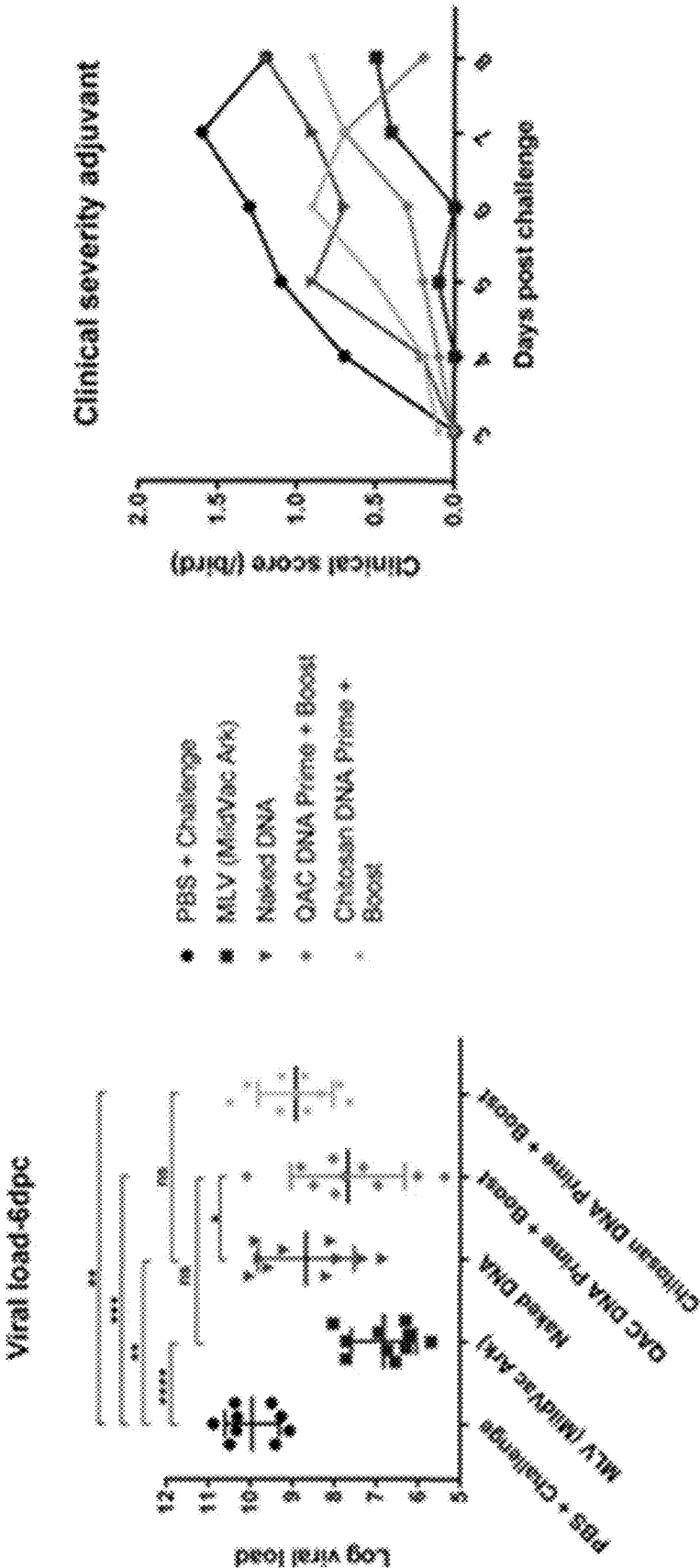


FIG. 4 CONTINUED

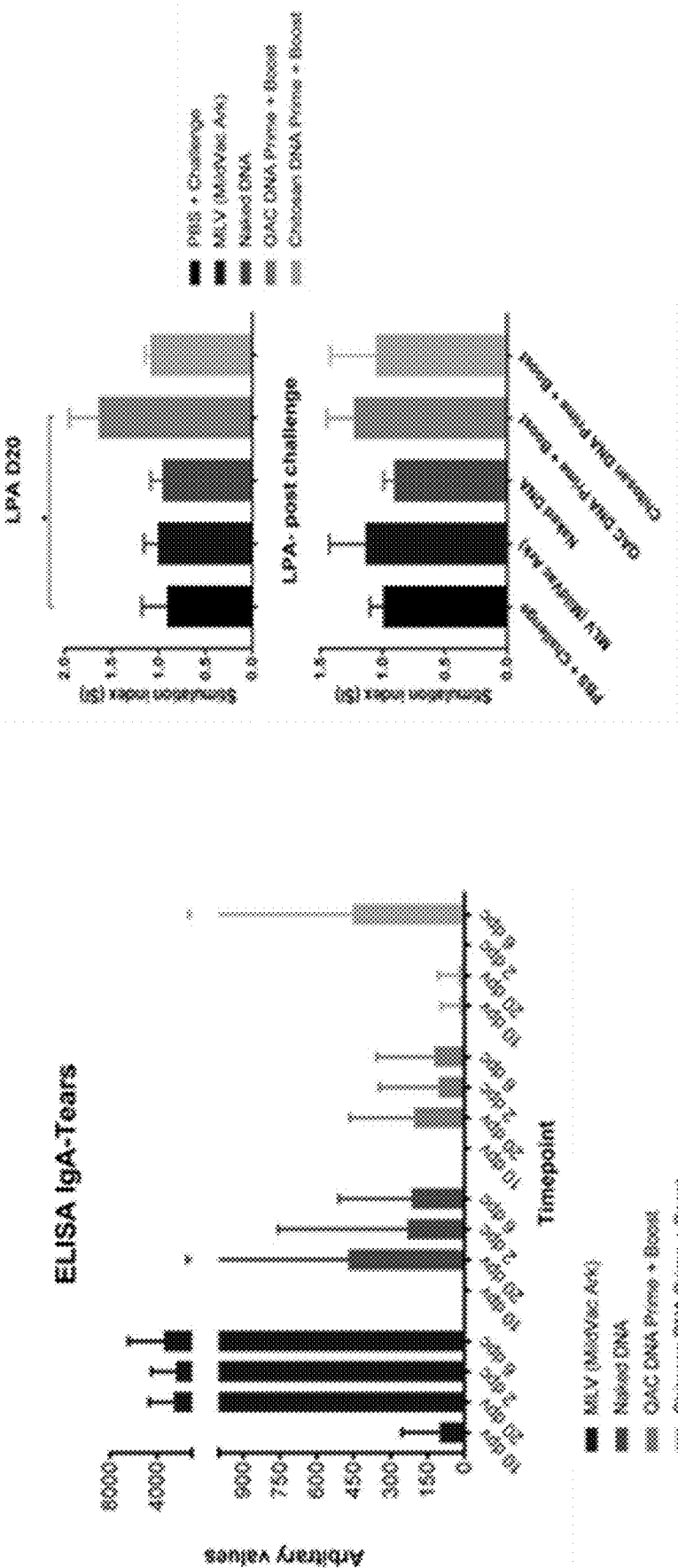


FIG. 5

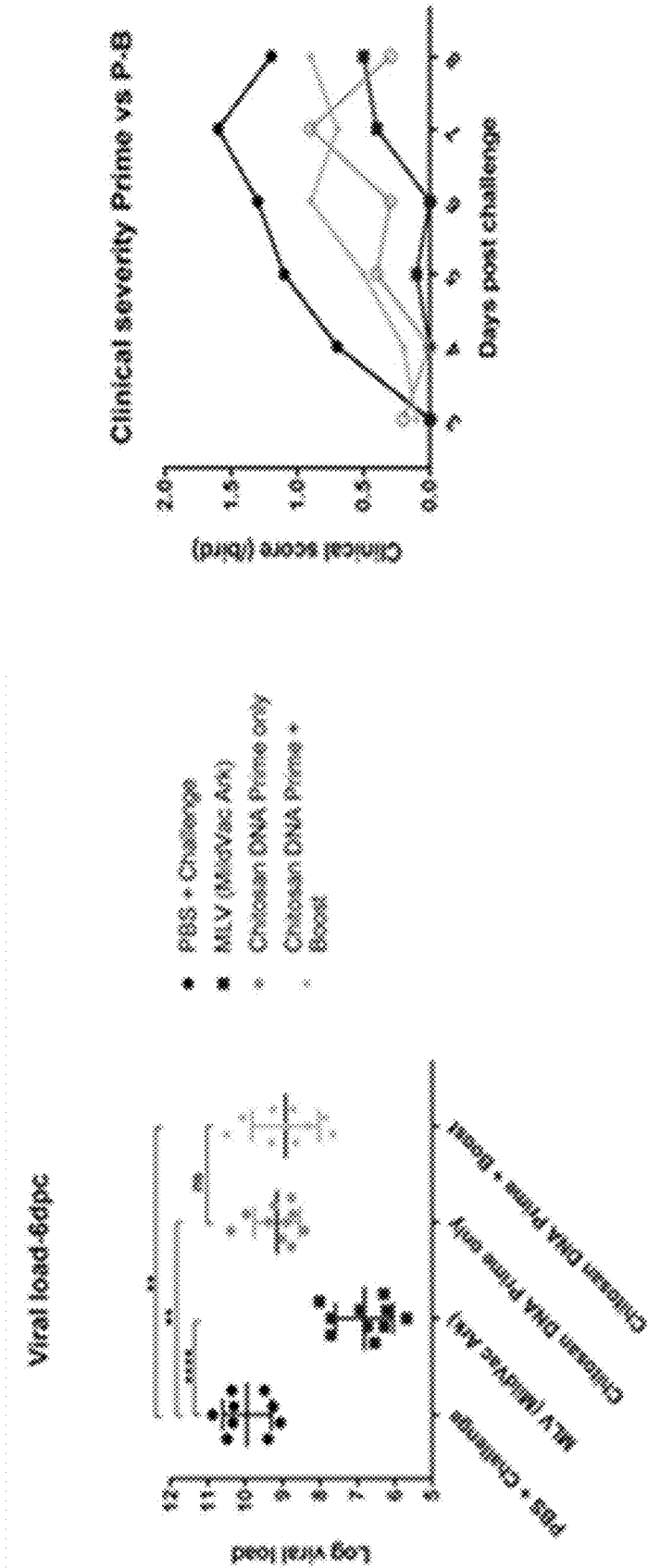


FIG. 5 CONTINUED

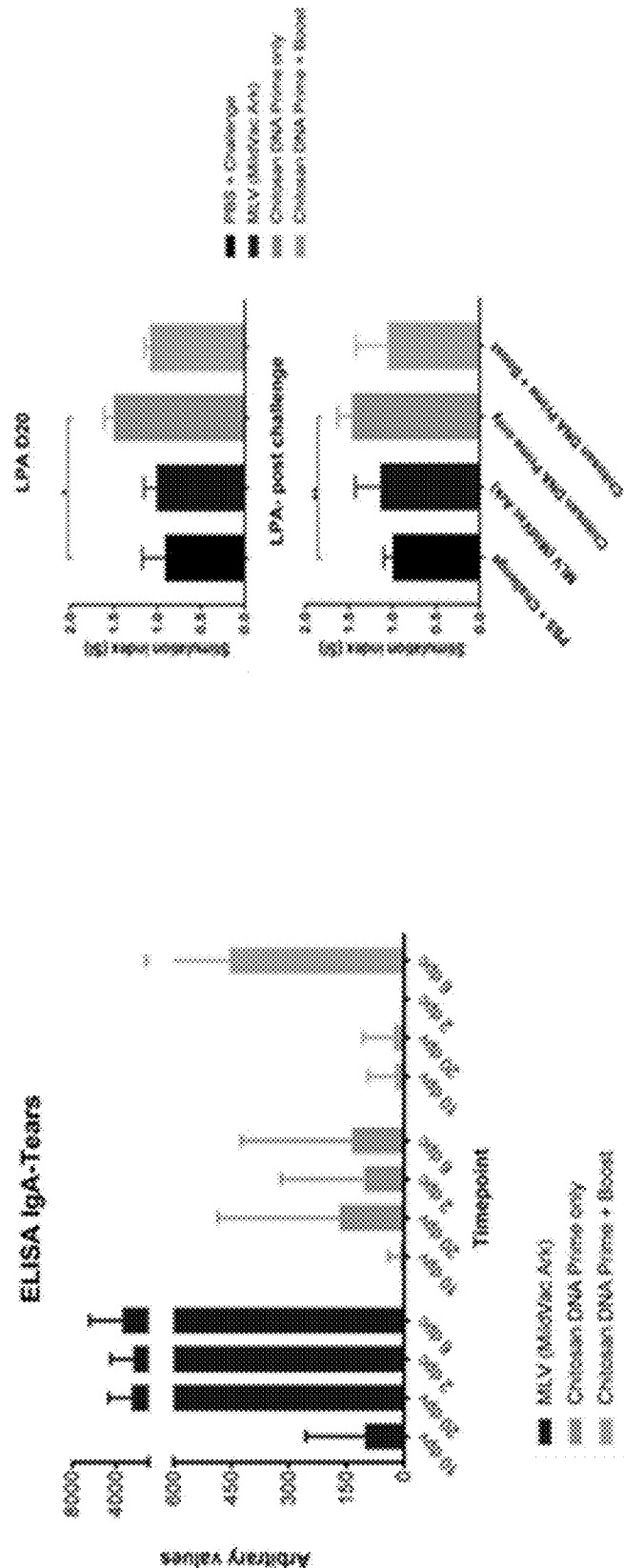


FIG. 6

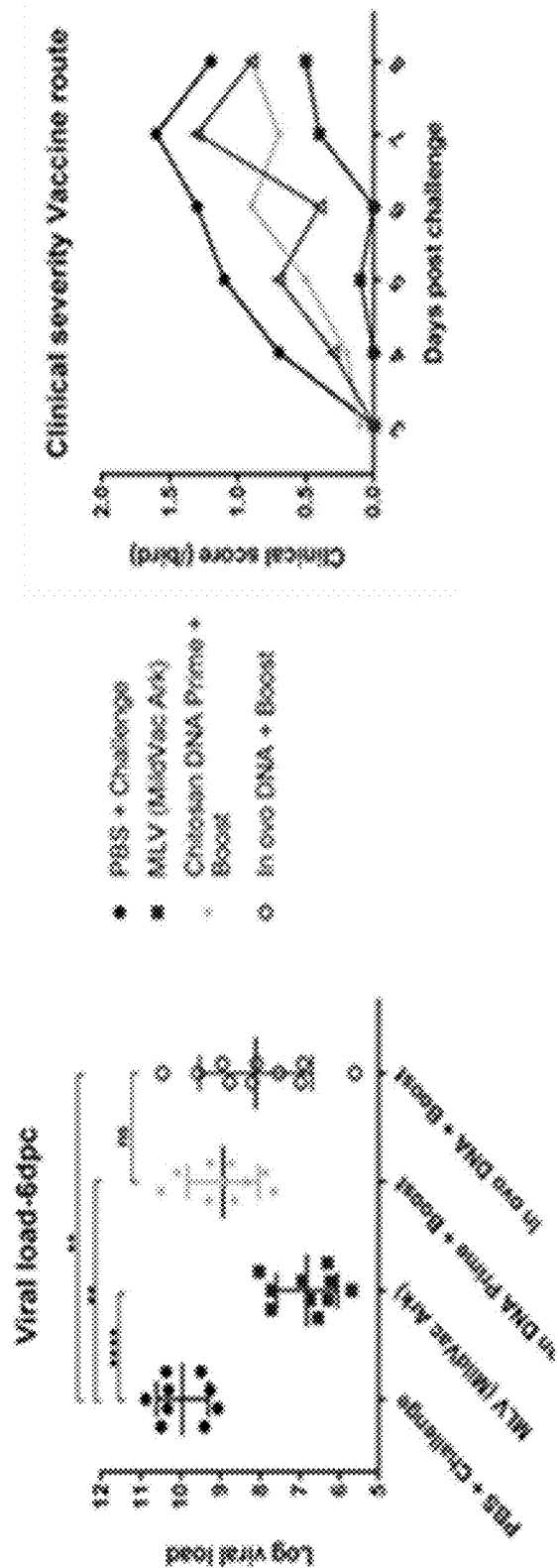
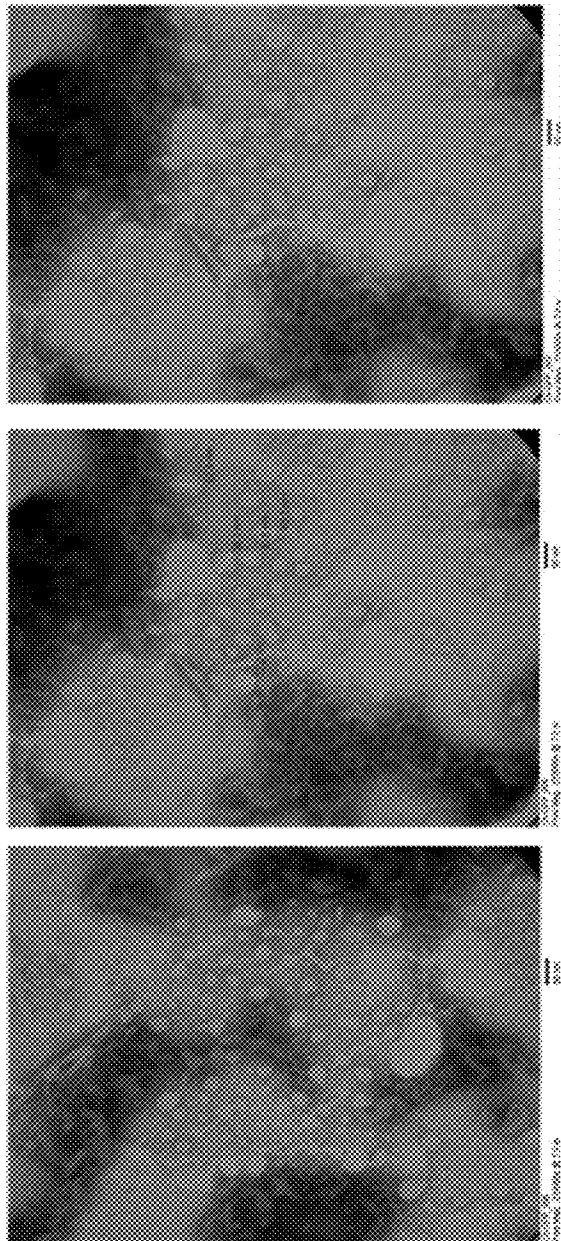


FIG. 7

TEM images (Chitosan-DNA complex 100 ug/ml)



DLS size measurement (Chitosan-DNA complex 100 ug/ml - left and 1000 ug/ml - right)

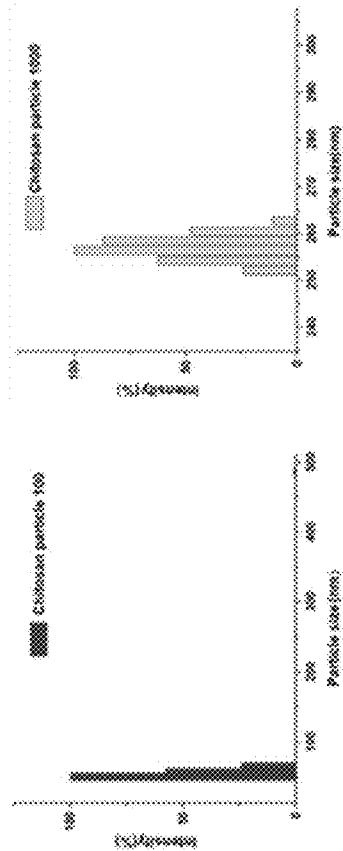


FIG. 8

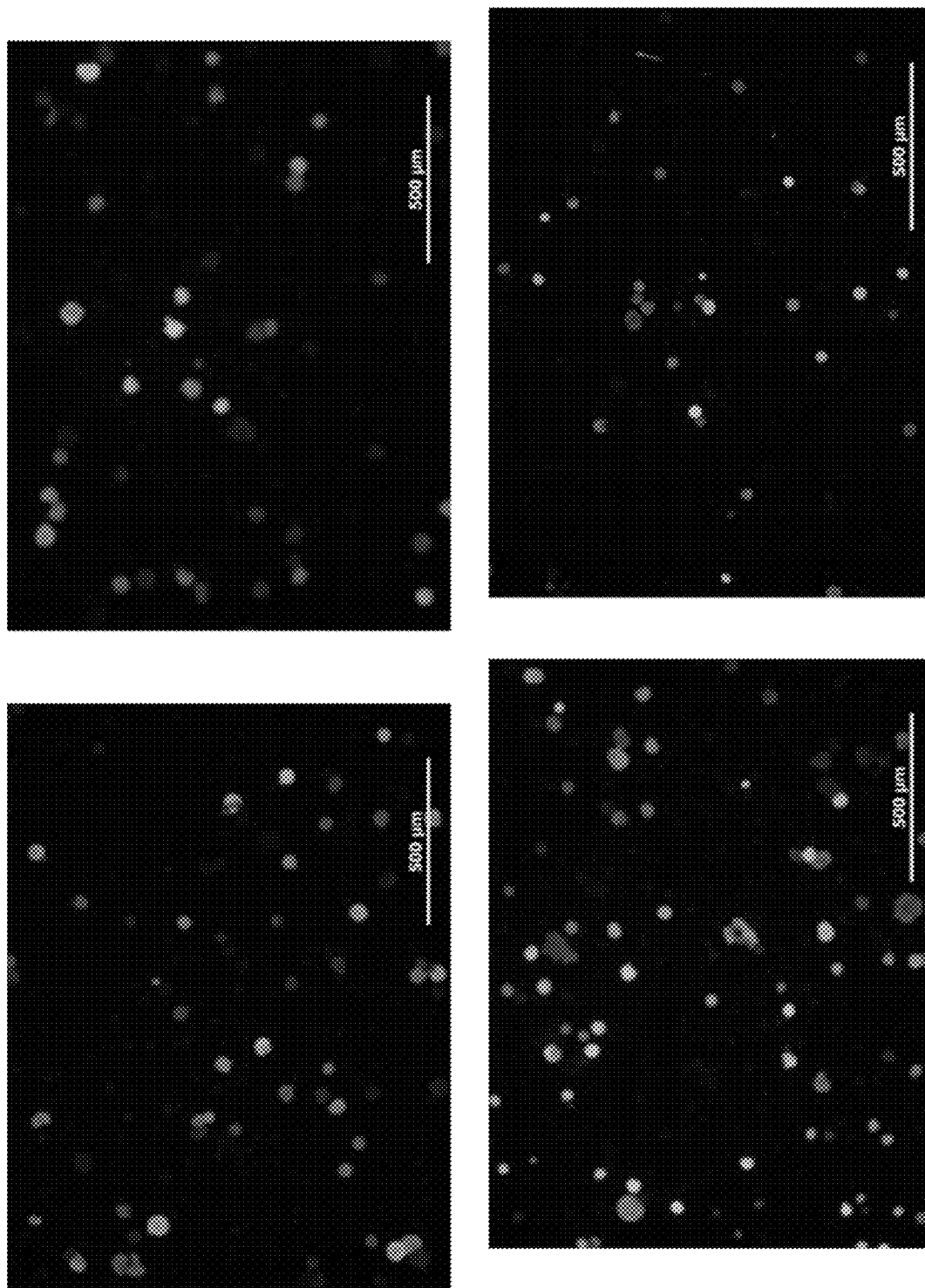


FIG. 9

DNA only control
DNA + Nuclease
Ct1 DNA + Nuclease method 1
Ct1 DNA + Nuclease method 2



FIG. 10

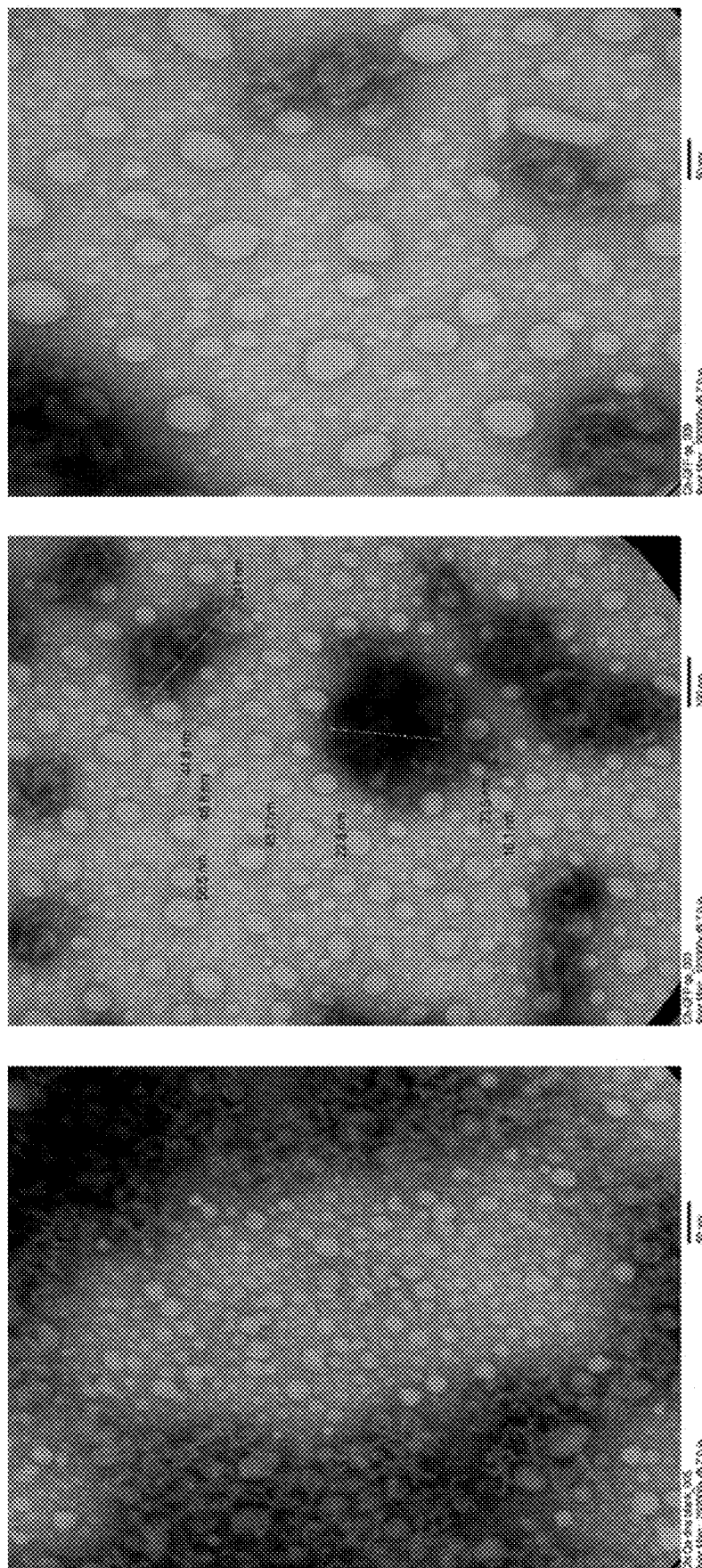


FIG. 11

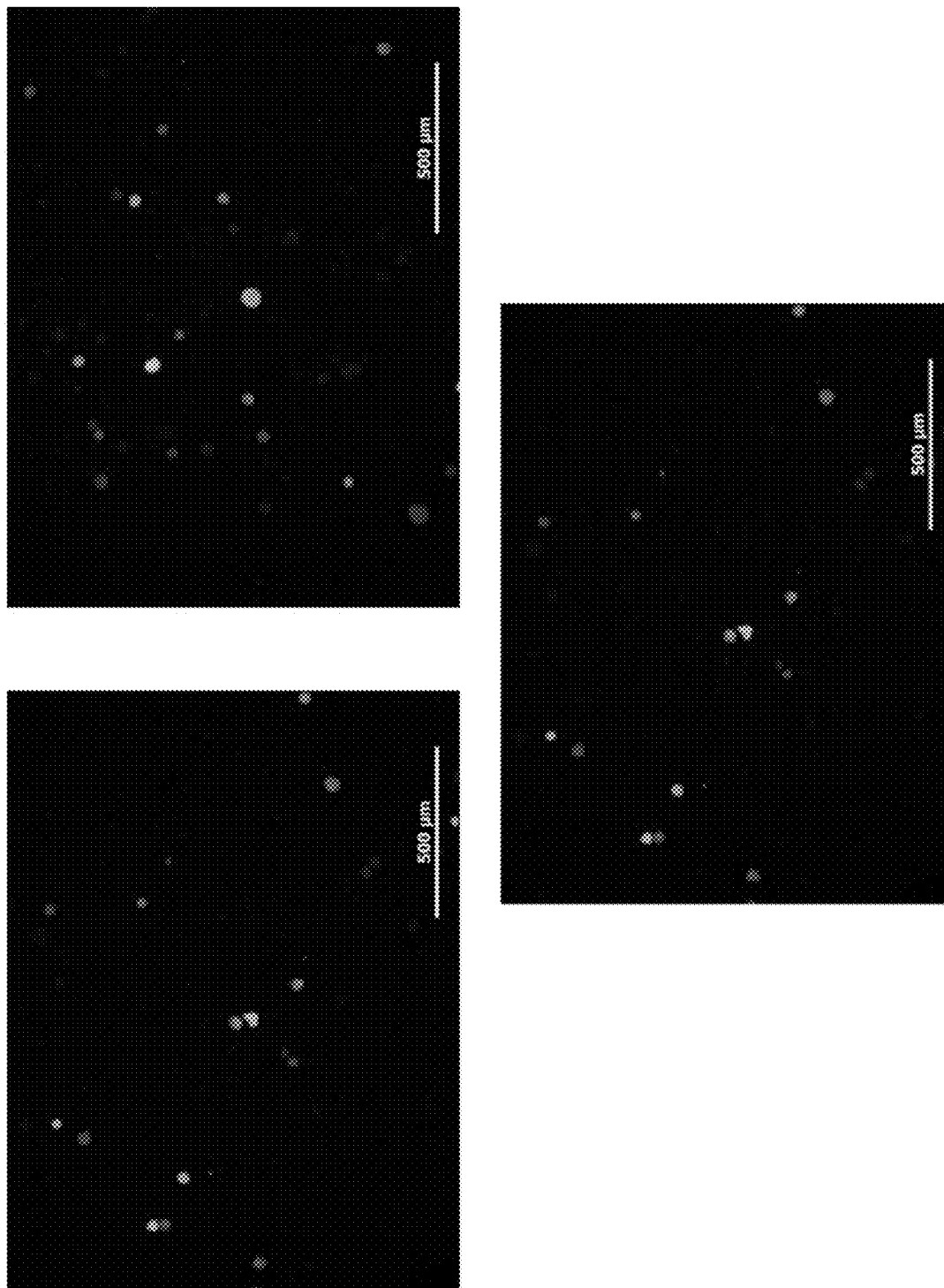


FIG. 12

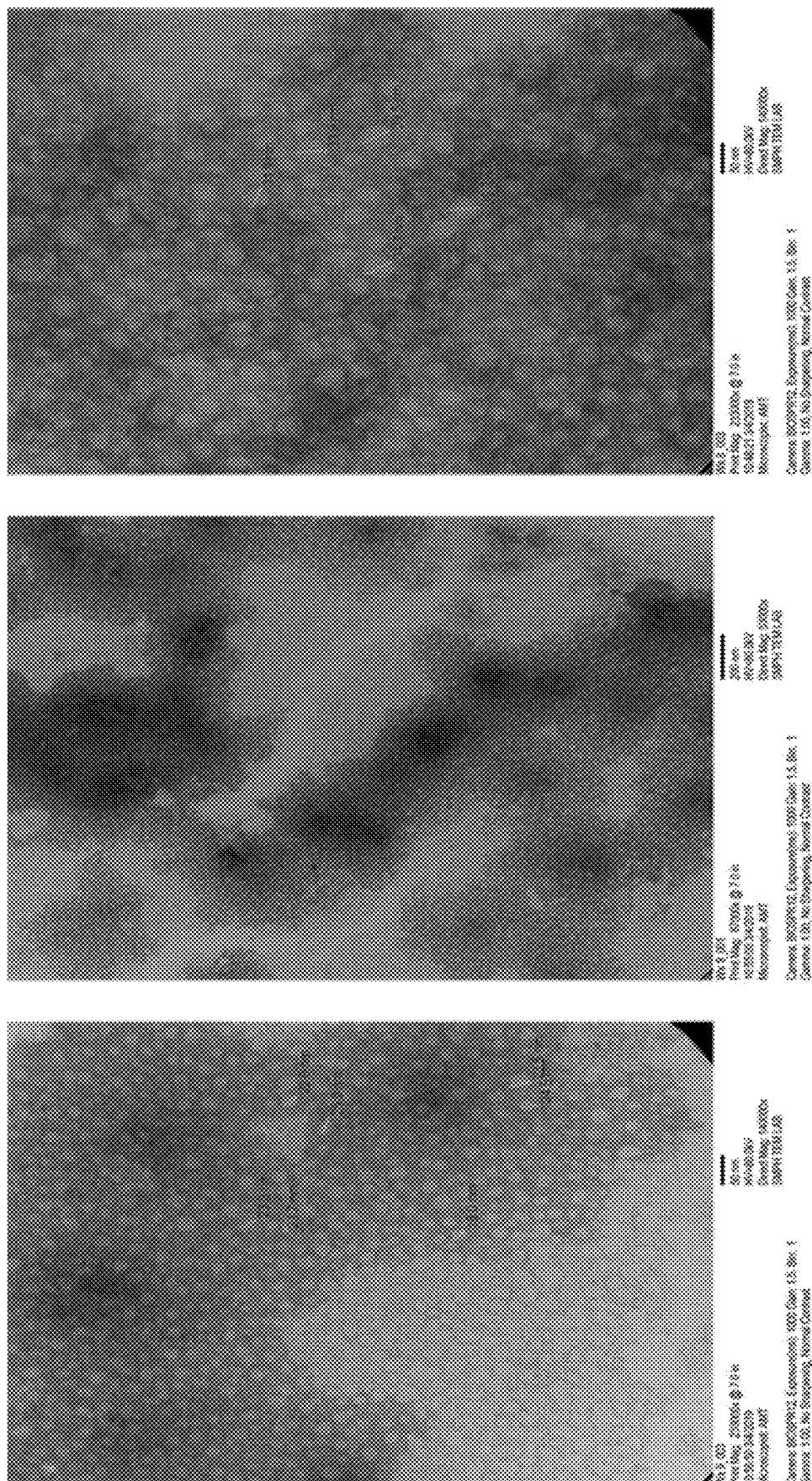


FIG. 13

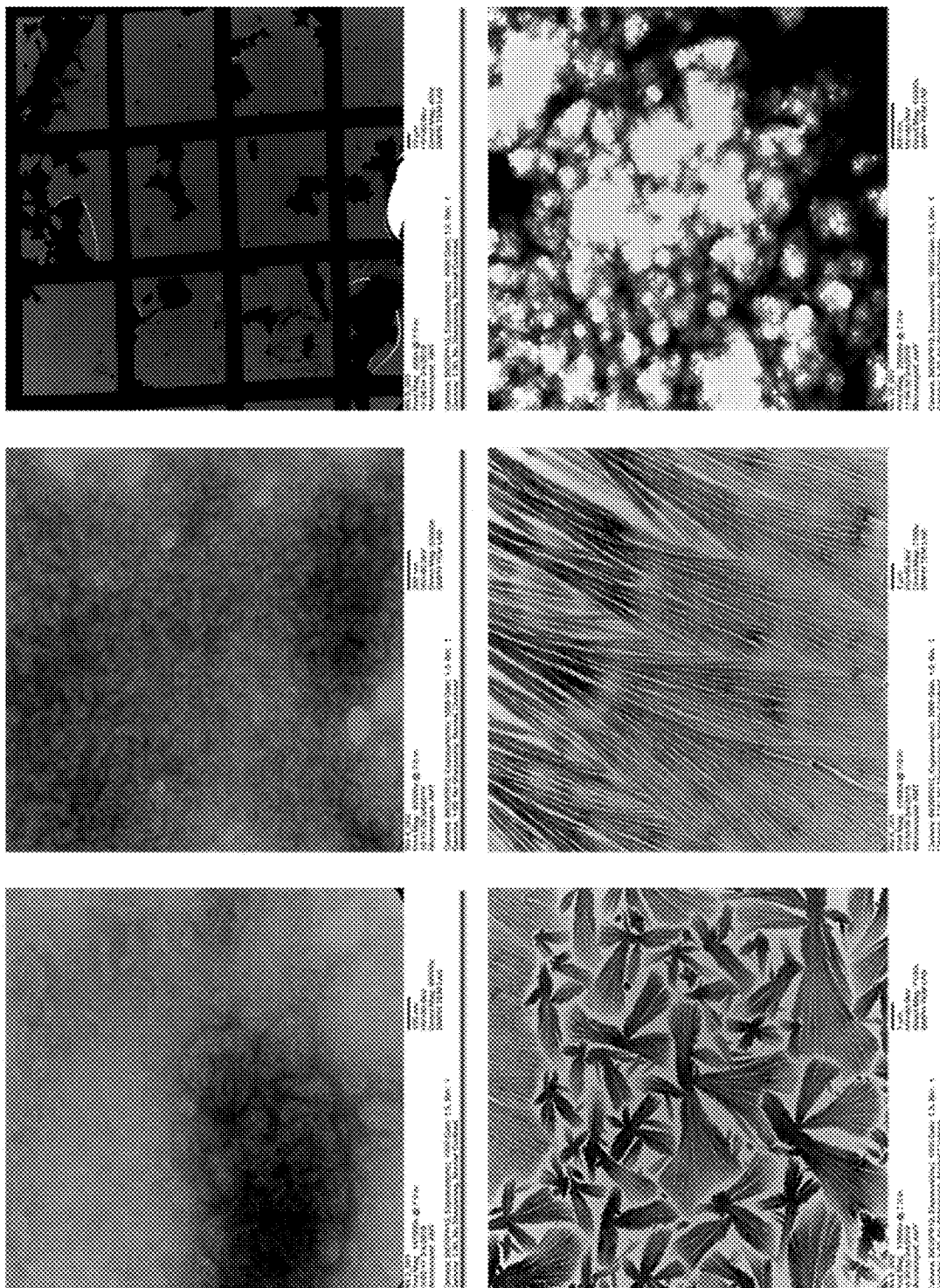
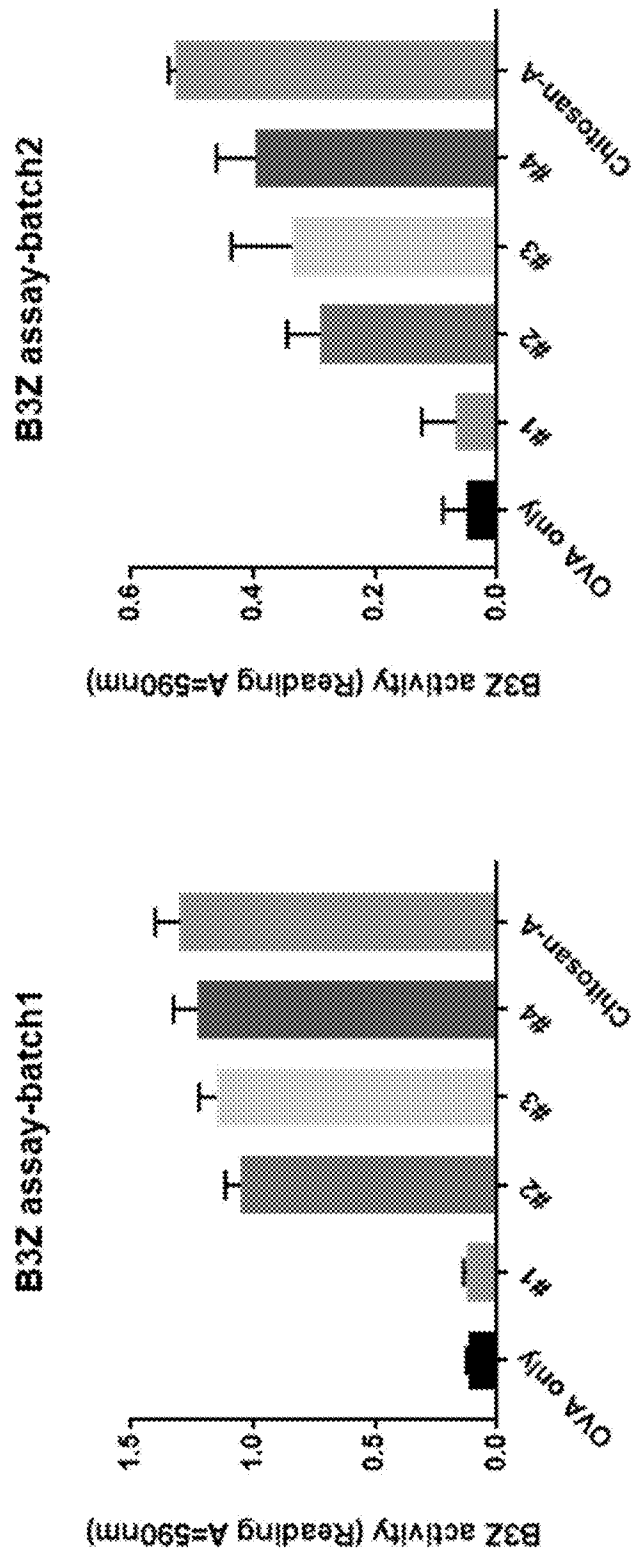


FIG. 14



Construct #	Quil-A conc (%)	Chitosan conc (%)
1	0.001	0.02
2	0.00025	
3	0.0000625	
4	0.000015625	

FIG. 15

Groups (n=4) BL6 mice

Adjuplex (control)

QAC conc. 1

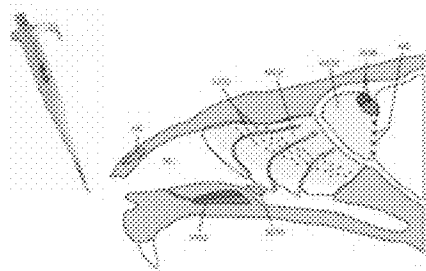
QAC conc. 2

Chitosan

Quil A

All with 30 μ g OVA protein

IN vaccinations 50ul



Mice

9 days

Examine phenotype
and functional
responses in BAL,
lungs, spleen

4 days

Data collection,
analysis, and report
complete

FIG. 16

Groups (n=7) BL6 mice

Adjuplex (control)

QAC conc. 1

QAC conc. 2

Chitosan

Quil A

-All with 30μg OVA protein for T cell responses

-All with 30μg DQ-OVA protein for innate cell processing

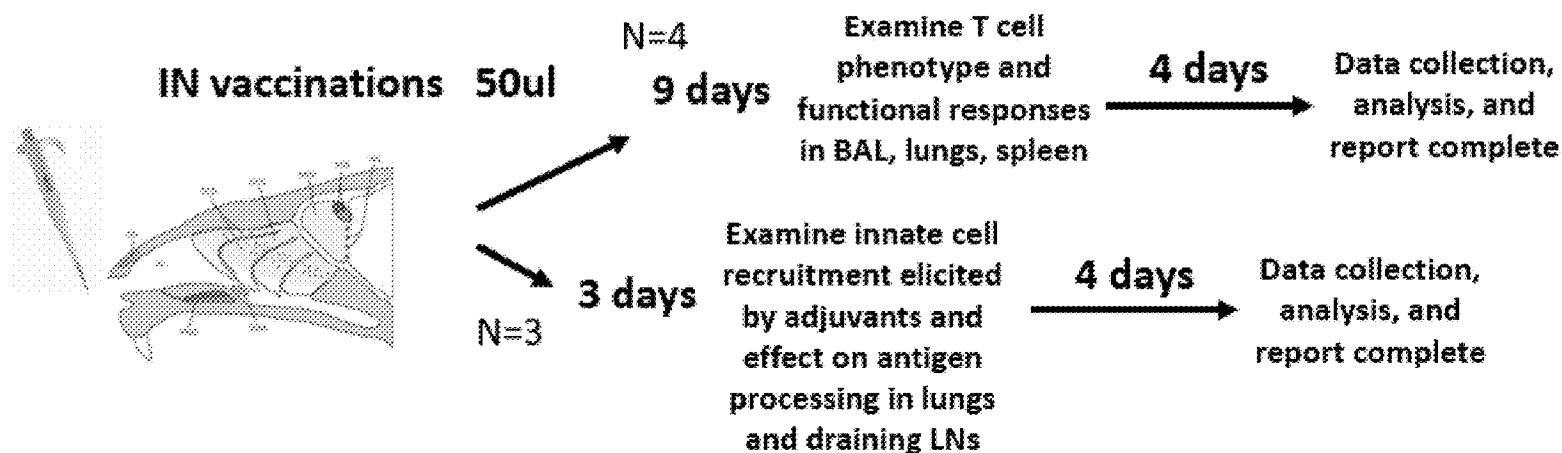
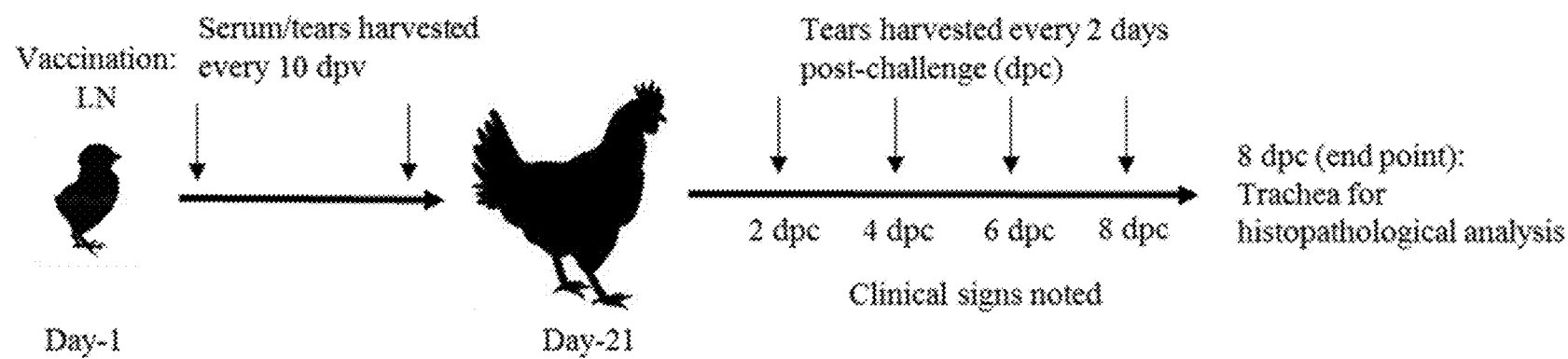
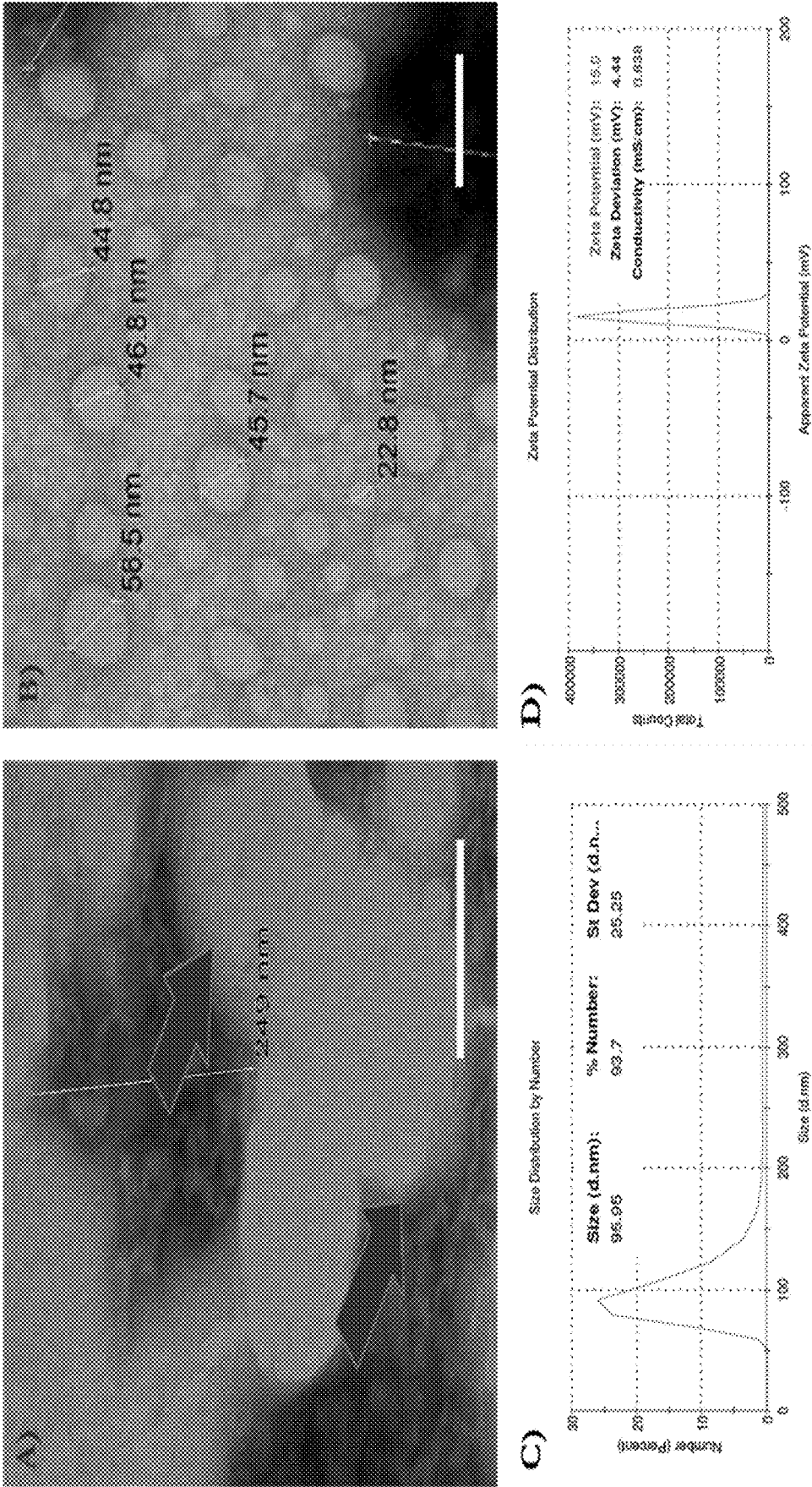


FIG. 17

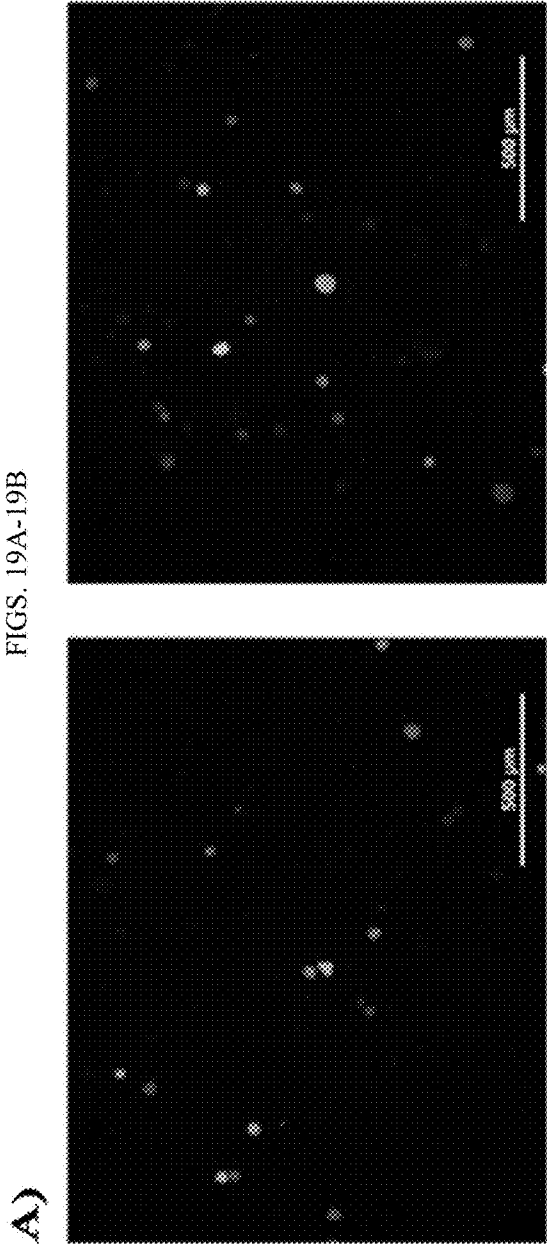


Groups	Vaccine construct (N=6)	Dosage
Group-1	Neg control	-
Group-2	Inactivated whole virus	30 ug
Group-3	Chitosan- IBV whole virus	30 ug
Group-4	QAC- IBV whole virus	30 ug

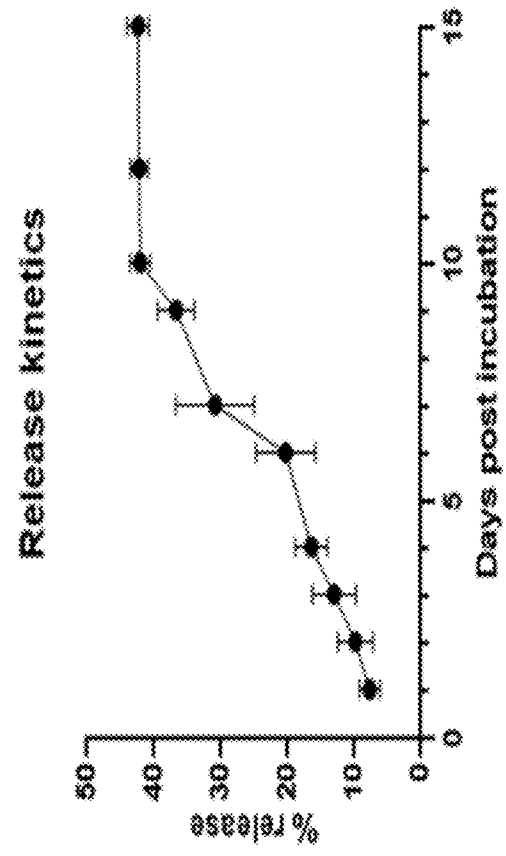
FIGS. 18A-18D



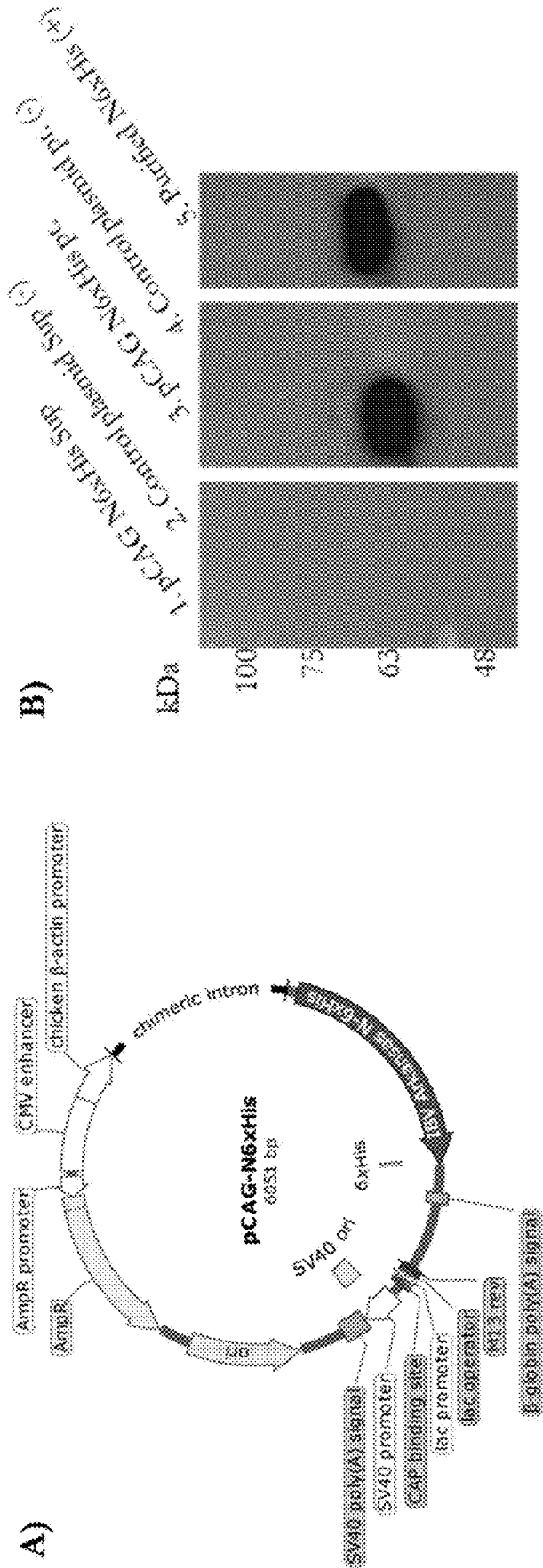
FIGS. 19A-19B



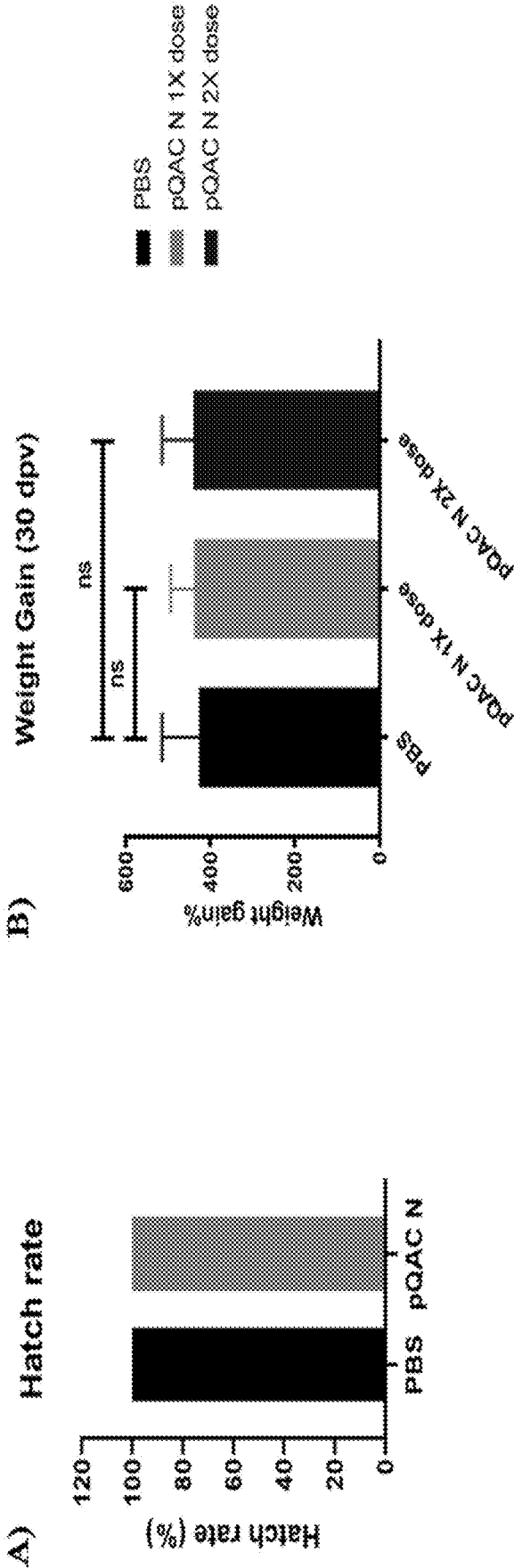
B)



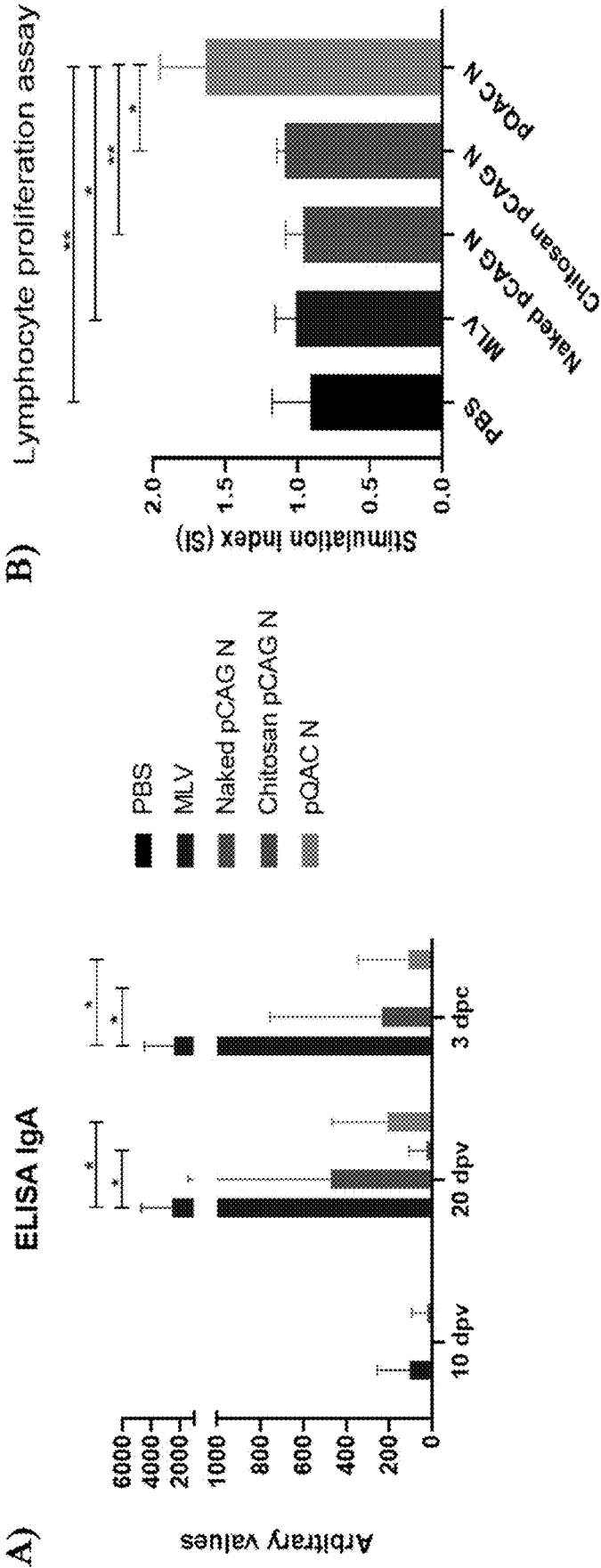
FIGS. 20A-20B



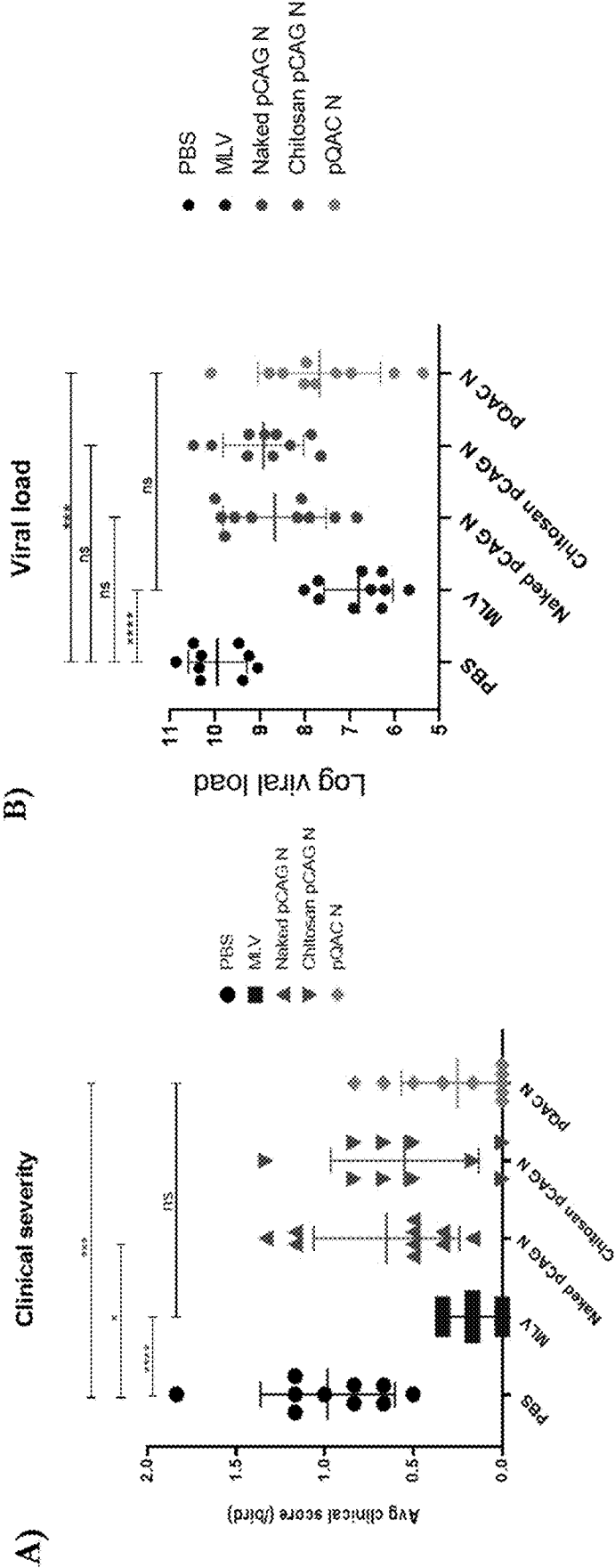
FIGS. 21A-21B



FIGS. 22A-22B

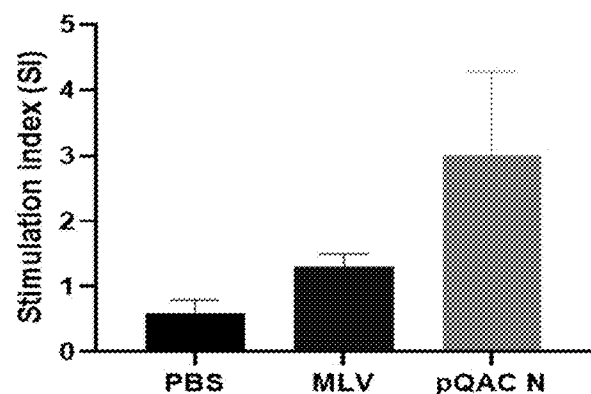


FIGS. 23A-23B

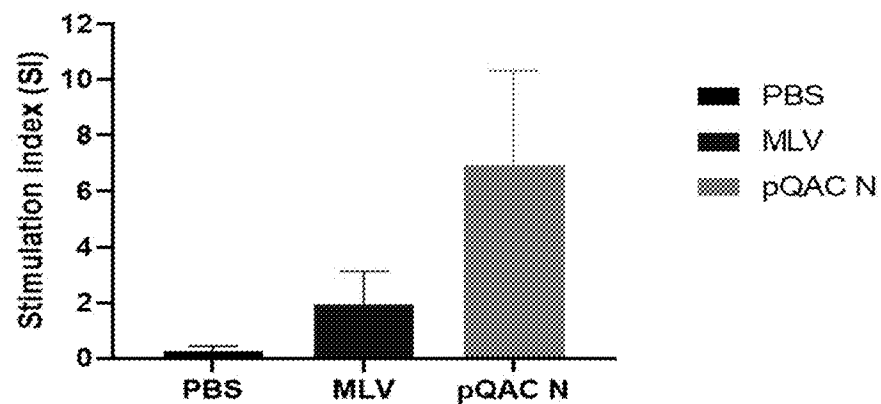


FIGS. 24A-24D

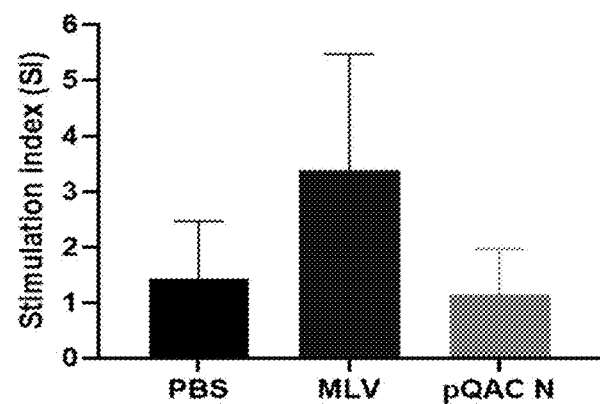
A) Lung lymphocyte proliferation



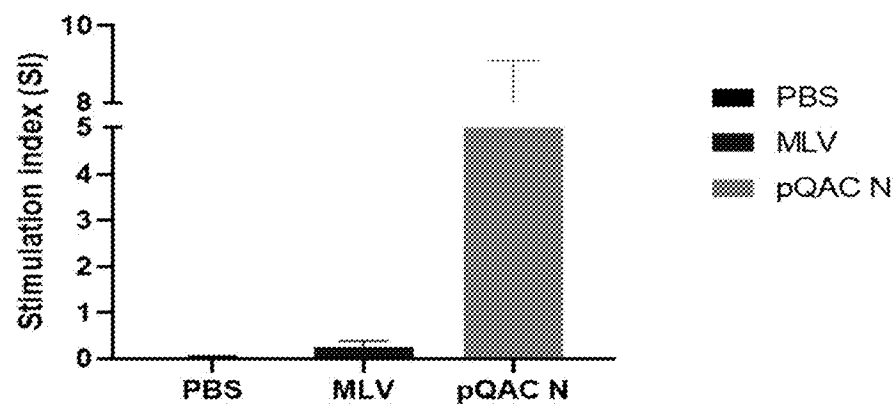
B) Lung CD8⁺ T-cell



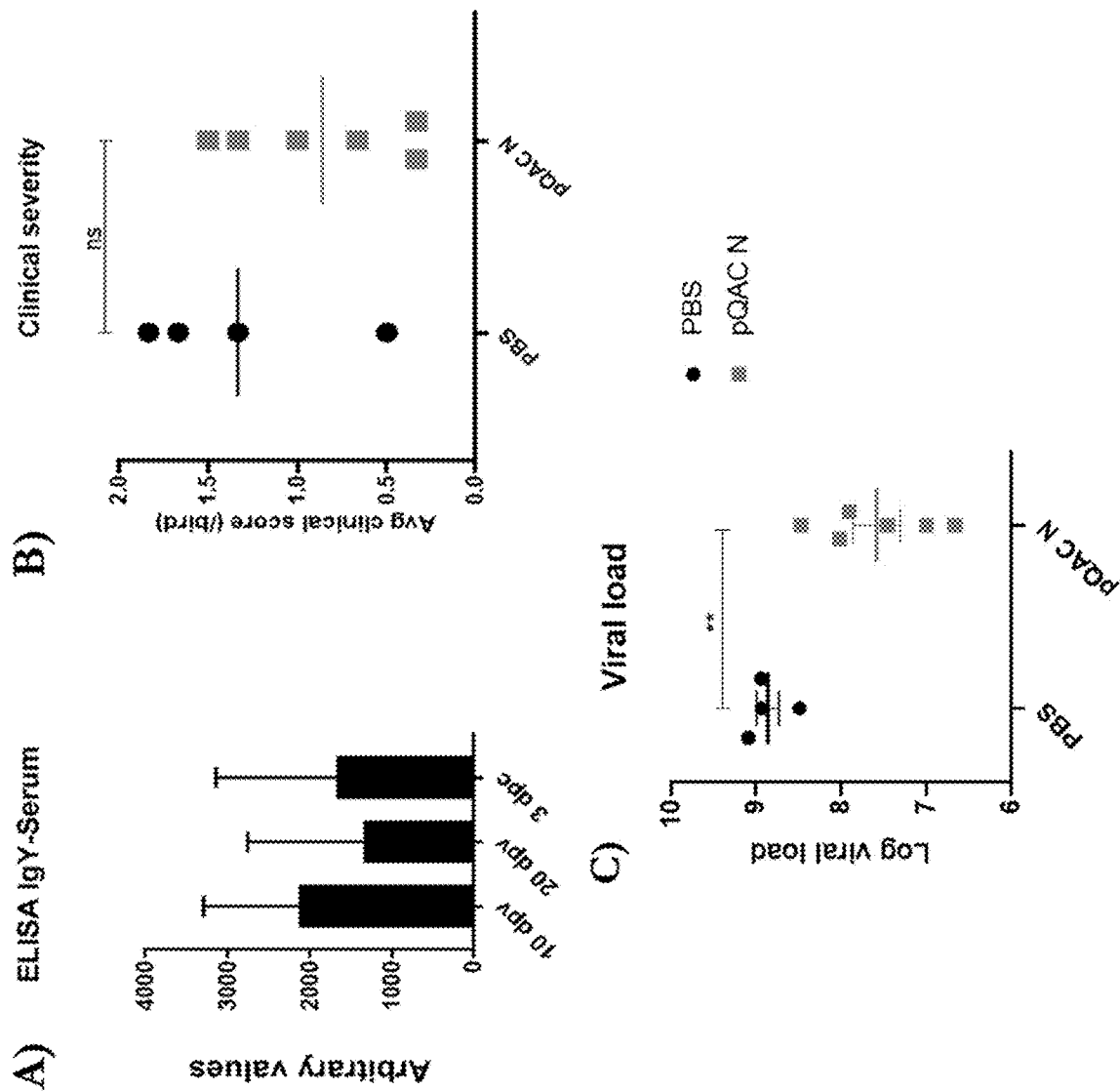
C) Lung CD4⁺ T-cell



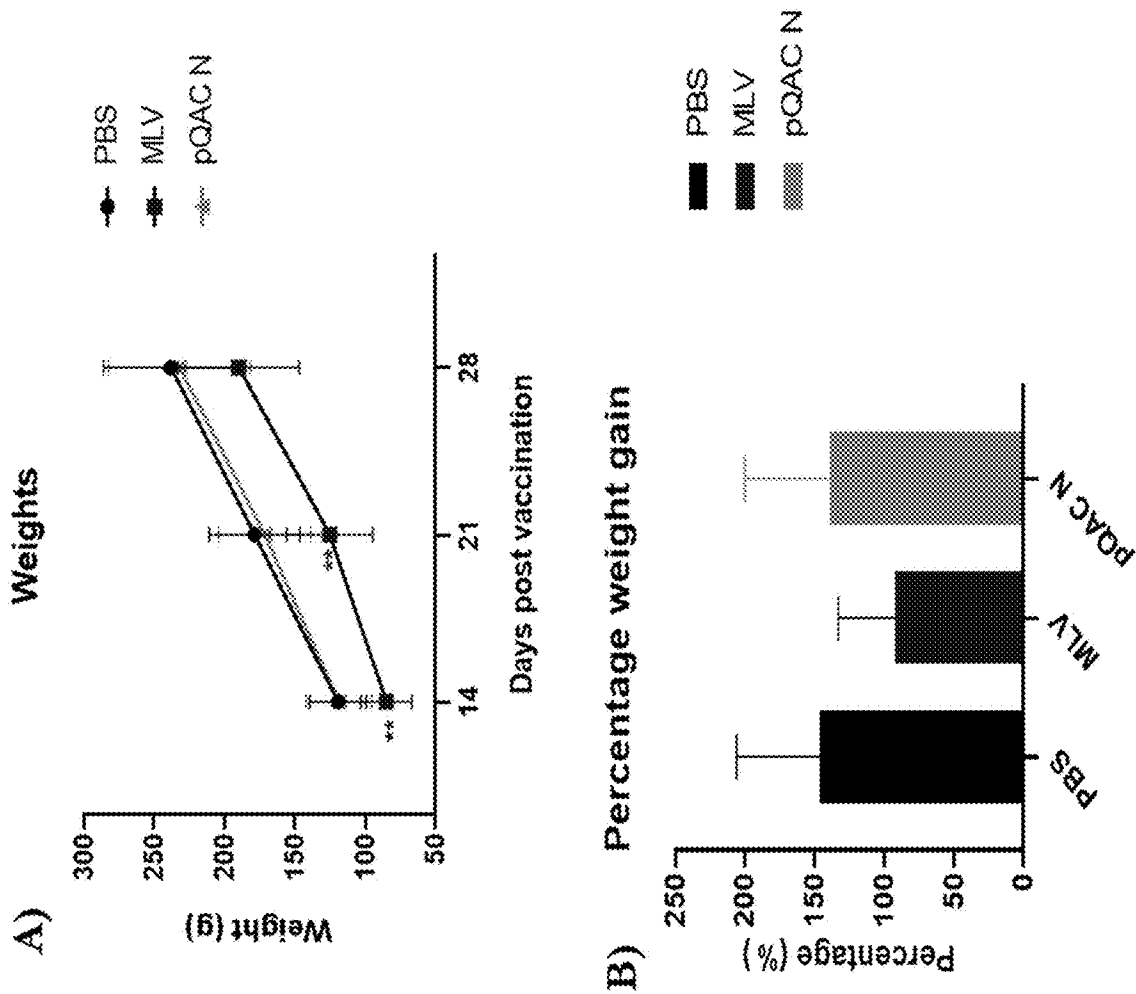
D) Lung TCRγδ cell



FIGS. 25A-25C



FIGS. 26A-26B



FIGS. 27A-27D

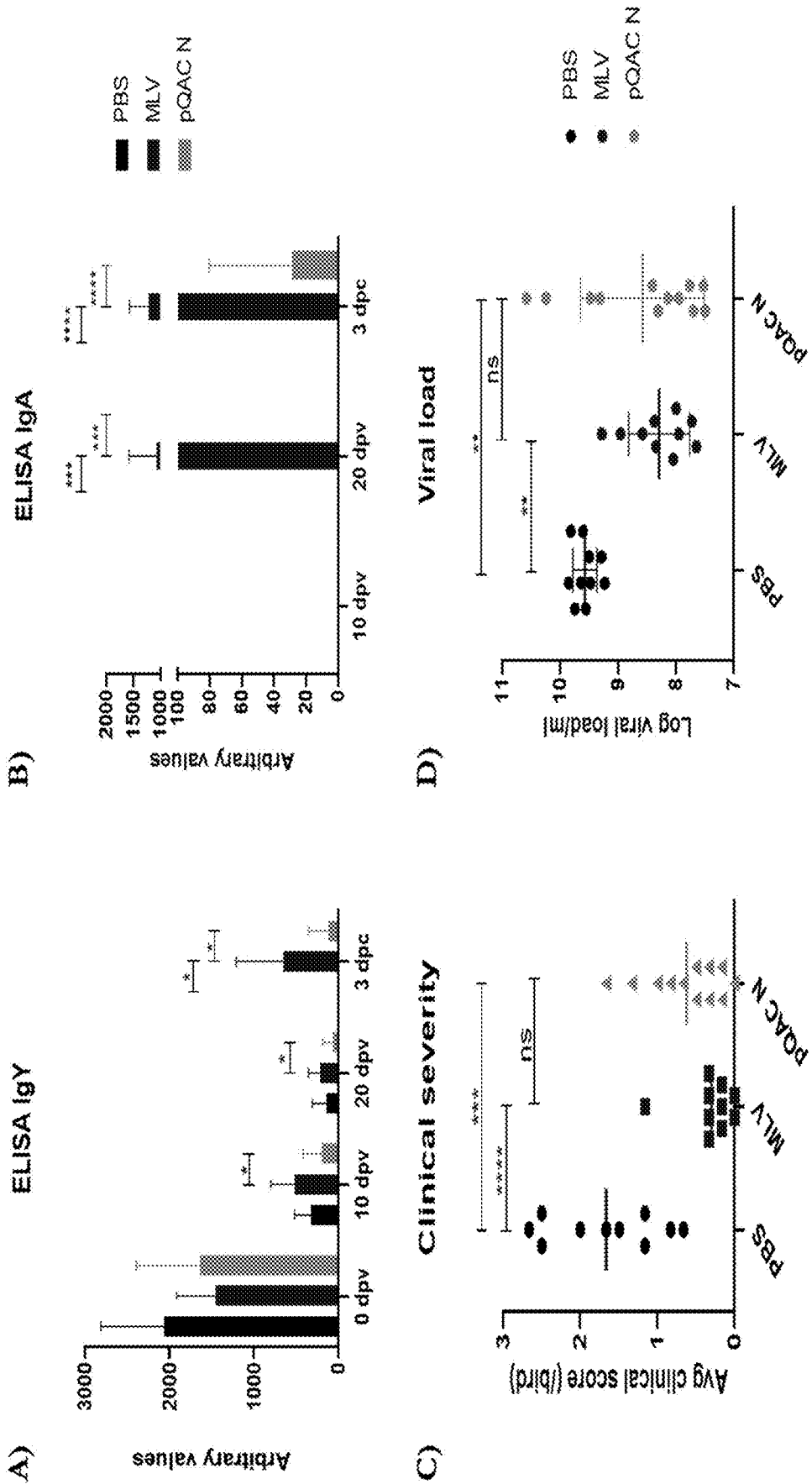


FIG. 28

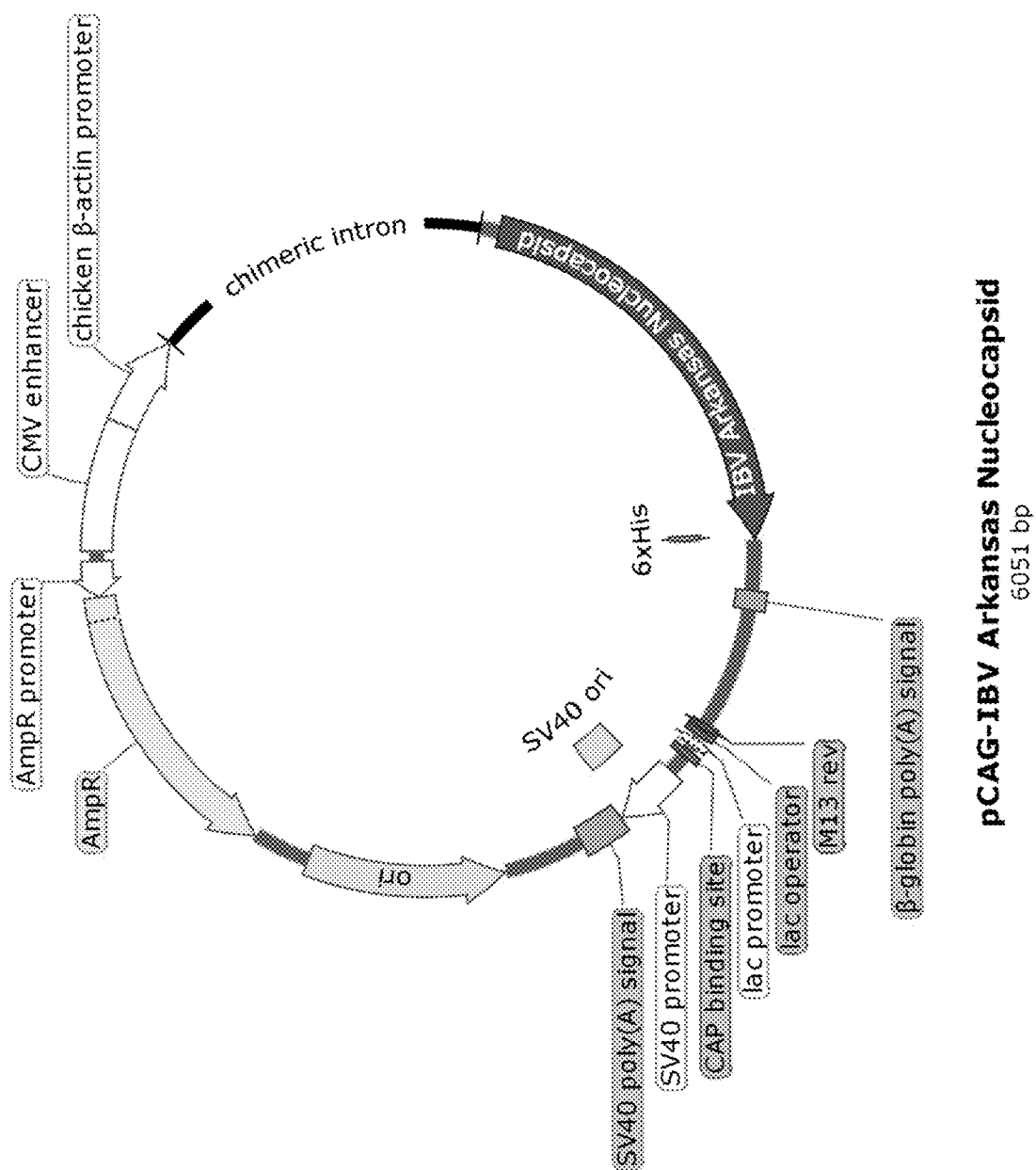
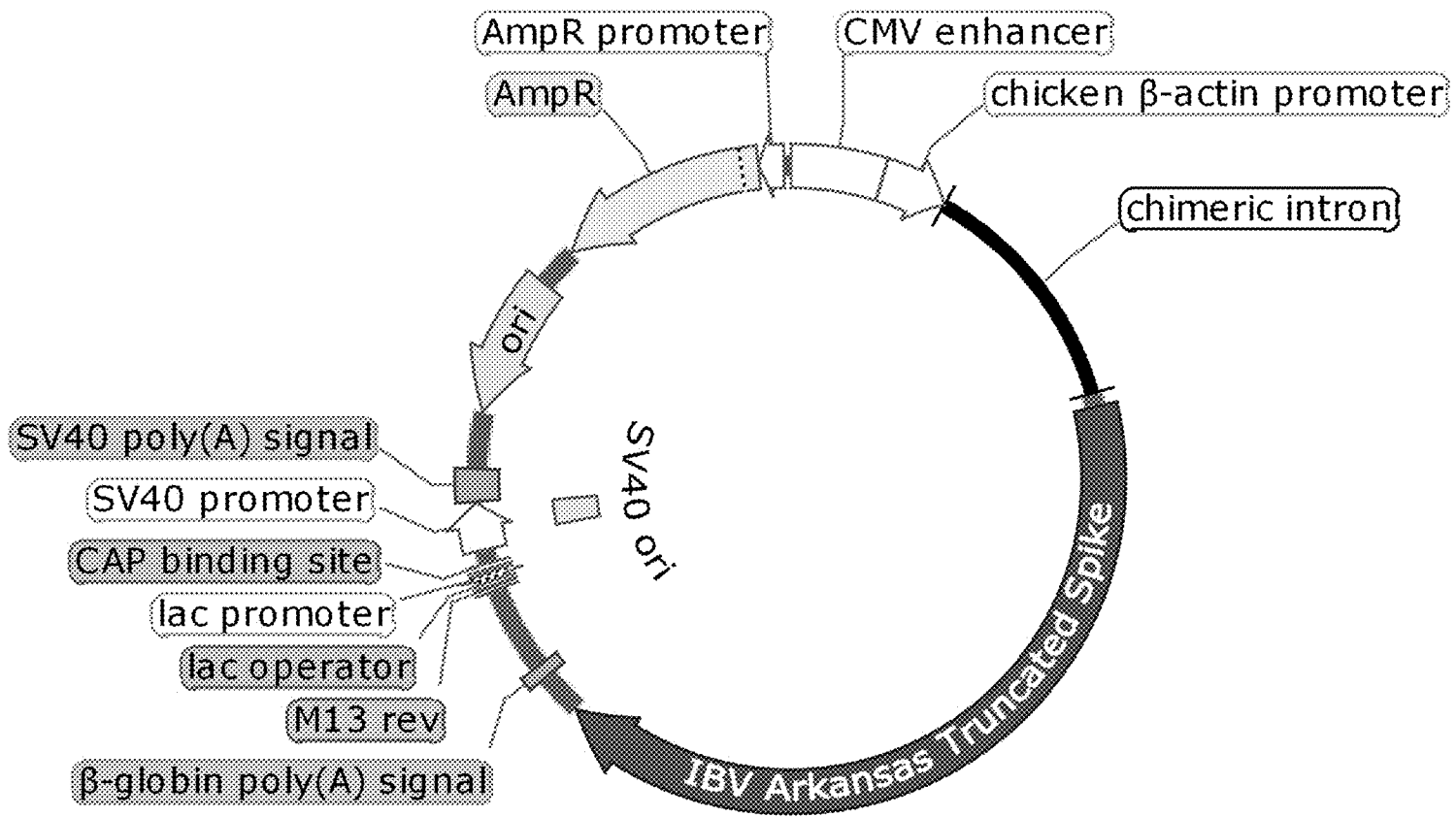
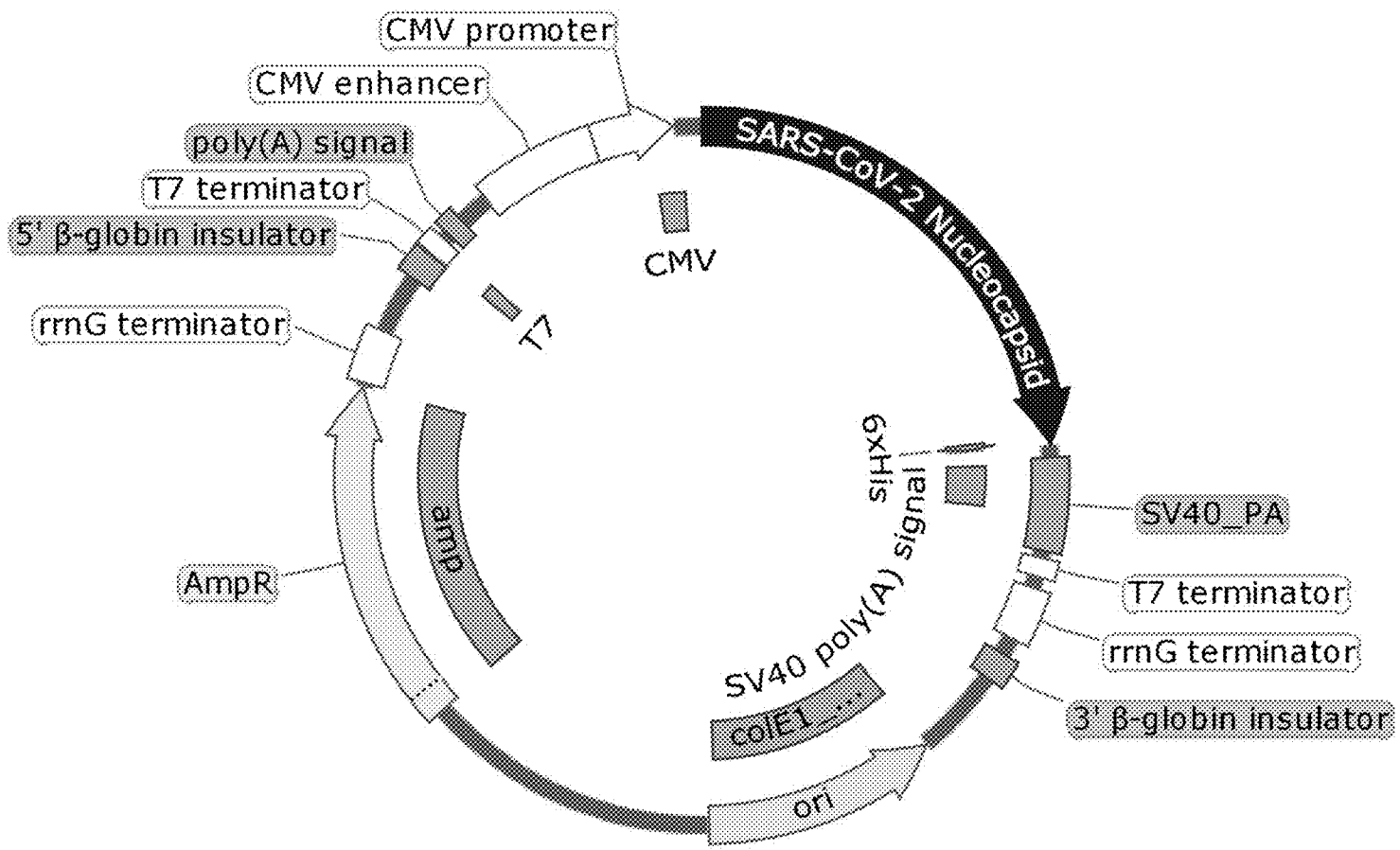


FIG. 29



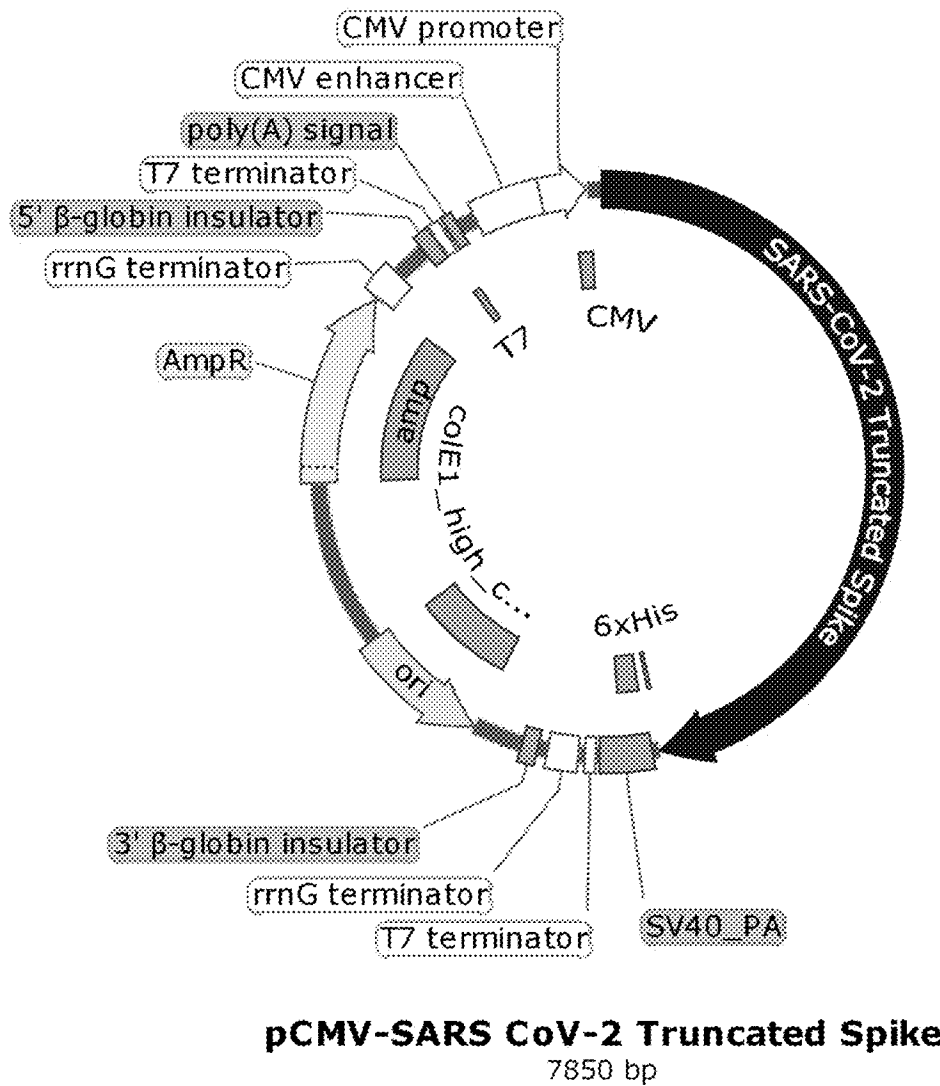
pCAG- IBV Arkansas Truncated Spike
8121 bp

FIG. 30



pCMV-SARS-CoV-2 Nucleocapsid
5471 bp

FIG. 31



NOVEL ADJUVANT FOR ANIMAL AND HUMAN VACCINES

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority to U.S. Provisional Application No. 62/860,640, filed Jun. 12, 2019, which is incorporated herein by reference in its entirety.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH

[0002] This invention was made with government support under 2016-67021-25042 awarded by the USDA/NIFA. The government has certain rights in the invention.

REFERENCE TO A SEQUENCE LISTING SUBMITTED VIA EFS-WEB

[0003] The content of the ASCII text file of the sequence listing named "960296_04039_ST25.txt" which is 126 kb in size was created on Jun. 5, 2020 and electronically submitted via EFS-Web herewith the application is incorporated herein by reference in its entirety.

BACKGROUND

[0004] Vaccines have been hailed as one of the greatest achievements in public health during the past century. Vaccines have been a key factor for fighting infectious diseases that afflict humans and animals, with corresponding increases in human average life expectancy. The global eradication of Smallpox virus in humans and Rinderpest virus in animals, and the near eradication or successful prevention of other viral or bacterial infections, for example meningitis in children due to *Hemophilus influenza* Type B, offer compelling examples.

[0005] Adjuvants play a key role in the successful use of vaccines in human and animal medicines. However, only a handful of such adjuvants are approved for human and animal use. Needed in the art are additional vaccine adjuvant compositions for the improvement of human and animal medicines.

SUMMARY OF THE INVENTION

[0006] In a first aspect, provided herein is a composition comprising disaggregated spherical nanostructures comprising Quil-A and chitosan. The disaggregated spherical nanostructures may additionally comprising a payload molecule.

[0007] In some embodiments, the payload molecule is selected from the group consisting of a DNA molecule, an RNA molecule, a polynucleotide, a protein, a polypeptide, a virus, a microbe, an attenuated virus, an attenuated microbe, a small molecule, an antibody, or a mixture thereof. In some embodiments, the payload molecule is negatively charged. In some embodiments, the payload is functionalized. In some embodiments, the payload molecule is an antigen specific for *Mycobacterium avium* subspecies *paratuberculosis*, *Mycobacterium bovis*, *Mycobacterium tuberculosis*, or *Mycobacterium avium* subspecies *avium*.

[0008] In some embodiments, the chitosan is functionalized by treatment with 5-formyl-2-furan sulfonic acid and sodium borohydride such that the chitosan surface is negatively charged.

[0009] In some embodiments, the spherical nanostructures are between about 5 nm and about 100 nm in diameter in the absence of a payload molecule.

[0010] In a second aspect, provided herein is a vaccine formulation comprising an antigen, the disaggregated spherical nanostructures comprising Quil-A and chitosan described herein as an adjuvant, and a pharmaceutically acceptable carrier.

[0011] In a third aspect, provided herein is a vaccine formulation comprising an antigen specific for *Mycobacterium avium* subspecies *paratuberculosis*, *Mycobacterium bovis*, *Mycobacterium tuberculosis*, or *Mycobacterium avium* subspecies *avium* and the disaggregated spherical nanostructures comprising Quil-A and chitosan described herein.

[0012] In a forth aspect, provided herein is a method of forming a composition comprising Quil-A chitosan spherical nanostructures, comprising the steps of heating a first solution comprising Quil-A at about 55° C. for about 30 minutes; heating a second solution comprising chitosan at about 55° C. for about 30 minutes; mixing equal volumes of the first and second solution dropwise to form a combined solution; vortex mixing the combined solution for about 30 seconds to form a combined, vortexed solution; and incubating the combined vortexed solution whereby a composition comprising Quil-A chitosan spherical nanostructures is formed.

[0013] In some embodiments, the combined vortexed solution is incubated at room temperature for about 1 hour. In some embodiments, the first solution additionally comprises a DNA antigen. In some embodiments, the combined vortexed solution is incubated at about 37 C with shaking at about 110 rpm for about 1 hour. In some embodiments, the first solution additionally comprises a protein antigen.

[0014] In some embodiments, the first solution comprises about 0.002% Quil-A and the second solution comprises about 0.04% chitosan. In some embodiments, the first solution and the second solution each have a pH between 5.5 and 7.0.

[0015] In a fifth aspect, provided herein is a composition comprising Quil-A chitosan spherical nanostructures produced by the methods described herein. In some embodiments the composition additionally comprises an antigen payload molecule.

[0016] In a sixth aspect, provided herein is a method of immunizing a subject against an antigen comprising the step of administering to the subject a vaccine formulation comprising a composition comprising Quil-A chitosan spherical nanostructures produced by the methods described herein and an antigen payload molecule. In some embodiments, the subject is selected from the group consisting of a human, a mouse, a rat, a cow, a horse, a pig, a goat, a sheep, a cat, a dog, or a bird.

INCORPORATION BY REFERENCE

[0017] All publications, patents, and patent applications mentioned in this specification are herein incorporated by reference to the same extent as if each individual publication, patent, and patent application was specifically and individually indicated to be incorporated by reference.

BRIEF DESCRIPTION OF DRAWINGS

[0018] The patent or patent application file contains at least one drawing in color. Copies of this patent or patent application publication with color drawings will be provided by the Office upon request and payment of the necessary fee.

[0019] FIG. 1 shows the formation of adjuvant nanostructures using Quil-A and chitosan with a DNA immunogen.

[0020] FIG. 2 shows parenteral immunization with naked DNA constructs. Partial protection was observed with naked DNA vaccines for parenteral administration. A partial reduction in viral burden and clinical signs of severity were observed with the naked IBV N vaccine construct, higher levels of circulating IBV specific IgY was observed in naked IBV N vaccinated groups vs spike subunit 1 glycoprotein (51), and mucosal IgA was not detected.

[0021] FIG. 3 shows intra nasal immunization with naked DNA constructs. Partial protection was observed with naked DNA vaccines for intra nasal administration. Partial reduction in viral burden and clinical signs of severity were observed with the naked IBV N vaccine construct, IBV specific mucosal IgA was detected in naked DNA vaccinated birds, and no appreciable circulating IgY responses were detected in birds vaccinated intra nasally.

[0022] FIG. 4 shows immunization with adjuvanted DNA constructs. Partial reduction in viral burden and clinical signs of severity observed were with chitosan adjuvanted DNA constructs compared with the naked DNA construct. Chitosan alone does not boost protection. More robust induction of mucosal IBV-specific IgA were observed with QAC adjuvanted DNA constructs. Higher reduction in viral burden and clinical severity were observed with QAC adjuvanted DNA construct with levels comparable to commercial vaccines. Significant proliferation of T-lymphocytes in response to antigen observed with birds vaccinated with QAC adjuvanted DNA construct. DNA constructs are more immunogenic and protective when adjuvanted with QAC. This demonstrated that the QAC complex adjuvant boosts protection and immunogenicity compared to that observed with naked DNA vaccines alone without adjuvant.

[0023] FIG. 5 shows a comparison of the prime (single dose of adjuvanted vaccine as day 1) and prime-boost (two doses of the adjuvanted vaccine 2 weeks apart at day 1 and day 14) strategies. No difference in reduction of viral burden and clinical severity, antibody and cell-mediated responses were observed between birds immunized with prime or prime-boost strategy.

[0024] FIG. 6 shows data comparing intra nasal and in ovo vaccination administration routes. Higher reduction in viral load was observed with birds vaccinated via the in ovo route, but the difference was not statistically significant. Higher antibody levels and cellular response was observed post vaccination and post challenge with in ovo group when compared to the intra nasal group. In ovo vaccination route might be a better route for vaccination because similar level of protection was observed (vs intra nasal) but this route is more easily scalable for large scale applications and can overcome interference by maternal derived antibodies (MDA) in commercial birds.

[0025] FIG. 7 shows TEM images of chitosan-DNA complexes and DLS size measurement of chitosan-DNA complexes.

[0026] FIG. 8 shows cells transfected with chitosan-pCAG-GFP complex.

[0027] FIG. 9 shows the results of a nuclease protection assay. DNA encapsulated in a chitosan-DNA complex was partially protected when treated with 5×DNase I.

[0028] FIG. 10 shows TEM images of empty QAC particles (left) and QAC particles with encapsulated DNA (middle and right, 30-60 nm).

[0029] FIG. 11 shows cells transfected with QAC-pCAG-GFP complex (100 ug/ml).

[0030] FIG. 12 shows images of QAC nanostructures at varying ratios of Quil-A:chitosan (0.001:0.02-.01).

[0031] FIG. 13 shows images of other structures formed by varying the ratio of Quil-A:chitosan in QAC complexes.

[0032] FIG. 14 shows the results of an in vitro antigen cross presentation assay (B3Z assay) using the QAC complex. QAC complex in vitro antigen cross presentation assay (B3Z assay)—Antigen cross presentation is important for effective immune responses to viral infections. Dendritic cells are professional antigen presenting cells and are unique in their ability to cross-present exogenous antigens on MHC class I molecules and activate antigen specific cytotoxic T cells. We tested the ability of Quil-A:Chitosan (QAC) particles encapsulating ovalbumin (OVA) protein to efficiently cross present by dendritic cells (DCs) in an in vitro system using soluble OVA. Ag cross-presentation of OVA257-264 was detected using the CD8 T cell hybridoma cell line B3Z that expresses β -galactosidase under control of the IL-2 promoter. Our preliminary results indicate that QAC can efficiently mediate cross presentation as seen across multiple Quil-A:Chitosan ratios. However, Quil-A is toxic to cells in vitro which could potentially explain a drop in absorbance values with increasing Quil-A concentrations. Adjuvants in vivo have been shown to work by inducing basal local cell damage releasing damage-associated pattern molecules (DAMPs). These DAMPs can further recruit immune cells enhancing antigen and presentation leading to a robust immune response.

[0033] FIG. 15 shows an in vivo cross presentation study in mice using the QAC complex loaded with ovalbumin protein (OVA).

[0034] FIG. 16 shows an in vivo study to measure innate and adaptive immune responses following treatment with the QAC complex loaded with OVA.

[0035] FIG. 17 shows an in vivo study measuring protein immunogen immune response in chickens treated with the QAC complex loaded with whole inactivated infectious bronchitis virus (IBV).

[0036] FIGS. 18A-18D show the nano structure of QAC adjuvant system. A) Aggregates of chitosan-pCAG-GFP preparation (arrows) were seen with TEM. B) Nanoparticles of QAC-pCAG-GFP preparation (arrows) with TEM. Scale bar=100 nm. C) Number-based DLS data and (D) Zeta potential on QAC-pCAG-GFP nanoparticles at 25° C. with Zetasizer® software.

[0037] FIGS. 19A-19B show QAC nanoparticle payload delivery and release. (A) GFP+Expi293F cells post addition of QAC-pCAG-GFP (B) Sustained release kinetics observed in vitro.

[0038] FIGS. 20A-20B show DNA vaccine construct. A) Plasmid map of pCAG-IBV Ark N, 6×His construct (pQAC-N) generated using Snapgene software. B) Western blot analysis with anti 6×His-HRP antibody confirming expression of N6×His from pCAG-N6×His plasmid. Lanes are as follows. Supernatant (lane 2) and pellet (lane 4) from Expi293F cells transfected with control pCAG plasmid,

Supernatant (lane 1) and pellet (lane 3) from Expi29F3 cells transfected with pCAG-N6xHis plasmid and purified N6xHis protein (lane 5).

[0039] FIGS. 21A-21B show safety of pQAC-N. A) Hatch rate % in ECEs inoculated with pQAC-N vaccine. B) Weight gain of chicks immunized with pQAC N construct after 30 days post vaccination (dpv). Data show means \pm SD. Significance (*, $P<0.05$) or non-significance (ns) was determined by one-way ANOVA with multiple comparisons.

[0040] FIGS. 22A-22B show pQAC-N vaccine immunogenicity. Groups of white leghorn SPF chicks were either unvaccinated (PBS) or immunized with MLV (day-1) or naked unadjuvanted pCAG-N or Chitosan complexed pCAG-N or pQAC-N vaccine (100 ug pQAC-N) at day-1 and day-14. (A) IBV specific IgA in tears, significance (*, $P<0.05$) was determined by two-way ANOVA and (B) lymphocyte proliferation assay on PBMCs harvested at day 20 post vaccination, significance (*, $P<0.05$; **, $P<0.01$) or non-significance (ns) was determined by one-way ANOVA with multiple comparisons. Data show means \pm SD.

[0041] FIGS. 23A-23B show protective efficacy of pQAC-N vaccine. Groups of white leghorn SPF chicks were either unvaccinated (PBS) or immunized with MLV (day-1) or naked pCAG-N or Chitosan pCAG-N or pQAC-N vaccine (100 ug) at day-1 and day-14. (A) Clinical sign severity represented as average score/bird over 8 days post challenge in each group (B) IBV log viral load/10 ul lachrymal fluid at 6 days post challenge. Significance (*, $P<0.05$; **, $P<0.01$; ***, $P<0.001$; ****, $P<0.0001$) or non-significance (ns) was determined by one-way ANOVA with multiple comparisons. Data show means \pm SD.

[0042] FIGS. 24A-24D show pQAC-N induces a robust T-cell response. (A) Lung cell proliferative capacity measured by CellTrace Violet dye dilution in unvaccinated, MLV and pQAC N vaccinated chickens. Proliferation was measured in (A) total lung cells, (B) CD8 α +, (C) CD4+ and (D) TCR $\gamma\delta$ + lung T cells after 4 days in culture post antigen stimulation.

[0043] FIGS. 25A-25C show protective efficacy of pQAC-N vaccine in commercial birds. Groups of commercial white leghorn chicks were either unvaccinated (PBS) or immunized with pQAC N vaccine (100 ug) at day-1 and day-14. (A) Serum IgY ELISA titres indicating presence of high levels of MDA (B) Clinical sign severity represented as average score/bird over 8 days post challenge in each group (C) IBV log viral load/10 ul lachrymal fluid at 6 days post challenge. Significance (*, $P<0.05$; **, $P<0.01$) or non-significance (ns) was determined by unpaired t test. Data show means \pm SD.

[0044] FIGS. 26A-26B show reduced weight gain in MLV vaccinated commercial birds. Groups of commercial white leghorn chicks were either unvaccinated (PBS) or immunized with MLV (day-1) or pQAC N vaccine (100 ug) at day-1 and day-14. (A) Absolute weight of birds in grams at 14, 21 and 28 dpv, significance (**, $P<0.01$) was determined by two-way ANOVA and compared with both PBS and pQAC N groups (B) Percentage weight gain of birds between 14 and 28 dpv. Data show means \pm SD.

[0045] FIGS. 27A-27D show Reduced tracheal viral shedding in pQAC-N vaccinated commercial birds. Groups of commercial white leghorn chicks were either unvaccinated (PBS) or immunized with MLV (day-1) or pQAC N vaccine (100 ug) at day-1 and day-14. IBV specific IgY in serum (A) and IgA in lachrymal fluid (B) significance (*, $P<0.05$; **, $P<0.01$; ***, $P<0.001$; ****, $P<0.0001$) was determined by two-way ANOVA (C) Clinical sign severity represented as average score/bird over 8 days post challenge in each group (D) IBV viral load in tracheal swabs at 6 days post challenge. Significance (**, $P<0.01$; ***, $P<0.001$; ****, $P<0.0001$) or non-significance (ns) was determined by one-way ANOVA with multiple comparisons. Data show means \pm SD.

[0046] FIG. 28 shows a vector map of the pCAG-IBV Arkansas nucleocapsid plasmid (SEQ ID NO:1).

[0047] FIG. 29 shows a vector map of the pCAG-IBV Arkansas Truncated Spike plasmid (SEQ ID NO:2).

[0048] FIG. 30 shows a vector map of the pCMV-SARS-CoV-2 nucleocapsid plasmid (SEQ ID NO:3).

[0049] FIG. 31 shows a vector map of the pCMV-SARS-CoV-2 truncated spike plasmid (SEQ ID NO:4).

DETAILED DESCRIPTION OF THE DISCLOSURE

In General

[0050] The present disclosure broadly relates to a Quil-A chitosan complex as well as methods of making and using such a complex.

[0051] In some embodiments, the present disclosure describes an adjuvant for use in a vaccine. The adjuvant is a Quil-A chitosan complex (QAC complex), which stimulates an immune response when administered in a vaccine composition.

[0052] In some embodiments, Quil-A and chitosan are combined to form a nanostructure complex which may be used as an adjuvant in a vaccine composition. The QAC complex may be loaded with a payload molecule, such as the antigen or immunogen with which the QAC complex stimulates an immune response. The QAC complex may be formulated into a vaccine composition with a pharmaceutically acceptable carrier.

[0053] The use of the term “or” in the claims is used to mean “and/or” unless explicitly indicated to refer to alternatives only or the alternatives are mutually exclusive. It is specifically contemplated that any listing of items using the term “or” means that any of those listed items may also be specifically excluded from the related embodiment.

[0054] Throughout this application, the term “about” means within 5% of a stated concentration range, density, temperature, or time frame.

[0055] As used herein the specification, “a” or “an” may mean one or more, unless clearly indicated otherwise. As used herein in the claims, when used in conjunction with the word “comprising,” the words “a” or “an” may mean one or more than one.

[0056] The terms “comprise,” “have,” and “include” are open-ended linking verbs. Any forms or tenses of one or more of these verbs, such as “comprises,” “comprising,” “has,” “having,” “includes,” and “including,” are also open-ended. For example, any method that “comprises,” “has” or “includes” one or more steps is not limited to possessing only those one or more steps and also covers other unlisted steps.

[0057] The terms “polypeptide,” “peptide,” and “protein,” as used herein, refer to a polymer comprising amino acid residues predominantly bound together by covalent amide bonds. By the term “protein,” we mean to encompass all the above definitions. The terms apply to amino acid polymers in which one or more amino acid residue may be an artificial

chemical mimetic of a naturally occurring amino acid, as well as to naturally occurring amino acid polymers and non-naturally occurring amino acid polymers. As used herein, the terms may encompass amino acid chains of any length, including full length proteins, wherein the amino acids are linked by covalent peptide bonds. The protein or peptide may be isolated from a native organism, produced by recombinant techniques, or produced by synthetic production techniques known to one skilled in the art.

[0058] The term “therapeutically effective amount,” as used herein, refers to an amount of an antigen or vaccine that would induce an immune response in a subject receiving the antigen or vaccine which is adequate to prevent signs or symptoms of disease, including adverse health effects or complications thereof, caused by infection with a pathogen, such as a virus or a bacterium. Humoral immunity or cell mediated immunity or both humoral and cell mediated immunity may be induced. The immunogenic response of an animal to a vaccine may be evaluated, e.g., indirectly through measurement of antibody titers, lymphocyte proliferation assays, or directly through monitoring signs and symptoms after challenge with wild-type strain. The protective immunity conferred by a vaccine may be evaluated by measuring, e.g., reduction in clinical signs such as mortality, morbidity, temperature number, overall physical condition, and overall health and performance of the subject. The amount of a vaccine that is therapeutically effective may vary depending on the particular virus used, or the condition of the subject, and may be determined by a physician.

[0059] The term “protected,” as used herein, refers to immunization of a patient against a disease. The immunization may be caused by administering a vaccine comprising an antigen. Specifically, in the present invention, the immunized patient is protected from a fungal, bacterial, or viral infection.

[0060] The term “vaccine,” as used herein, refers to a composition that includes an antigen. Vaccine may also include a biological preparation that improves immunity to a particular disease. A vaccine may typically contain an agent, referred to as an antigen, that resembles a disease-causing microorganism, and the agent may often be made from weakened or killed forms of the microbe, its toxins or one of its surface proteins. The antigen may stimulate the body's immune system to recognize the agent as foreign, destroy it, and “remember” it, so that the immune system can more easily recognize and destroy any of these microorganisms that it later encounters.

[0061] Vaccines may be prophylactic, e.g., to prevent or ameliorate the effects of a future infection by any natural or “wild” pathogen, or therapeutic, e.g., to treat the disease. Administration of the vaccine to a subject results in an immune response, generally against one or more specific diseases. The amount of a vaccine that is therapeutically effective may vary depending on the particular virus used, or the condition of the patient, and may be determined by a physician. The vaccine may be introduced directly into the subject by the subcutaneous, oral, oronasal, or intranasal routes of administration.

[0062] A vaccine of the present invention will include a suitable antigen to stimulate an immune response in a subject or patient. It is envisioned that vaccines of the present invention are not limited to a specific antigen or disease target, except where specifically specified. In some

embodiments, the vaccine of the present invention provides immunity against a fungus, a parasite, a bacteria, a microbe, or a virus.

[0063] In some embodiments, the vaccine of the present disclosure provides immunity against bacteria. In one embodiment of the invention, the vaccine comprises an antigen for a *Mycobacterium* species, such as, but not limited to, *Mycobacterium avium* subspecies *paratuberculosis*, *Mycobacterium bovis*, *Mycobacterium tuberculosis*, and *Mycobacterium avium* subspecies *avium*. A non-limiting example of an antigen of the present disclosure are the compositions described in U.S. Patent Publication No. 2018/0147272 (“Vaccine Candidates Against Johne’s Disease”), U.S. Pat. No. 9,663,758 (“Global Gene Regulators (GGR) as vaccine candidates against paratuberculosis”), and U.S. Pat. No. 9,220,764 (“Immunogenic compositions against tuberculosis”).

[0064] In some embodiments, the vaccine of the present disclosure provides immunity against a virus. In some embodiments, the vaccine comprises an antigen for infectious bronchitis virus. In some embodiments, the vaccine comprises an antigen for Severe Acute Respirator Syndrome Coronavirus 2 (SARS-CoV-2).

Vaccine Administration

[0065] The term “administration,” as used herein, refers to the introduction of a substance, such as a vaccine, into a subject’s body. The administration, e.g., parenteral administration, may include subcutaneous administration, intramuscular administration, transcutaneous administration, intradermal administration, intraperitoneal administration, intraocular administration, intranasal administration and intravenous administration.

[0066] The vaccine or the composition according to the invention may be administered to an individual according to methods known in the art. Such methods comprise application e.g. parenterally, such as through all routes of injection into or through the skin: e.g. intramuscular, intravenous, intraperitoneal, intradermal, mucosal, submucosal, or subcutaneous. Also, the vaccine may be applied by topical application as a drop, spray, gel or ointment to the mucosal epithelium of the eye, nose, mouth, anus, or vagina, or onto the epidermis of the outer skin at any part of the body.

[0067] Other possible routes of application are by spray, aerosol, or powder application through inhalation via the respiratory tract. In this last case, the particle size that is used will determine how deep the particles will penetrate into the respiratory tract.

[0068] Alternatively, application may be via the alimentary route, by combining with the food, feed or drinking water e.g. as a powder, a liquid, or tablet, or by administration directly into the mouth as a liquid, a gel, a tablet, or a capsule, or to the anus as a suppository.

[0069] The term “immune status” or “immunocompetence,” as used herein, refers to the ability of the body to produce a normal immune response following exposure to an antigen. Immunocompetence is the opposite of immunodeficiency or immuno-incompetent or immuno-compromised.

[0070] The present disclosure is generally applied to mammals, including but not limited to humans, cows, horses, sheep, pigs, goats, rabbits, dogs, cats, mice and rats. In some embodiments, the present disclosure can be applied to birds. In certain embodiments, non-human mammals, such as mice

and rats, may also be used for the purpose of demonstration. One may use the present invention for veterinary purpose. For example, one may wish to treat commercially important farm animals, such as cows, horses, pigs, rabbits, goats, sheep, and birds, such as chickens. One may also wish to treat companion animals, such as cats and dogs.

Adjuvants

[0071] As used herein “Quil-A chitosan complex” or “QAC complex” refers to a composition of Quil-A and chitosan which forms distinct disaggregated spherical nanostructures. FIG. 12 shows an example of these disaggregated spherical nanostructures. As used herein, “disaggregated,” refers to the formation of discrete observable particles as opposed to aggregated non-discrete assemblies with non-distinct boundaries. The QAC complex nanostructures are less 100 nm in diameter when measured in the absence of any payload molecules. For example, between about 5 nm and about 100 nm, between about 10 nm and about 95 nm, between about 15 nm and about 90 nm, between about 20 nm and about 90 nm, or between about 25 nm and about 85 nm. The QAC complex may be loaded with one or more payload molecules. The payload-QAC complex may be between about 20 nm and about 1000 nm in diameter. The specific size of the payload-QAC complex will vary depending on the size and amount of payload in the nanostructure.

[0072] In one embodiment, the QAC complex is formed by mixing a first solution of Quil-A into a second solution of chitosan to form a final mixed solution including the QAC complex. In the final mixed solution, the Quil-A and the chitosan are typically present at a ratio of between about 1:15 to about 1:100, between about 1:15 and about 1:75, between about 1:15 and about 1:50, between about 1:15 and about 1:25, between about 1:17 and about 1:25, or between about 1:18 and about 1:25. In some embodiments, the Quil-A and the chitosan are present at a ratio of about 1:20 (e.g., 1:15, 1:16, 1:17, 1:18, 1:19, 1:20, 1:21, 1:22, 1:23, 1:24, or 1:25) in the final mixed solution. In some embodiments, in the final solution Quil-A is at a concentration of 0.001% and chitosan is at a concentration of between about 0.02% and about 0.1%. In some embodiments, in the final solution Quil-A is at a concentration between about 0.00001% and about 0.5%. In some embodiments, in the final solution the chitosan is at a concentration between about 0.00015% and about 7.5%.

[0073] In some embodiments, the Quil-A solution and the chitosan solution are mixed drop-wise. In some embodiments, the Quil-A solution and the chitosan solution are mixed by vortex mixing for about 15-90 seconds (15 seconds, 20 seconds, 25 seconds, 30 seconds, 35 seconds, 40 seconds, 45 seconds, 50 seconds, 60 seconds, 70 seconds, 80 seconds or 90 seconds). In some embodiments, the Quil-A solution and the chitosan solution are mixed drop-wise followed by vortex mixing for about 15-90 seconds (15 seconds, 20 seconds, 25 seconds, 30 seconds, 35 seconds, 40 seconds, 45 seconds, 50 seconds, 60 seconds, 70 seconds, 80 seconds or 90 seconds).

[0074] The Quil-A solution and the chitosan solution may be heated prior to mixing. In some embodiments, the Quil-A solution and the chitosan solution are heated to a temperature between about 20° C. and about 60° C. (e.g., 20° C., 25° C., 30° C., 35° C., 40° C., 45° C., 50° C., 55° C., or 60° C.) for between about 20 to about 40 minutes (20 minutes, 25 minutes, 30 minutes, 35 minutes, or 40 minutes) prior to

mixing. In some embodiments, after mixing the final solution is incubated at room temperature for about 1 hour to promote QAC complex formation. In some embodiments, after mixing the final solution is incubated for 1 hour at between about 20° C. and about 45° C. (e.g., 20° C., 21° C., 22° C., 23° C., 24° C., 25° C., 26° C., 27° C., 28° C., 29° C., 30° C., 31° C., 32° C., 33° C., 34° C., 35° C., 36° C., 37° C., 38° C., 39° C., 40° C., 41° C., 42° C., 43° C., 44° C., or 45° C.) with shaking at between about 90 rpm and about 150 rpm (e.g., 90 rpm, 95 rpm, 100 rpm, 105 rpm, 110 rpm, 115 rpm, 120 rpm, 125 rpm, 130 rpm, 135 rpm, 140 rpm, 145 rpm, or 150 rpm). The pH of the solutions may be between 5.5 and 7.0.

[0075] In some embodiments, the Quil-A solution is prepared by creating a stock solution of Quil-A in water and diluting the stock Quil-A solution with a buffered solution. The buffer may be any suitable buffer known in the art to buffer a solution at a pH between 5.5 and 7.0. In some embodiments the buffer is sodium sulfate buffer.

[0076] In some embodiments, the chitosan solution is prepared by creating a stock solution of chitosan in acetic acid and diluting the stock acetic acid solution with a buffered solution. The buffer may be any suitable buffer in the art to buffer a solution at a pH between 5.5 and 7.0. In some embodiments the buffer is sodium acetate buffer at pH 5.5.

[0077] In some embodiments, a first solution of about 0.002% Quil-A in 50 mM sodium sulfate buffer pH 5.5 and a second solution of about 0.04% chitosan in 5 mM sodium acetate buffer pH 5.5 are each heated at 55° C. for about 30 min. Equal volumes of the first and second solution are mixed dropwise then vortex mixed for about 30 s followed by incubation at room temperature for about 1 hour for QAC complex formation.

[0078] As used herein “Quil-A” refers to the powdered saponin fraction isolated from extract of the bark of *Quillaja saponaria* trees. Quil-A is commercially available, for example from Desert King sold under the product name Vet-Sap™ (desertking.com/pharmaceutical-applications/#veterinary_adjuvant).

[0079] In some embodiments, the Quil-A is replaced with a surfactant or mild detergent. Surfactants and mild detergents may include but are not limited to, polyoxyethylene (20) sorbitan monolaurate (Tween™ 20), polyethylene glycol sorbitan monostearate (Tween™ 60), polyoxyethylene-sorbitan tristearate (Tween™ 65), polyoxyethylene (20) sorbitan monooleate (Tween™ 80), polyoxyethylenesorbitan trioleate (Tween™ 85), octyl oligoxyethylene (OPOE), N,N-dimethyldodecylamine (LDAO), and polyethylene glycol p-(1,1,3,3-tetramethylbutyl)-phenyl ether (Triton X-100). In general, when replacing Quil-A with a surfactant or mild detergent, the concentration of the surfactant or milk detergent will vary depending on the strength of the detergent or surfactant such that lower concentrations of stronger detergents and higher concentrations of weaker detergents are used.

[0080] As used herein “chitosan” refers to a linear polysaccharide composed of randomly distributed β -linked D-glucosamine and N-acetyl-D-glucosamine. Chitosan can be obtained from the chitin shells of shrimp and other crustaceans by treatment of the shells with an alkaline substance. Chitosan is a non-toxic, naturally occurring cationic polymer that readily complexes with DNA and negatively charged proteins. Chitosan is biocompatible and bio-

degradable. Compositions incorporating chitosan have sustained release kinetics and are immunomodulatory by enhancing the T-cell response. In some embodiments, chitosan is deacetylated chitosan, for example >75% deacetylated chitosan. Deacetylated chitosan (>75%) is available commercially from Sigma (C3646). Higher deacetylation percentages, for example about 90%, will mediate stronger binding with nucleic acids resulting in slower release kinetics from the nanoparticle structures of the Quil-A chitosan complex. In some embodiments, the chitosan is at least 70%, 75%, 80%, 85%, 90%, or 95% deacetylated. In some embodiments, the chitosan is between about 60% and about 90% deacetylated.

[0081] The QAC complex may be loaded with one or more payload molecules. The payload molecule may be an antigen of interest for use in a vaccine composition. The payload molecule may be an immunogen for use in a vaccine composition. The payload molecule may be, but is not limited to, a DNA molecule, an RNA molecule, a polynucleotide, a protein, a polypeptide, a virus, a microbe, an attenuated virus, an attenuated microbe, a small molecule, an antibody, or a mixture thereof.

[0082] In some embodiments, the payload is a live attenuated microbe. The pathogen of interest may be attenuated or reduced in virulence by any suitable means known in the art including but not limited to repeated passaging through a series of cell cultures, animal embryos (e.g., chicken embryos), or by genetic engineering to produce a mutated strain of the pathogen (e.g., mutant bacteria or mutant fungi).

[0083] In some embodiments, the payload is a recombinant protein. In some embodiments, the payload may be a subunit vaccine.

[0084] In some embodiments, the payload is an inactivated pathogen. The pathogen of interest may be inactivated by any suitable means known in the art including but not limited to, heat treatment, UV treatment, and chemical treatment (e.g., formaldehyde or glutaraldehyde).

[0085] In some embodiments, the payload is a recombinant viral vector. The recombinant viral vector may include, but is not limited to, an adeno viral vector or a poxvirus vector. Recombinant viral vectors may be used to deliver vaccine antigens by encoding immunogenic agents from a pathogen of interest.

[0086] In some embodiments, the payload is a recombinant nucleic acid. Recombinant nucleic acids may encode an immunogenic agent from a pathogen of interest such as, but not limited to, bacterial genes and fungal genes. In some embodiments, the payload is a recombinant RNA or DNA molecule encoding an immunogenic or antigenic polypeptide.

[0087] As used herein, the terms “polynucleotide,” “polynucleotide sequence,” “nucleic acid” and “nucleic acid sequence” refer to a nucleotide, oligonucleotide, polynucleotide (which terms may be used interchangeably), or any fragment thereof. These phrases also refer to DNA or RNA of natural or synthetic origin (which may be single-stranded or double-stranded and may represent the sense or the antisense strand). The polynucleotides may be cDNA or genomic DNA. Polynucleotides homologous to the polynucleotides described herein are also provided. Those of skill in the art understand the degeneracy of the genetic code and that a variety of polynucleotides can encode the same polypeptide.

[0088] In some embodiments, the payload is a polynucleotide construct. As used herein, the term “construct” refers to recombinant polynucleotides including, without limitation, DNA and RNA, which may be single-stranded or double-stranded and may represent the sense or the antisense strand. Recombinant polynucleotides are polynucleotides formed by laboratory methods that include polynucleotide sequences derived from at least two different natural sources or they may be synthetic. Constructs thus may include new modifications to endogenous genes introduced by, for example, genome editing technologies. Constructs may also include recombinant polynucleotides created using, for example, recombinant DNA methodologies.

[0089] The payload constructs provided herein may be prepared by methods available to those of skill in the art. Notably each of the constructs described are recombinant molecules and as such do not occur in nature. Generally, the nomenclature used herein and the laboratory procedures utilized in the present invention include molecular, biochemical, and recombinant DNA techniques that are well known and commonly employed in the art. Standard techniques available to those skilled in the art may be used for cloning, DNA and RNA isolation, amplification and purification. Such techniques are thoroughly explained in the literature.

[0090] The constructs provided herein may include a promoter operably linked to any one of the polynucleotides described herein. The promoter may be a heterologous promoter or an endogenous promoter associated with the antigenic or immunogenic payload polypeptide.

[0091] As used herein, the terms “heterologous promoter,” “promoter,” “promoter region,” or “promoter sequence” refer generally to transcriptional regulatory regions of a gene, which may be found at the 5' or 3' side of the polynucleotides described herein, or within the coding region of the polynucleotides, or within introns in the polynucleotides. Typically, a promoter is a DNA regulatory region capable of binding RNA polymerase in a cell and initiating transcription of a downstream (3' direction) coding sequence. The typical 5' promoter sequence is bounded at its 3' terminus by the transcription initiation site and extends upstream (5' direction) to include the minimum number of bases or elements necessary to initiate transcription at levels detectable above background. Within the promoter sequence is a transcription initiation site (conveniently defined by mapping with nuclease S1), as well as protein binding domains (consensus sequences) responsible for the binding of RNA polymerase.

[0092] As used herein, a polynucleotide is “operably connected” or “operably linked” when it is placed into a functional relationship with a second polynucleotide sequence. For instance, a promoter is operably linked to a polynucleotide if the promoter is connected to the polynucleotide such that it may effect transcription of the polynucleotide. In various embodiments, the polynucleotide may be operably linked to at least 1, at least 2, at least 3, at least 4, at least 5, or at least 10 promoters.

[0093] Heterologous promoters useful in the practice of the present invention include, but are not limited to, constitutive, inducible, temporally-regulated, developmentally regulated, chemically regulated, tissue-preferred and tissue-specific promoters. The heterologous promoter may be a plant, animal, bacterial, fungal, or synthetic promoter. Suitable promoters include, without limitation, promoters for

Rous sarcoma virus (RSV), human immunodeficiency virus (HIV-1), AmpR promoter, SV40, cytomegalovirus (CMV), SV40 virus, chicken beta actin (CAG), and the like as well as the translational elongation factor EF-1 α promoter or ubiquitin promoter. Those of skill in the art are familiar with a wide variety of additional promoters for use in various cell types.

[0094] Vectors including any of the constructs or polynucleotides described herein are provided. The term “vector” is intended to refer to a polynucleotide capable of transporting another polynucleotide to which it has been linked. In some embodiments, the vector may be a “plasmid,” which refers to a circular double-stranded DNA loop into which additional DNA segments may be ligated. Certain vectors are capable of autonomous replication in a host cell into which they are introduced (e.g., bacterial vectors having a bacterial origin of replication and episomal mammalian vectors). Other vectors can be integrated into the genome of a host cell upon introduction into the host cell, and thereby are replicated along with the host genome, such as some viral vectors or transposons. Vectors may carry genetic elements, such as those that confer resistance to certain drugs or chemicals. In some embodiments, the payload is a vector as described herein.

[0095] In some embodiments, the payload is polypeptide antigen specific for Infectious Bronchitis Virus (IBV) or a polynucleotide encoding a polypeptide antigen specific for IBV. IBV is a member of the genus gammacoronavirus, family Coronaviridae, order Nidovirales with a 27.6 Kb single stranded positive sense RNA genome encoding major structural proteins, spike glycoprotein (S), envelope (E), membrane (M) and nucleocapsid (N). In some embodiments, the payload is selected from the group consisting of the IBV S, E, M, and N proteins and fragments thereof. In some embodiments, the payload is a polynucleotide encoding the IBV S, E, M, or N proteins or fragments thereof. In some embodiments, the payload is a polypeptide at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, or at least 99% to the IBV nucleocapsid protein of SEQ ID NO:5. In some embodiments, the payload is the IBV nucleocapsid protein of SEQ ID NO:5. In some embodiments, the payload is a polynucleotide encoding a polypeptide at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, or at least 99% to the IBV nucleocapsid protein of SEQ ID NO:5. In some embodiments, the payload is a polynucleotide encoding the IBV nucleocapsid protein of SEQ ID NO:5. In some embodiments, the payload comprises the polynucleotide of SEQ ID NO:6 or a sequence at least 90% identical thereto.

[0096] In some embodiments, the payload is a polypeptide at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, or at least 99% to the IBV truncated spike protein of SEQ ID NO:7. In some embodiments, the payload is the IBV truncated spike protein of SEQ ID NO:7. In some embodiments, the payload is a polynucleotide encoding a polypeptide at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, or at least 99% to the IBV truncated spike protein of SEQ ID NO:7. In some embodiments, the payload is a polynucleotide encoding the IBV nucleocapsid protein of SEQ ID NO:7. In some embodiments, the payload comprises the polynucleotide of SEQ ID NO:8 or a sequence at least 90% identical thereto.

[0097] In some embodiments, the payload is a polypeptide antigen specific for Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2). SARS-CoV-2 includes the

major structural proteins spike (S), envelope (E), membrane (M), and nucleocapsid (N). In some embodiments, the payload is selected from the group consisting of the SARS-CoV-2 S, E, M, and N proteins and fragments thereof. In some embodiments, the payload is a polynucleotide encoding the SARS-CoV-2 S, E, M, or N protein or fragments thereof. In some embodiments, the payload is a polypeptide at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, or at least 99% to the SARS-CoV-2 nucleocapsid protein of SEQ ID NO:9. In some embodiments, the payload is the SARS-CoV-2 nucleocapsid protein of SEQ ID NO:9. In some embodiments, the payload is a polynucleotide encoding a polypeptide at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, or at least 99% to the SARS-CoV-2 nucleocapsid protein of SEQ ID NO:9. In some embodiments, the payload is polynucleotide encoding the SARS-CoV-2 nucleocapsid protein of SEQ ID NO:9. In some embodiments, the payload comprises the polynucleotide of SEQ ID NO:10 or a sequence at least 90% identical thereto.

[0098] In some embodiments, the payload is a polypeptide at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, or at least 99% to the SARS-CoV-2 truncated spike protein of SEQ ID NO:11. In some embodiments, the payload is the SARS-CoV-2 truncated spike protein of SEQ ID NO:11. In some embodiments, the payload is a polynucleotide encoding a polypeptide at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, or at least 99% to the SARS-CoV-2 truncated spike protein of SEQ ID NO:11. In some embodiments, the payload is polynucleotide encoding the SARS-CoV-2 truncated spike protein of SEQ ID NO:11. In some embodiments, the payload comprises the polynucleotide of SEQ ID NO:12 or a sequence at least 90% identical thereto.

[0099] In some embodiments, the payload is functionalized prior to loading into the QAC complex. Proteins, DNA molecules, and RNA molecules that are negatively charged at neutral pH are generally readily taken up by QAC particles due to the electrostatic interactions between the negatively charged payload and the positively charged QAC particles. To improve the loading capacity of neutral and positively charged payloads, the payload may be functionalized to alter the surface charge of the payload. For example, chemical modifications such as amination of protein molecules can introduce negatively charged amino groups. Chemical modifications such as carboxylation of protein molecules can increase the number of free carboxylic acid groups on the protein surface to enhance loading of the protein into the QAC complex. The isoelectric point of the protein molecule can be reduced by protein surface modification with malonic acid moieties to increase interaction with the chitosan component of the QAC complex.

[0100] In some embodiments, the chitosan is functionalized. Chitosan may be functionalized with negatively charged sulfonate groups by reaction of the amino group of chitosan with 5-formyl-2-furan sulfonic acid (FFSA) followed by treatment using sodium borohydride to form a negatively charged chitosan surface. Use of the negatively charged chitosan in the formation of the QAC complex will generally be favorable for loading of positively charged payload molecules.

[0101] In some embodiments, the QAC complex is loaded with a DNA molecule payload. The QAC-DNA loaded complex is formed by mixing a solution of Quil-A and DNA

into a solution of chitosan to form a final mixed solution including the QAC-DNA complex. In the final mixed solution, the Quil-A and the chitosan are present at a ratio of between 1:15 to 1:100. In some embodiments, the Quil-A and the chitosan are present at a ratio of about 1:20 (e.g., 1:15, 1:16, 1:17, 1:18, 1:19, 1:20, 1:21, 1:22, 1:23, 1:24, or 1:25) in the final mixed solution. In some embodiments, in the final solution Quil-A is at a concentration of 0.001% and chitosan is at a concentration between about 0.02% and about 0.1%. In some embodiments, the DNA payload in the DNA Quil-A solution is at a concentration between about 10 µg/ml and about 1000 µg/ml.

[0102] In some embodiments, the Quil-A DNA solution and the chitosan solution are mixed drop-wise. In some embodiments, the Quil-A DNA solution and the chitosan solution are mixed by vortex mixing for about 15-90 seconds (15 seconds, 20 seconds, 25 seconds, 30 seconds, 35 seconds, 40 seconds, 45 seconds, 50 seconds, 60 seconds, 70 seconds, 80 seconds or 90 seconds). In some embodiments, the Quil-A DNA solution and the chitosan solution are mixed drop-wise followed by vortex mixing for about 15-90 seconds (15 seconds, 20 seconds, 25 seconds, 30 seconds, 35 seconds, 40 seconds, 45 seconds, 50 seconds, 60 seconds, 70 seconds, 80 seconds or 90 seconds). The Quil-A DNA solution and the chitosan solution may be heated prior to mixing. In some embodiments, the Quil-A DNA solution and the chitosan solution are heated to a temperature between about 20° C. and about 60° C. (e.g., 20° C., 25° C., 30° C., 35° C., 40° C., 45° C., 50° C., 55° C., or 60° C.) for between about 20 to about 40 minutes (20 minutes, 25 minutes, 30 minutes, 35 minutes, or 40 minutes) prior to mixing. In some embodiments, after mixing the final solution is incubated at room temperature for about 1 hour to promote QAC complex formation. In some embodiments, after mixing the final solution is incubated for 1 hour at between about 20° C. and about 45° C. (e.g., 20° C., 21° C., 22° C., 23° C., 24° C., 25° C., 26° C., 27° C., 28° C., 29° C., 30° C., 31, 32° C., 33° C., 34° C., 35° C., 36° C., 37° C., 38° C., 39° C., 40° C., 41° C., 42° C., 43° C., 44° C., or 45° C.) with shaking at between about 90 rpm and about 150 rpm (e.g., 90 rpm, 95 rpm, 100 rpm, 105 rpm, 110 rpm, 115 rpm, 120 rpm, 125 rpm, 130 rpm, 135 rpm, 140 rpm, 145 rpm, or 150 rpm). The pH of the solutions may be between 5.5 and 7.0.

[0103] In some embodiments, a first solution of about 0.002% Quil-A and between about 1 µg/ml and about 5,000 µg/ml DNA in 50 mM sodium sulfate buffer and a second solution of about 0.04% chitosan in 5 mM sodium acetate buffer pH5.5 are each heated at 55° C. for about 30 min. Equal volumes of the first and second solution are mixed dropwise then vortex mixed for about 30 s followed by incubation at room temperature for about 1 hour to form the QAC DNA loaded complex.

[0104] In some embodiments, the QAC complex is loaded with a protein molecule payload. The QAC-protein loaded complex is formed by mixing a solution of Quil-A and protein into a solution of chitosan to form a final mixed solution including the QAC-protein complex. In the final mixed solution, the Quil-A and the chitosan are present at a ratio of between 1:15 to 1:100. In some embodiments, the Quil-A and the chitosan are present at a ratio of about 1:20 (e.g., 1:15, 1:16, 1:17, 1:18, 1:19, 1:20, 1:21, 1:22, 1:23, 1:24, or 1:25) in the final mixed solution. In some embodiments, in the final solution Quil-A is at a concentration of

0.001% and chitosan is at a concentration between about 0.02% and about 0.1%. In some embodiments, the protein payload in the protein Quil-A solution is at a concentration between about 10 µg/ml and about 1000 µg/ml.

[0105] In some embodiments, the Quil-A protein solution and the chitosan solution are mixed drop-wise. In some embodiments, the Quil-A protein solution and the chitosan solution are mixed by vortex mixing for about 15-90 seconds (15 seconds, 20 seconds, 25 seconds, 30 seconds, 35 seconds, 40 seconds, 45 seconds, 50 seconds, 60 seconds, 70 seconds, 80 seconds or 90 seconds). In some embodiments, the Quil-A protein solution and the chitosan solution are mixed drop-wise followed by vortex mixing for about 15-90 seconds (15 seconds, 20 seconds, 25 seconds, 30 seconds, 35 seconds, 40 seconds, 45 seconds, 50 seconds, 60 seconds, 70 seconds, 80 seconds or 90 seconds). The Quil-A protein solution and the chitosan solution may be heated prior to mixing. In some embodiments, the Quil-A protein solution and the chitosan solution are heated to a temperature between about 20° C. and about 60° C. (e.g., 20° C., 25° C., 30° C., 35° C., 40° C., 45° C., 50° C., 55° C., or 60° C.) for between about 20 to about 40 minutes (20 minutes, 25 minutes, 30 minutes, 35 minutes, or 40 minutes) prior to mixing. In some embodiments, after mixing the final solution is incubated at room temperature for about 1 hour to promote QAC complex formation. In some embodiments, after mixing the final solution is incubated for 1 hour at between about 20° C. and about 45° C. (e.g., 20° C., 21° C., 22° C., 23° C., 24° C., 25° C., 26° C., 27° C., 28° C., 29° C., 30° C., 31, 32° C., 33° C., 34° C., 35° C., 36° C., 37° C., 38° C., 39° C., 40° C., 41° C., 42° C., 43° C., 44° C., or 45° C.) with shaking at between about 90 rpm and about 150 rpm (e.g., 90 rpm, 95 rpm, 100 rpm, 105 rpm, 110 rpm, 115 rpm, 120 rpm, 125 rpm, 130 rpm, 135 rpm, 140 rpm, 145 rpm, or 150 rpm). The pH of the solutions may be between 5.5 and 7.0.

[0106] In some embodiments, a first solution of about 0.002% Quil-A and between about 1 µg/ml and about 5,000 µg/ml protein in 50 mM sodium sulfate buffer and a second solution of about 0.04% chitosan in 5 mM sodium acetate buffer pH5.5 are each heated at 55° C. for about 30 min. Equal volumes of the first and second solution are mixed dropwise then vortex mixed for about 30 s followed by incubation at about 37° C. for about 1 hour with shaking at about 110 rpm to form the QAC protein loaded complex.

[0107] A vaccine comprising a QAC complex adjuvant as described herein may also comprise other suitable agents or ingredients. Suitable agents may include a suitable carrier or vehicle for delivery. As used herein, the term “carrier” refers to a pharmaceutically acceptable solid or liquid filler, diluent or encapsulating material. A water-containing liquid carrier can contain pharmaceutically acceptable additives such as acidifying agents, alkalizing agents, antimicrobial preservatives, antioxidants, buffering agents, chelating agents, complexing agents, solubilizing agents, humectants, solvents, suspending and/or viscosity-increasing agents, tonicity agents, wetting agents or other biocompatible materials. A tabulation of ingredients listed by the above categories, may be found in the U.S. Pharmacopeia National Formulary, 1857-1859, (1990).

[0108] Some examples of the materials which can serve as pharmaceutically acceptable carriers are sugars, such as lactose, glucose and sucrose; starches such as corn starch and potato starch; cellulose and its derivatives such as

sodium carboxymethyl cellulose, ethyl cellulose and cellulose acetate; powdered tragacanth; malt; gelatin; talc; excipients such as cocoa butter and suppository waxes; oils such as peanut oil, cottonseed oil, safflower oil, sesame oil, olive oil, corn oil and soybean oil; glycols, such as propylene glycol; polyols such as glycerin, sorbitol, mannitol and polyethylene glycol; esters such as ethyl oleate and ethyl laurate; agar; buffering agents such as magnesium hydroxide and aluminum hydroxide; alginic acid; pyrogen free water; isotonic saline; Ringer's solution, ethyl alcohol and phosphate buffer solutions, as well as other nontoxic compatible substances used in pharmaceutical formulations. Wetting agents, emulsifiers and lubricants such as sodium lauryl sulfate and magnesium stearate, as well as coloring agents, release agents, coating agents, sweetening, flavoring and perfuming agents, preservatives and antioxidants can also be present in the compositions, according to the desires of the formulator.

[0109] Examples of pharmaceutically acceptable antioxidants include water soluble antioxidants such as ascorbic acid, cysteine hydrochloride, sodium bisulfate, sodium metabisulfite, sodium sulfite and the like; oil-soluble antioxidants such as ascorbyl palmitate, butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), lecithin, propyl gallate, alpha-tocopherol and the like; and metal-chelating agents such as citric acid, ethylenediamine tetraacetic acid (EDTA), sorbitol, tartaric acid, phosphoric acid and the like.

[0110] In another embodiment, the present formulation may also comprise other suitable agents such as a stabilizing delivery vehicle, carrier, support or complex-forming species. The coordinate administration methods and combinatorial formulations of the instant invention may optionally incorporate effective carriers, processing agents, or delivery vehicles, to provide improved formulations for delivery of the QAC complex and other biologically active agents and antigens of the composition.

[0111] The vaccine formulation may additionally include a biologically acceptable buffer to maintain a pH close to neutral (7.0-7.3). Such buffers preferably used are typically phosphates, carboxylates, and bicarbonates. More preferred buffering agents are sodium phosphate, potassium phosphate, sodium citrate, calcium lactate, sodium succinate, sodium glutamate, sodium bicarbonate, and potassium bicarbonate. The buffer may comprise about 0.0001-5% (w/v) of the vaccine formulation, more preferably about 0.001-1% (w/v). Other excipients, if desired, may be included as part of the final vaccine formulation.

[0112] The remainder of the vaccine formulation may be an acceptable diluent, to 100%, including water. The vaccine formulation may also be formulated as part of a water-in-oil, or oil-in-water emulsion.

[0113] The vaccine formulation may be separated into vials or other suitable containers. The vaccine formulation herein described may then be packaged in individual or multi-dose ampoules, or be subsequently lyophilized (freeze-dried) before packaging in individual or multi-dose ampoules. The vaccine formulation herein contemplated also includes the lyophilized version. The lyophilized vaccine formulation may be stored for extended periods of time without loss of viability at ambient temperatures. The lyophilized vaccine may be reconstituted by the end user, and administered to a patient.

[0114] The term "lyophilization" or "lyophilized," as used herein, refers to freezing of a material at low temperature followed by dehydration by sublimation, usually under a high vacuum. Lyophilization is also known as freeze drying. Many techniques of freezing are known in the art of lyophilization such as tray-freezing, shelf-freezing, spray-freezing, shell-freezing and liquid nitrogen immersion. Each technique will result in a different rate of freezing. Shell-freezing may be automated or manual. For example, flasks can be automatically rotated by motor driven rollers in a refrigerated bath containing alcohol, acetone, liquid nitrogen, or any other appropriate fluid. A thin coating of product is evenly frozen around the inside "shell" of a flask, permitting a greater volume of material to be safely processed during each freeze drying run. Tray-freezing may be performed by, for example, placing the samples in lyophilizer, equilibrating 1 hr at a shelf temperature of 0° C., then cooling the shelves at 0.5° C./min to -40° C. Spray-freezing, for example, may be performed by spray-freezing into liquid, dropping by ~20 µl droplets into liquid N₂, spray-freezing into vapor over liquid, or by other techniques known in the art.

[0115] The vaccine of the present invention may be either in a solid form or in a liquid form. Preferably, the vaccine of the present invention may be in a liquid form. The liquid form of the vaccine may have a concentration of about 0.5-20 µg/ml Quil-A and about 100-250 µg/ml chitosan. In some embodiments, the liquid form of the vaccine includes 10 µg/ml Quil-A and 200 µg/ml chitosan. The liquid form of the vaccine may have a concentration of about 10-1000 µg/ml DNA payload or 5-500 µg/ml RNA payload.

[0116] To vaccinate a patient, a therapeutically effective amount of vaccine comprising the QAC complex adjuvant or a QAC complex loaded with a payload antigen or immunogen may be administered to a patient. The therapeutically effective amount of vaccine may typically be one or more doses, preferably in the range of about 0.01-10 mL, most preferably 0.1-1 mL, containing 1-200 micrograms, most preferably 1-100 micrograms of vaccine formulation/dose. The therapeutically effective amount may also depend on the vaccination species. For example, for smaller animals such as mice, a preferred dosage may be about 0.01-1 mL of a 1-50 microgram solution of antigen. For a human patient, a preferred dosage may be about 0.1-1 mL of a 1-50 microgram solution of antigen. The therapeutically effective amount may also depend on other conditions including characteristics of the patient (age, body weight, gender, health condition, etc.), characteristics of the antigen or pathogen of interest, and others. In one embodiment the vaccine formulation of the present invention comprises the QAC complex adjuvant or a QAC complex loaded with a payload antigen or immunogen with Quil-A at a concentration of 10 µg/ml and chitosan at a concentration of 200 µg/ml.

[0117] A vaccine of the present invention may be administered by using any suitable means as disclosed above. Preferably, a vaccine of the present invention may be administered by intranasal delivery, transmucosal administration, subcutaneous or intramuscular administration, e.g., needle injection. In some embodiments, vaccine compositions for protection against a viral infection are formulated for transmucosal delivery. In some embodiments, vaccine compositions for protection against a bacterial infection are formulated for subcutaneous administration.

[0118] After vaccination using a vaccine of the present invention comprising the QAC complex adjuvant, a patient may be immunized against at least one type of fungi, bacteria, or virus. In one specific embodiment, a patient after vaccination may be immunized against at least one species of bacteria. In one preferred embodiment, a patient after vaccination may be immunized from *Mycobacterium avium* subspecies *paratuberculosis*, *Mycobacterium bovis*, *Mycobacterium tuberculosis*, and *Mycobacterium avium* subspecies *avium*.

[0119] The instant invention may also include kits, packages and multicontainer units containing the above described pharmaceutical compositions, active ingredients, and/or means for administering the same for use in the prevention and treatment of diseases and other conditions in mammalian subjects. Briefly, these kits include a container or formulation that contains the QAC complex adjuvant or a QAC complex loaded with a payload antigen or immunogen with mucosal or subcutaneous delivery enhancing agents disclosed herein formulated in a pharmaceutical preparation for delivery. In some embodiments, the kit includes a Quil-A solution as described herein and a chitosan solution as described herein for the preparation of QAC complex using a user supplied payload molecule.

[0120] As used herein, the term “pharmaceutically acceptable carrier” refers to any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like that are physiologically compatible. Preferably, the carrier is suitable for intravenous, intramuscular, subcutaneous, parenteral, spinal or epidermal administration (e.g., by injection or infusion).

[0121] A “pharmaceutically acceptable salt” refers to a salt that retains the desired biological activity of the parent compound and does not impart any undesired toxicological effects (see e.g., Berge, S. M., et al. (1977) *J. Pharm. Sci.* 66:1-19). Examples of such salts include acid addition salts and base addition salts. Acid addition salts include those derived from nontoxic inorganic acids, such as hydrochloric, nitric, phosphoric, sulfuric, hydrobromic, hydroiodic, phosphorous and the like, as well as from nontoxic organic acids such as aliphatic mono- and dicarboxylic acids, phenyl-substituted alkanic acids, hydroxy alkanic acids, aromatic acids, aliphatic and aromatic sulfonic acids and the like. Base addition salts include those derived from alkaline earth metals, such as sodium, potassium, magnesium, calcium and the like, as well as from nontoxic organic amines, such as N,N'-dibenzylethylenediamine, N-methylglucamine, chlorprocaine, choline, diethanolamine, ethylenediamine, procaine and the like.

[0122] In one embodiment, the composition may also comprise suitable stabilizing delivery vehicle, carrier, support or complex-forming species, such as those as discussed above. For example, the composition may additionally comprise at least one of a stabilizer, a buffer, or an adjuvant.

[0123] The present invention has been described in terms of one or more preferred embodiments, and it should be appreciated that many equivalents, alternatives, variations, and modifications, aside from those expressly stated, are possible and within the scope of the invention.

Example 1

[0124] The embodiment described here demonstrates the preparation of loaded Quil-A chitosan complexes.

[0125] Infectious Bronchitis (IB) is an acute respiratory illness of domestic fowl caused by Infectious Bronchitis Virus (IBV) (1). IBV is a member of the genus gammacoronavirus, family Coronaviridae, order Nidovirales with a 27.6 Kb single stranded positive sense RNA genome encoding major structural proteins, spike glycoprotein (S), envelope (E), membrane (M) and nucleocapsid (N) (2). IB associated clinical signs include tracheal rales, frequent sneezing with nasal exudate, lethargy, labored breathing, etc. Uncomplicated IB infections are not lethal and virus with associated symptoms are cleared within 10 days (3). Apart from replicating and causing pathology in the upper respiratory tract, IBV can also affect the oviducts and kidneys depending on the strain of the virus. IBV control is of great economic importance to the poultry industry, infected broilers are subjected to increased condemnation at the slaughterhouses and layers are plagued with a drop in egg quality and production (4-6). High antigenic variation in the spike glycoprotein (S) is a hallmark of different IBV serotypes (6-8). Multiple serotypes co-circulate in birds, which complicates diagnosis and control of IBV. Current commercial modified live virus (MLV) vaccines confer homologous protection but fail to cross-protect against multiple and newly emerging serotypes. Sequence difference even as little as 4% in the 51 subunit of S protein can lead to vaccine failure (9, 10). Unfortunately, MLVs have been shown to persist and transmit in vaccinated birds potentially mediating recombination with virulent circulating serotypes leading to the emergence of new serotypes (11-13). With the emergence of new serotypes like GA98 linked to the excessive use of MLV (14), there is an urgent need to develop a safe and effective vaccine against IBV, the focus of the current study.

[0126] The use of plasmid DNA as potential immunogens was described almost 30 years ago (15), however, only 5 DNA-based vaccines have been licensed for veterinary use (16). Nucleic acid based vaccines have significant advantages over MLV as they have a superior safety profile, invoke robust cell mediated immunity (CMI) with potent adjuvants, cost less to produce and are thermostable obviating the need for cold chain (17). DNA-based vaccines encoding IBV S1, M and N genes administered in ovo and intramuscularly have been studied with variable protection levels against IBV (18-26). The use of plasmid DNA vaccines in the field despite having practical advantages and being safe has been limited owing to poor immunogenicity and cellular availability. Nanocarriers increase bioavailability of antigen cargo generating an immediate uptake by immune cells and hence, are potent adjuvants (27). Nanocarriers can protect plasmid DNA and antigen cargo from degradation in vivo facilitating delivery in vaccine hostile mucosal surfaces (28, 29). To provide a safer alternative to current MLVs, we detail the development of nano carriers composed of natural adjuvants, Quil-A and chitosan (QAC) for the delivery of IBV plasmid DNA immunogen. Natural adjuvants (such as Quil-A and chitosan) are inexpensive to produce, making them ideal candidates for animal vaccines. Chitosan is a nontoxic, biodegradable, biocompatible natural polysaccharide. Chitosan is cationic in nature and can readily complex with negatively charged nucleic acids and proteins through electrostatic interactions (30). Chitosan is immunomodulatory, forms stable DNA/protein complexes and has mucoadhesive properties because of which they are widely applied for mucosal routes of administration (31).

Quil-A is a potent adjuvant with mild surfactant properties (32) produced from the plant *Quillaja saponaria*, which can form nanoparticle compounds like ISCOMs and those formed with chitosan (QAC) as we detail below.

[0127] The sequence similarity of IBV Nucleocapsid (N) protein between diverse serotypes is greater than 90%, for this reason IBV N protein is an ideal immunogen candidate in an effort to develop a cross-protective vaccine. Immunization with N protein elicited a robust cytotoxic T-lymphocyte (CTL) response, an important correlate of protection against IBV (20, 33, 34). Adoptively transferred IBV reactive CD8⁺ T-cells protect against IBV challenge in naïve chickens (35, 36). In the present study, we evaluated the ability of a stable plasmid DNA construct expressing the IBV N protein complexed with the QAC adjuvant system given intra nasally to protect immunized birds against challenge with virulent strain of IBV. Our results indicate that pQAC-N vaccine elicits a CD8⁺ T-cell response which protect vaccinated birds against IBV challenge. Levels of protection in pQAC-N vaccinated birds were higher when compared to unadjuvanted or chitosan complexed plasmid DNA vaccine. Our data demonstrate that intra nasal immunization with pQAC-N induced a strong cell mediated immune response that protect vaccinated birds with a significant reduction in clinical signs and viral load to levels seen with commercial MLV vaccinated birds.

Results

[0128] Synthesis and Characterization of pQAC-N Nanovaccine.

[0129] The green fluorescent protein (GFP) gene was inserted into the pCAG plasmid and was used as a DNA payload for nanoparticle (NP) characterization. Transmission electron microscopy (TEM) analysis of chitosan-plasmid DNA complexes indicated presence of aggregated structures (FIG. 18A). However, when Quil-A was added to chitosan-DNA complex, defined particles were formed with the disaggregation of chitosan-DNA complexes (FIG. 18B). Size estimations using TEM analysis indicated that QAC-DNA nanoparticles were <100 nm (FIG. 18B). Dynamic light scattering (DLS) was also used to measure the hydrodynamic size and zeta potential of QAC-pDNA particles. As expected, particles were 95 ± 25 nm in size (94%) with a net positive zeta potential of 15 ± 4.44 mV (FIGS. 18C-18D).

[0130] The ability of QAC adjuvant system to deliver plasmid DNA payload was evaluated in vitro to examine its potential for immunization programs. The QAC nanoparticles encapsulating total 5 ug pCAG-GFP construct were added to a suspension of Expi293F cells. After 72 hrs post addition, presence of fluorescent cells was observed using fluorescence microscopy (FIG. 19A), indicating the delivery and expression of the GFP from the construct. The release kinetics of the GFP protein from the pCAG-GFP construct was evaluated in phosphate buffered saline (PBS) at pH 7.4 by quantifying the amount of starting and released plasmid DNA in buffer using spectrophotometry. The analysis showed that almost 42% of plasmid DNA within the nanoparticles was released within 15 days, the end point for our analysis. Overall, a biphasic release kinetics was observed with sustained release of DNA cargo for the first 10 days followed by a plateau over the next 5 days (FIG. 19B). The encapsulation efficiency of DNA (percentage of encapsulated DNA relative to the starting DNA) in QAC nanoparticles ranged from 70-90%.

[0131] Finally, following successful encapsulation and sustained release of plasmid DNA with QAC, we used the same nanocarrier (QAC) to encapsulate plasmid DNA encoding N protein from IBV-Arkansas strain with a C-terminal 6xHis tag, henceforth referred to as pQAC-N. The expression of antigen was confirmed using western blot analysis with an anti-6xHis antibody (FIGS. 20A-20B).

[0132] QAC-Based Nanovaccine is Well Tolerated by Chickens—

[0133] In ovo and spray vaccinations (intranasal) are two strategies used in the field for mass vaccinations of poultry flocks. The safety of pQAC-N in chicken hosts was evaluated through two routes of administration, In ovo and intranasal, a proxy for field spray vaccinations. Embryo development and hatch rate of pQAC-N (100 ug) inoculated specific pathogen free (SPF) embryonated chicken eggs (ECE) was similar to ECEs inoculated with PBS (100%, FIG. 21A). In addition, 1-day-old SPF chicks were immunized with pQAC-N construct intra nasally and monitored for general or respiratory distress, depression or in appetite and weight gain over the course of 30 days post vaccination. No signs for respiratory distress were observed in chicks immunized at 1 day of age and weight gain over 30 days was not statistically different from chicks inoculated with PBS (FIG. 21B). Overall, our analysis in chickens and chicken embryos indicated that pQAC-N is well-tolerated.

[0134] Immunization with pQAC-N Induces a Robust Immune Response—

[0135] Harderian glands play a critical role in the control of IBV infection in the upper respiratory tract by secreting IBV specific IgA antibodies into the lachrymal fluid (tears) (34). Accordingly, we examined the ability of pQAC-N vaccine to elicit IBV specific immune responses in birds following intranasal delivery. Lachrymal fluid samples collected at different time points, 10, 20 days post vaccination (DPV, pre-challenge) and 3 days post challenge (DPC) were examined using ELISA plates coated with IBV Arkansas 51 and N proteins. IBV specific IgA titers were detectable in pQAC-N immunized birds at 20 DPV at levels higher than seen in chitosan pCAG-N immunized birds (FIG. 22A). Albeit detectable, IgA levels were not significantly higher than levels seen in other groups, naked (unadjuvanted) and chitosan complexed pCAG-N. IgA levels were 10 folds higher in birds immunized with commercial MLV when compared to other experimental vaccine groups (FIG. 22A), most likely because of the IgA against 51 which was not included in the pQAC-N but present in MLV.

[0136] To assess the effect of IBV specific cellular immune responses induced by pQAC-N, we measured the ability of PBMCs from immunized chickens to respond to IBV antigen stimulation. PBMCs were harvested from vaccinated birds at 20 DPV (pre-challenge time point) and processed for antigen specific cell proliferation assay (MTT assay). The stimulation of PBMCs from chickens vaccinated with pQAC-N resulted in significantly higher proliferation ($p < 0.05$) than that of PBMCs from other groups including MLV (FIG. 22B), suggesting a better cellular immunity following pQAC-N immunization.

[0137] Reduced Clinical Severity and Viral Burden in Immunized Birds—

[0138] To investigate the protective efficacy of pQAC-N vaccine, all immunized birds were challenged with virulent IBV Arkansas-DPI serotype at 3 weeks post first vaccination (21 DPV) and the clinical severity of bird groups was scored

up to 8 DPC (FIG. 23A). Vaccination with naked pCAG-N (unadjuvanted) and chitosan complexed pCAG-N conferred partial protection against clinical signs associated with IBV. On the other hand, pQAC-N and commercial MLV immunized bird groups were relatively asymptomatic with a significant reduction in clinical severity when compared to unvaccinated birds (FIG. 23A).

[0139] In addition, we used RT-qPCR to assess the level of viral RNA in lachrymal fluid of all birds at 6 DPC. A significant reduction in viral load (~2.5 logs) was found in the pQAC-N immunized birds when compared to control birds at levels comparable to commercial MLV vaccinated birds (FIG. 23B). A partial reduction in viral load was also observed in naked pCAG-N and chitosan pCAG-N administered birds (FIG. 23B). Overall, reduction in viral load was higher in the pQAC-N than other DNA-based vaccine constructs suggesting an important role played by Quil-A in the induced immunity in chickens.

[0140] Localized IBV-Specific Cellular Responses in Immunized Birds—

[0141] Induction of robust T-cell responses has been identified as relevant correlates of protection against IBV infection in previous studies (34). An antigen specific T cell proliferation assay based on CellTrace™ Violet Cell dye staining of lung cells to trace proliferating T cells was developed. Different T-cell subsets responding to antigen stimulation was identified using flow cytometry assisted T-cell assay. Twenty days after first vaccination, the IBV Ark N protein specific proliferation was measured. The stimulation index (SI), which is the fold increase in stimulated to unstimulated cells calculated. Lung cells from pQAC-N vaccinated birds responded well to antigen stimulation which was higher when compared to negative and MLV control groups (FIG. 24A). An increase in the stimulation of proliferating CD8+ and TCRγδ+ T cells was observed in pQAC-N vaccinated birds in comparison to control birds (FIGS. 24B & 24D), suggesting a potential role for CD8+ and TCRγδ+ cell in pQAC-N immunity. On the other hand, the CD4+ T-cell proliferation was higher in MLV vaccinated birds (FIG. 24C).

[0142] Protective Efficacy of pQAC-N in the Presence of Maternally Derived Antibodies—

[0143] Maternally derived antibodies (MDA), mainly IgY are transferred from vaccinated hens to progeny via the yolk. Presence of IBV specific MDA has shown to protect against IBV challenge in SPF chickens (37-39). Although effective against IBV infections, MDA can interfere with MLV vaccination and dampen development of active immunity (39). In this pilot study, we investigated the ability of pQAC-N to mediate protection in the presence of interfering MDA. Commercial white leghorn chicks with high levels of circulating IBV specific IgY which persisted till about day-24 of age was used (FIG. 25A). As seen with the SPF chick, pQAC-N immunized commercial birds were also protected against IBV Arkansas DPI challenge with a significant reduction in viral load (FIG. 25C) and clinical severity scores (FIG. 25B) compared to unvaccinated birds, suggesting the ability of the pQAC-N to protect birds without interference by MDA.

[0144] After the pilot study, the ability of pQAC-N to reduce viral shedding in trachea was evaluated in comparison to MLV vaccinated birds. Similar to the previous trials, pQAC-N vaccinated birds had significantly reduced viral shedding in tracheal swabs and clinical severity post chal-

lenge on par with MLV vaccinated birds (FIGS. 27C & 27D). Interestingly, MLV vaccinated birds showed signs of respiratory distress as late as 15 dpv, with one bird dying at 10 dpv which was not observed in the other groups and with SPF birds in the previous trial. Diagnostic analysis including necropsy with bacteriology and virology analysis was performed by the Wisconsin Veterinary Diagnostic Laboratory on the dead bird and on a euthanized control bird. Investigation revealed the presence of *Staphylococcus aureus* and *Escherichia coli* superinfection in the dead MLV vaccinated bird which was not detected in the euthanized control bird. Severe locally extensive pulmonary edema was reported in the lungs of the MLV vaccinated bird which was a result of bacteremia/sepsis, the likely cause of death. MLV vaccinated birds were significantly lighter in weight (FIG. 26A) and had reduced weight gain between 14-28 dpv (FIG. 26B), a potential consequence of bacterial superinfection.

Discussion

[0145] Many experimental subunit and plasmid DNA vaccines against IBV have been developed and its efficacy detailed in previous studies. Intra muscularly (I.M) and in ovo administered experimental vaccines without adjuvants or complemented with natural and molecular adjuvants like IL-2 and GM-CSF have been shown to reduce viral shedding, clinical signs and improve protection rates against IBV (18-26). Alternative routes such as in ovo administration of S1 plasmid DNA vaccine has also been shown to elicit a modest immune response (19). Compared to S1 protein, higher protection rates against IBV challenge and increased total T-cells were shown with I.M immunization with plasmid encoding N protein (20, 23, 25). In this study, we detail the development of a safe mucosal vaccine adjuvant (QAC) for intranasal immunization, a highly desired feature of mass vaccinations under field condition. The safety of QAC nanocarrier was examined by two common routes of field immunization, I.N and in ovo. Birds intranasally immunized by a single dose or double doses (at 1 and 14 days of age) did not show any signs of respiratory distress, and weight gain was same as observed with control birds. The in ovo immunization also did not affect embryo development and egg hatch rates. Previously, both Quil-A and chitosan were shown to be biocompatible with no adverse effects reported when administered to animals (30-32, 40). Similarly, our results indicate that the QAC adjuvant is safe, tolerable and biocompatible in chicken hosts.

[0146] For vaccine efficacy, our experimental vaccine (pQAC-N) was compared to the current commercial MLV in a challenge model adopted based on recommended guidelines from the food and drug administration with modifications (FDA-9CFR). The superiority of the QAC adjuvant system was shown in a challenge model where birds were challenged immediately at 7 days following vaccine boost with a relatively high dose of IBV Ark-DPI (6.5E9 genome copy no or 10^{6.5} EID₅₀). Specifically, we demonstrated the ability of QAC adjuvant system to enhance immunogenicity and protective efficacy of pCAG-N plasmid vaccine which otherwise conferred partial protection when administered independently (naked pCAG-N). It is possible that N protein uptake and processing by antigen-presenting cells (APCs) is a key step in the downstream activation of B and T-cells for developing a robust memory immune response (41) that was shown in the pQAC-N immunized birds. Factors that influence APC uptake of antigens includes particle surface

charge, size, hydrophobicity and others (42). Soluble viral immunogens by themselves owing to their small size (<20 nm) are poorly taken up by APCs. Particulate adjuvant systems with a size range of 20-200 nm have been shown to promote APC uptake by endocytosis (42). Our findings have shown that QAC-based formulations formed spherical disaggregated particles of optimal size (<100 nm) for efficient APC uptake and processing, as expected when the Quil-A surfactant is added. Delivery systems with net positive surface charge like QAC (positive zeta potential 15 ± 4.4 mV), temporarily disrupt membrane of cells causing membrane flipping and/or fusion at cell surfaces mediating payload delivery or enter cells via clathrin-mediated endocytic pathway, a potential pathway for effective antigen uptake (43). Chitosan DNA particles promote the slow release of packaged DNA and similarly, we observed the sustained release of DNA payload complexed with QAC in vitro (44). Our in vitro analysis indicated that QAC could promote targeted delivery of payload into cells and/or act as antigen depots maintaining a sustained release of payload priming immune cells continually. Overall, encapsulation of plasmid DNA by QAC mediated slow release of immunogen which could help in continuous priming of antigen presenting cells and overcome the need for multiple immunizations.

[0147] pQAC-N vaccinated SPF birds had a significantly lower viral burden when compared to unvaccinated birds. Chitosan by itself did not reduce viral shedding observed with naked unadjuvanted pCAG-N construct, suggesting a key role of the nanocarrier size and composition used in our hands. Interestingly, a strong correlation between reduced clinical severity and reduction in viral load was observed. The protective efficacy of pQAC-N was comparable to MLV with similar levels of reduced clinical severity and non-significant reduction in viral load observed between both groups. To our knowledge, with the exception of one study, most experimental IBV DNA vaccines have been tested against serotypes not endemic to US, via I.M route and without a comparable commercial live virus group (20-26). Here we observed that the pQAC-N vaccine when administered I.N was able to protect vaccinated birds against a field-relevant IBV Arkansas-DPI, most likely because of the induced localized immunity as suggested before (34, 45). PBMCs harvested from pQAC-N vaccinated birds responded to antigen stimulation ex vivo with significantly higher proliferation than seen with control and other plasmid DNA vaccine groups. Moreover, the analysis of different immunological parameters indicated that pQAC-N induces strong CMI responses in contrast to MLV, which induced potent antibody responses. The induction of CMI responses could be a hallmark for the pQAC-N nanovaccine. IBV N protein is a highly immunogenic antigen with mapped CTL epitopes in the C-terminal which mediate potent CTL memory responses. IBV specific memory CD8+ T-cell responses restrict IBV replication efficiently and are strong correlates of protection for IBV control (34). It is noteworthy here that birds vaccinated with an experimental IBV N-based vaccines generally have a higher percentage and proliferation of CD3+ CD8+ T-cells, albeit not specific to IBV, a limitation of assaying total T-cell numbers (20, 23, 25).

[0148] To decipher QAC-DNA mediated IBV specific immunity, we used flow cytometry assisted lymphocyte proliferation assay to identify and quantify subsets of T-cells responding to IBV antigen. Similar to results with MTT

assay, we noticed lung cells from pQAC-N vaccinated birds had higher stimulation ex vivo when compared to the control groups. More reactive CD8+ and TCR $\gamma\delta$ + T-cells were present in pQAC-N vaccinated birds, albeit non-significant. Large variations in recall proliferation within pQAC-N vaccinated group was observed, a phenomenon that has been reported in other published studies investigating chicken immune responses (46). Presented results suggest that vaccination with pQAC-N confers protection against IBV challenge to levels similar to MLV vaccination and that protection might be attributed to an induction of CD8+ and TCR $\gamma\delta$ + memory T cell responses, rather than CD4+ induction observed with MLV vaccination. Further studies to elucidate the exact mechanism of pQAC-N mediated immunity are needed.

[0149] Most commercial breeders are immunized with IBV vaccines and transfer IBV specific IgY to their progeny via egg yolk which can interfere with vaccine efficacy. Prior evidence suggests that DNA vaccination could overcome limitations of early vaccinations by priming the immune system even in the presence of interfering MDA (47-49). Our results indicate that pQAC-N can mediate protection in the presence of MDA reducing viral shedding in lachrymal fluid and in the trachea. As observed in the trial with SPF chicks, IBV specific IgA and IgY were detectable in pQAC N vaccinated birds albeit significantly lower than levels in MLV vaccinated birds. Mortality associated with IBV outbreaks in the field is usually low unless compounded by secondary bacterial infections (50). Interestingly, in the latest trial conducted, MLV vaccinated commercial birds had active bacterial superinfection leading to mortality in one bird (~8%), reduced weight gain and presence of respiratory clinical signs which was not seen in pQAC-N vaccinated birds. This observation underscores the inferior safety profile of commercial MLV vaccines.

[0150] Although protective, addition of other inexpensive biocompatible adjuvants to generate a complementing humoral response could be used to the current pQAC-N construct. In summary, we detailed the development of a safe plasmid DNA vaccine complimented by a mucosal adjuvant system (QAC) which protects SPF and commercial birds against IBV challenge by eliciting a strong T-cell immune response. We postulate that the QAC nano-adjuvant system can be used as a vaccine adjuvant for the delivery of plasmid DNA and protein immunogens against other respiratory viruses and intracellular pathogens for poultry and other animals.

Materials and Methods

[0151] Cells and Viruses—

[0152] Expi293F cells obtained from ThermoFisher Scientific was used for confirming expression of IBV Ark N6xHis protein from vaccine construct. The cells were cultured in Expi293 medium at 37° C., 125 rpm, 8% CO₂ atmosphere in plastic flasks with ventilated caps. The virulent IBV Arkansas DPI strain (a kind gift from Dr Ladman and Dr Gelb) was propagated in 9-day old SPF ECEs and allantoic fluid harvested four days after infection. The stock virus titre was determined using RT-qPCR (see below) and also titrated and expressed as 50% embryo infectious dose (EID₅₀) (51). IBV S1 gene sequence of Ark DPI challenge isolate is AF006624.

[0153] Preparation of Plasmids—

[0154] Arkansas Nucleocapsid (N6×His) and S1 gene (S1 6×His) was amplified from reverse transcribed cDNA synthesized from IBV Arkansas 99 (ATCC VR-841) with a C-terminus 6×His tag. The forward primer (5'-ATCACT-GAATTCACCATGGCAAGCGGTAAAGCAG-3') and reverse primer (5'-ATCACTGCGGCCGCTTAGTGGT-GATGGTGATGATGACCTCCTCCAAGTTCATTCTCTCCTAGAGCTGC-3') were employed for amplifying N6×His. The forward primer (5'-ATCACTGAATTCAC-CATGTTGGTGAAGTCACTGTTTCTAGTG-3') and reverse primer (5'-ATCACTGCGGCCGCTCAGTGGT-GATGGTGATGATGCCCTCCGCCGAGGATCCAGTTCATTAGTGATCTTAATGTAAAACTGGTTTTC') were employed for amplifying S1 6×His. Amplified gene fragments were cloned into EcoRI and NotI restriction sites of pCAG-GFP plasmid, a gift from Connie Cepko (addgene plasmid #11150, FIG. 3A). To confirm insertion of genes in the correct orientation, DNA sequencing was performed at the UW-Madison Biotechnology Center with an ABI Prism 3730XL DNA analyzer using BigDye terminators (Applied Biosystems, CA). To confirm expression of N6×His and S1 6×His protein, Expi293F cells seeded in 6-well format was transfected with an optimized ratio of DNA (4 ug): TransT PRO transfection reagent (2 ul) according to manufacturer's instructions (Mirus Bio, WI, USA). Three days post transfection, cells were harvested for western blot analysis. Cell fractions were boiled in Laemmli sample buffer (BioRad, Hercules, Calif., USA) and resolved on a 4-20% SDS-PAGE gel by electrophoresis using a Mini-PROTEAN 3 system (BIO-RAD, CA). Polyacrylamide gels were electroblotted onto nitrocellulose membranes using a Turboblott® system. Membranes were blocked in 5% (W/V) skim milk and probed with polyclonal anti-6×His HRP antibody (ThermoFisher Scientific, MA1-21315-HRP). Membranes were developed using a solid phase 3,30,5,50-tetramethylbenzidine (TMB) substrate system.

[0155] Characterization of Nanoparticles—

[0156] Quil-A (VET-SAP, Desert King) stock solution of 0.2% was made in nuclease free water. Chitosan stock solution ($\geq 75\%$ deacetylated, Sigma) of 0.4% in 1% glacial acetic acid was prepared and diluted to 0.04% in 5 mM sodium acetate buffer, pH 5.5. Both components were heated separately for 30 mins in a 55 C water bath. Equal volume of Quil-A-plasmid DNA solution was added drop by drop to the chitosan solution and the mixture was vortexed for 30 s. The solution was left at room temperature for 1 hour to promote QAC-DNA particle formation. Plasmid DNA was diluted to 100 ug/ml in 50 mM sodium sulphate buffer and Quil-A was added to a final concentration of 0.002%. Size distribution and zeta potential of QAC-NPs in aqueous dispersion was measured by dynamic light scattering (DLS) on a Malvern zetasizer instrument at 25° C. For size distribution, 50 ul of QAC-NPs in solution was placed in a low volume cuvette and analyzed directly. For zeta potential measurement, approximately 1 mL of the QAC-NPs in solution was placed in a disposable capillary zeta potential cell available from the Zetasizer Nano series. TEM experiments were performed at the Medical school Electron Microscopy facility of the University of Wisconsin-Madison using a Philips CM120 transmission electron microscope (FEI, Eindhoven, the Netherlands) at 80 kV. The size and morphology of vaccine preparations was reexamined via negative staining using the drop method. QAC NPs loaded

with 1 mg total DNA was resuspended in 600 μ L of 0.05 M phosphate buffered saline (PBS, pH 7.4) at 37° C. At each time point, suspensions were removed and centrifuged at 14,000 rcf for 20 min. The supernatant was removed and replaced with PBS and returned to incubation. Supernatant samples were quantified for released DNA from the QAC using a NanoDrop™ Spectrophotometer and compared to the total DNA used. QAC-GFP DNA (5 ug) was added to 3×10^7 Expi293F cells seeded into a 6 well plate. 72 hours post addition the presence of GFP+ cells was identified using an upright fluorescence microscope. Background fluorescence was normalized using GFP-Expi293F control cells.

[0157] Nanoparticle Characterization—

[0158] The QAC complex forms distinct nanoparticles. Various ratios of Quil-A and chitosan were tested as recited in Table 1 and Table 2 below. Also see FIGS. 12 and 13.

TABLE 1

Quil-A conc (%)	Chitosan conc (%)	Comments
0.00004	0.02	Aggregated structure
0.0002		Aggregated structure
0.001		Distinct nanostructure
0.005		Aggregated structure - thick sheets
0.025		Aggregated structure - thick sheets

TABLE 2

Quil-A conc (%)	Chitosan conc (%)	Comments
0.001	0.0008	Distinct filamentous structures
	0.004	Aggregated structure
	0.02	Distinct nanostructures
	0.1	Distinct nanostructures (size - 20 nm \pm 2 nm)
	0.5	Aggregated structure - thick sheets

[0159] QAC-Protein Protocol—

[0160] Quil-A stock solution of 0.2% was made in nuclease free water. Chitosan stock solution of 0.4% in 1% glacial acetic acid was prepared and diluted to 0.04% in 5 mM sodium acetate buffer, pH 5.5. Protein was diluted to 100 μ g/ml in 50 mM sodium sulfate buffer and Quil-A was added to a final concentration of 0.002%. Equal volume of Quil-A-protein solution was added drop by drop to the chitosan solution and the mixture was vortexed for 30 s. The solution was vigorously mixed in shaking conditions (110 rpm) for 1 hour at 37 C to promote QAC-protein particle formation.

[0161] Vaccine Safety Study—

[0162] In this study, the tolerability and biocompatibility of pQAC-N was evaluated in 1-day-old white leghorn SPF chicks and ECEs. A total of 30 chicks was divided into 3 groups of 10 each. Chicks from the first group was inoculated with PBS (negative control). Chicks from the other groups were inoculated with either a single dose (100 ug) or 2× the dose (200 ug) of pQAC-N at day-1 via the intranasal route. Chicks were monitored for general or respiratory distress, depression or in appetite and weight gain over the course of 30 days post inoculation. In another experiment, 6 embryonated chicken eggs (ECEs) was divided into 2 groups of 3 each. At 18.5 days post incubation, ECEs were either inoculated with PBS (negative control) or with 100 ug

of pQAC-N construct into the allantoic cavity. Embryo development and hatch rate of inoculated ECEs was monitored.

[0163] Vaccine Efficacy Studies—

[0164] For all the vaccine experiments, birds were challenged with a dose of 6.5×10^6 genome copy no or $10^{6.5}$ EID₅₀/bird of virulent IBV Arkansas DPI strain via direct intranasal instillations. The challenge dose was determined in an independent infection experiment wherein the challenge dose resulted in discernable clinical signs as early as 3 dpc and peak viral load replication was observed at 6 dpc. The protective efficacy of pQAC-N construct was evaluated in 1-day-old white leghorn SPF chicks (Charles River Laboratories). A total of 50 chicks was divided equally into 5 groups (n=10 each) and used for the efficacy study, first 2 groups were inoculated with PBS (negative control) or commercial Arkansas MLV (Mildvac-Ark®, Merck Animal Health USA, positive control) via direct intranasal instillations (dose according to manufacturer's instructions). The other groups were either vaccinated with naked (no adjuvant), chitosan complexed or pQAC-N at day-1 and followed by a booster dose at day-14 via intranasal (IN) route. A vaccine dose (100 ng/bird) was administered at each vaccination time point. At 20 days post first vaccination time point (DPV), PBMCs were harvested from blood collected using previously described protocols for proliferation assay (see below). At 10, 20 DPV & 3 days post challenge (DPC) lachrymal fluid (tears) samples were harvested for ELISA and at 6 DPC for viral load estimation (see below). Lachrymation was induced by placing sodium chloride (salt) crystals on the eyes and tears were collected using micropipettes (52). Clinical severity was noted everyday post challenge for 8 days. The severity scores of clinical signs of IBV were as follows; 0=normal, 1=Infrequent sneezing (single event during observation), 2=frequent sneezing (more than one event during observation), 1=mild rales, 2=severe rales, 2=presence of nasal exudate. The severity scores of IBV clinical signs, described in the figure legends were recorded once a day for each chicken for 8 days after challenge. The severity score represents as average score of clinical signs measured for each chicken over 8 days (53). Lachrymal fluid harvested at 6 dpc was analyzed for viral RNA using IBV N gene specific RT-qPCR.

[0165] In another experiment, pQAC-N was used to immunize 1-day-old commercial white leghorn chicks (Cackle Hatchery®, MO, USA). A total of 10 chicks was divided into 2 groups. Chicks from the first group (n=4) was inoculated with PBS (negative control) while the second group (n=6) was immunized with pQAC-N construct at day-1 and followed by a booster dose at day-14 via intranasal (IN) route. Blood was collected from birds in the negative control group at day-10, 20 and 24 in age to quantitate MDA IgY. All birds were sampled, challenged and followed for clinical scores and virus titers as described above for the SPF chicks.

[0166] In the final experiment, pQAC-N was used to immunize 3-day-old commercial white leghorn chicks (Welp Hatchery, IA, USA). A total of 35 chicks was divided into 3 groups. Chicks were either inoculated with PBS (negative control, n=11) or commercial Arkansas MLV (Mildvac-Ark, Merck Animal Health USA, positive control, n=12) or with pQAC-N construct at day-3 and followed by a booster dose at day-17 (14 dpv) via intranasal (IN) route as described above. Blood was collected from all the birds

at 0, 10, 20 and 24 dpv to quantitate MDA IgY and lachrymal fluid from all the birds at 10, 20 and 24 dpv to quantitate IBV specific IgA. All birds were sampled, challenged and followed for clinical scores as described above for the SPF chicks. Tracheal swabs were taken at 6 dpc and analyzed for viral RNA using IBV N gene specific RT-qPCR. One bird in MLV group died at 10 dpv, one bird from MLV and PBS control groups each were euthanized at 12 dpv for necropsy and diagnosis by the Wisconsin Veterinary Diagnostic Laboratory (WVDL), Madison, Wis., USA.

[0167] Recombinant Protein Purification—

[0168] The pCAG constructs with S1 6xHis and N6xHis were transfected into Expi293F cells as described above using TransIT PRO transfection reagent according to manufacturer's instructions (Mirus Bio, WI, USA). For S1 6xHis purification, supernatant was harvested and for N6xHis purification, cells were harvested 3 days after transfection. The samples were purified using Thermo Scientific™ His-Pur™ Ni-NTA Resin according to manufacturer's instructions (batch method). The protein was eluted with elution buffer (50 mM sodium phosphate, 0.3 M sodium chloride, 250 mM imidazole pH 8.0). The eluate was concentrated using PEG-20 solution (800 mg/ml) hygroscopically and dialyzed using Thermo Scientific™ SnakeSkin™ Dialysis Tubing (7K MWCO) against PEG free PBS. Protein concentration was determined by Thermo Scientific™ Pierce™ BCA Protein Assay Kit.

[0169] IBV Specific ELISA—

[0170] Sera and lachrymal fluid from different time-points were screened for humoral response against IBV Arkansas serotype. In order to measure IgY and IgA antibody levels in plasma and tears of chicken respectively, an IBV-specific enzyme-linked immunosorbent assay (ELISA) was developed as described previously with modifications(54). Briefly, ELISA plates were coated with inactivated IBV Arkansas (100 ng/well, IgY) or IBV Arkansas S1 and N6xHis protein (50 ng total/well, IgA) diluted in carbonate/bicarbonate buffer, pH 9.6 and incubated overnight at 4 C followed by blocking with 5% Skim milk to reduce background. A 50 ul of diluted serum (1/200) or tears (1/50) harvested at different time-points from immunized chickens was added to the wells and incubated at 37 C for 1 hour. Post washing (PBS-TritonX 100, 0.1%), either HRP conjugated anti-chicken IgY (NBP1-74778, NOVUS Bio) or anti-chicken IgA (NB7284, NOVUS Bio) at dilutions of 1/1000 was added to the wells and incubated at 37° C. for 1 hr. Post washing, 50 ul of TMB substrate solution was added and incubated for 20 minutes or until color developed. The reaction was stopped by the addition of 1M sulphuric acid and plates are read at 450 nm. To generate standard curves, sera and tears from severely IBV infected chickens from previous experiments was used. Two-fold serial dilutions was assigned and arbitrary value and used for analysis.

[0171] Assessment of IBV Specific Lymphocyte Proliferation Assay—

[0172] PBMCs were prepared from harvested blood as described previously (46). PBMCs were adjusted to 10^7 cells/ml in RPMI 1640 (Invitrogen) supplemented with 10% inactivated fetal calf serum and 100 µl cells per well were transferred into flat-bottomed 96-well plates. Equal volumes of medium containing stimulant (IBV Ark DPI live virus, MOI=1) was added in triplicate and cultures were incubated for 2 days at 41° C., 5% CO₂. Negative controls received 100 µl RPMI 1640 medium only. After incubation, to each

well, 15 μ l of MTT reagent (CellTiter 96® Non-Radioactive Cell Proliferation Assay, Promega) and cells incubated for a further 4 hrs at 41° C., 5% CO₂ until development of MTT (3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl-tetrazolium bromide) formazan was observed. Post incubation, Dimethyl sulfoxide (DMSO) was added to dissolve the formazan crystals and incubated for an hour at 41° C., 5% CO₂. The absorbance of the purple color was taken at 570 nm in an ELISA plate reader. PBMCs from 4 chicks/group was used to assess proliferative capacity. The output stimulation index (SI) is the ratio of absorbance post stimulation to the absorbance in unstimulated conditions.

[0173] Viral Load Measurement—

[0174] RNA was extracted from lachrymal fluid (10 μ l) or Tracheal swabs (100 μ l) collected from chickens using Zymo Direct-Zol™ RNA mini prep kit (Zymo Research, CA, USA) according to manufacturer's instructions. RT-qPCR was conducted in two steps: cDNA synthesis (Invitrogen™ SuperScript™ II First-Strand Synthesis System) and qPCR reactions. cDNA synthesis was performed with 0.5 μ l (50 ng/ μ l) random hexamers, 0.5 μ l of 10 mM dNTPs, and 4 μ l RNA and heated at 65° C. for 5 min and chilled on ice followed by addition of 1 μ l of 10 \times RT buffer, 1 μ l of 0.1 M DTT, 1 μ l of 25 mM MgCl₂, 0.5 μ l of RNaseOUT and 0.5 μ l of SuperScript III enzyme in final volume of 10 μ l. The reaction conditions include 25° C. for 5 min, 50° C. for 60 min and 70° C. for 15 min. SYBR green RT-qPCR was performed using an IBV N gene specific primer pair set forward primer: 5' ATGCTCAACCTAGTCCCTAGCA 3' and reverse primer: 5' TCAAACCTGCGGATCATCACGT 3' amplifying 128 nt of N gene of IBV Arkansas DPI. PCRs were performed using a StepOnePlus™ Real-Time PCR System (Applied Biosystems, Foster City, Calif., U.S.A.) under the following conditions: one cycle 95 C for 2 min followed by 40 cycles of 95 C for 3 sec and 60 C for 30 sec. Each 20 μ l reaction was carried out using 1 μ l of diluted cDNA (1/10), 10 μ l of GoTaq® qPCR mastermix (Promega), 2 μ l of forward and reverse primers and 7 μ l of nuclease free water. A serial 10-fold dilution of pCAG-IBV Ark N6 \times His plasmid was used to establish the standard curve. Temperature melt curve analysis was used to confirm the specificity of the product.

[0175] Flow Cytometric Assessment of IBV Specific Proliferation—

[0176] A subgroup of additional chicks (N=4 each) from each vaccine group in the SPF chick vaccine efficacy study were used for flow cytometric assessment. All chicks were euthanized at 20 DPV and single cell suspensions from lungs were prepared using standard techniques and used for T-cell proliferation assay. Briefly, lungs were excised and placed in a gentleMACS dissociator M tube (Miltenyi 130-093-236) with 5 mL collagenase B (2 mg/ml, Roche). Lung tissue was processed using the gentleMACS dissociator followed by incubation for 30 min at 37° C. Single-cell suspensions lung were prepared by gently squeezing through a 70-mm cell strainer (Falcon) after lysing RBCs using 1 \times BD Biosciences BD Pharm Lyse™. Total of 10⁷ cells/ml were stained with CellTrace™ Violet Cell Proliferation dye (Thermo Scientific C34557) according to manufacturer's instructions and 100 μ l of cells plated/well in RPMI 1640 with 10% chicken immune serum. After overnight incubation at 41° C., 5% CO₂, cells were stimulated with 130 ng of IBV Arkansas N6 \times His protein complexed with chitosan per well in 100 μ l of RPMI 1640 with 10% chicken immune serum. Four days

post stimulation, cells were stained for surface markers, CD4-AF647 (clone CT-4), CD8 α -FITC (clone 3-298) together and TCR γ δ -FITC (clone TCR-1) independently for flow cytometry analysis. All antibodies were purchased from SouthernBiotech (Birmingham, Ala., USA). All samples were acquired on an BD LSR Fortessa flow cytometer. Data were analyzed with FlowJo software (BD Biosciences). The strategy for gating on proliferating CD4+ and CD8 α + T cells was debris exclusion on the Forward Scatter (FSC)—Side Scatter (SSC) dot plot followed by exclusion of dead cells by fixable viability dye eFluor 780 (Invitrogen™, #65-0865-14) staining. Out of the live cells, total proliferated cells were gated positive using a histogram plot with eF450 on the x-axis (for CellTrace™ Violet). Finally, CD4 cells were gated positive at the AF647 axis and CD8 α cells were gated positive at the FITC axis in a FITC-AF647 dot plot. A similar approach was used for identifying proliferating TCR γ δ + T-cells. The output, stimulation index (SI) is the ratio of % proliferating cells post stimulation to the % proliferating cells in unstimulated condition.

[0177] Statistical Analysis—

[0178] Statistical analyses were performed using Graph-Pad software (La Jolla, Calif.). Weight gain, cellular immune assays, clinical severity scoring, viral loads were compared using an ordinary one-way ANOVA test with multiple comparisons where *, P<0.05; **, P<0.01; ***, P<0.001; ****, P<0.0001 were considered significantly different among groups. Antibody titers and absolute weight of birds were compared using a two-way ANOVA test where *, P<0.05; **, P<0.01; ***, P<0.001; ****, P<0.0001 were considered significantly different among groups.

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Example 2

[0233] This prophetic example outlines a QAC complex antigen in vivo cross presentation study. Antigen cross presentation is important for effective immune response to tumors and viral infections. Dendritic cells are antigen presenting cells and are unique in their ability to cross-present exogenous antigens on MHC class I molecules and activate antigen specific cytotoxic T cells. This study examines the function of the QAC complex to mediate antigen cross presentation by dendritic cells (DCs, specifically, bone marrow derived DCs and splenic DCs) in an in vivo assay system using soluble ovalbumin protein (OVA).

[0234] BL6 mice will be separated into groups (n=4) corresponding to treatment with Adjuvax control, a first concentration of QAC complex loaded with 30 µg OVA protein, a second concentration of QAC complex loaded with 30 µg OVA protein, chitosan loaded with 30 µg OVA protein, and Quail-A loaded with 30 µg OVA protein. Mice will receive a 50 µL intra nasal vaccination with the selected treatment. After 9 days, phenotype and functional responses in bronchoalveolar lavage (BAL), lungs, and spleen will be assessed. At 13 days post treatment, data collection and further analysis on the mice will be carried out. (FIG. 15) Phenotypic parameters measured include MHCI tetramers specific to ovalbumin peptide sequence SIINFEKL (SIINFEKL MHCI tetramers), CD103 and CD69 tissue residency, and KLRG1, CD127, CD44, and CD62L expression. Functional parameters measured include ovalbumin immunogenic peptide CD8 sequence SIINFEKL (OVA I CD8 SIINFEKL), ovalbumin immunogenic peptide CD4 sequence ISQAVHAAHAEINEAGR (OVA II CD4 ISQAVHAAHAEINEAGR), and expression of CD4, CD8, IL-2, IL-4, IL-6, IL-10, IL-13, IL-17, IFNγ, and TNFα.

[0235] This study will confirm that the cross presentation measured in vitro in primary DC cells using the QAC complex is mirrored in vivo. Additionally, the mouse experiment will generate a model immunologic system for QAC mediated cross presentation in vivo for other antigens and immunogens.

Example 3

[0236] This prophetic example outlines an in vivo study to measure innate and adaptive immune responses to the loaded QAC complex. The QAC complex can strongly induce cross presentation with OVA in vitro in DC cells. The mouse study presented provides a model immunologic system for the study of in vivo QAC complex induced cross presentation. Measurements including antigen presenting cells will strongly augment the adjuvant mechanism. Chitosan has been demonstrated to work in vitro, but will not function in vivo for protein antigens. The present studies will confirm the function of the QAC complex in antigen presenting cell recruitment and activation.

[0237] BL6 mice will be separated into groups (n=7) corresponding to treatment with Adjuvax control, a first concentration of QAC complex loaded with 30 µg OVA protein or DQ-OVA protein, a second concentration of QAC complex loaded with 30 µg OVA protein or DQ-OVA protein, chitosan loaded with 30 µg OVA protein or DQ-OVA, and Quail-A loaded with 30 µg OVA protein or DQ-OVA. Mice will receive a 50 µL intra nasal injection of the indicated treatment. After 3 days, mice treated with DQ-OVA will be examined for innate cell recruitment elic-

ited by adjuvants and its effect on antigen processing in lungs and draining lymph nodes (LNs). At 7 days post treatment data collection and further analysis on the mice will be carried out. (FIG. 16). At 4 days after treatment, mice treated with OVA will be examined for T cell phenotype and functional responses in BAL, lungs, and spleen. At 13 days post treatment, data collection and further analysis on the mice will be carried out.

[0238] Phenotypic parameters measured in T cells will include SIINFEKL MHC I tetramers, and expression of CD103, CD69, KLRG1, CD127, CD44, and CD62L. Functional parameters measured include OVA I CD8 SIINFEKL, OVA II CD4 ISQAVHAAHAEINEAGR, and expression of

CD4, CD8, IL-2, IL-4, IL-6, IL-10, IL-13, IL-17, IFN γ , and TNF α . Parameters measured in innate immune cells include antigen uptake, such as uptake of unprocessed Texas Red or digested GFP, and analysis of antigen presenting cells including neutrophils, Alv macs, Eosinophils, CD103 cDCs, monocyte derived DCs, monocyte, Natural Killer cells, and new cell subsets. Antigen presenting cells (APCs) will be analyzed using flow cytometry to assay for antigen uptake which is essentially a fluorophore encapsulated by QAC. If there is efficient antigen uptake, antigen presenting cells would have taken up the fluorophore. Potent adjuvants are able to efficiently deliver payload to APCs and mediate antigen uptake.

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<210> SEQ ID NO 4
<211> LENGTH: 7850
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: synthetic

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<400> SEQUENCE: 4

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<210> SEQ ID NO 5

<211> LENGTH: 412

<212> TYPE: PRT

<213> ORGANISM: Infectious Bronchitis Virus

<400> SEQUENCE: 5

Met Ala Ser Gly Lys Ala Thr Gly Lys Thr Asp Ala Pro Ala Pro Val

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Ile Lys Leu Gly Gly Pro Lys Pro Pro Lys Val Gly Ser Ser Gly Asn	20	25	30
Ala Ser Trp Phe Gln Ala Ile Lys Ala Lys Lys Leu Asn Ser His Pro	35	40	45
Pro Lys Phe Glu Gly Ser Gly Val Pro Asp Asn Glu Asn Leu Lys Thr	50	55	60
Ser Gln Gln His Gly Tyr Trp Arg Arg Gln Ala Arg Phe Lys Pro Val	65	70	75
Lys Gly Gly Arg Lys Pro Val Pro Asp Ala Trp Tyr Phe Tyr Tyr Thr	85	90	95
Gly Thr Gly Pro Ala Ala Asp Leu Asn Trp Gly Asp Ser Gln Asp Gly	100	105	110
Ile Val Trp Val Ala Ala Lys Gly Ala Asp Val Lys Ser Arg Ser His	115	120	125
Gln Gly Thr Arg Asp Pro Asp Lys Phe Asp Gln Tyr Pro Leu Arg Phe	130	135	140
Ser Asp Gly Gly Pro Asp Gly Asn Phe Arg Trp Asp Phe Ile Pro Leu	145	150	155
Asn Arg Gly Arg Ser Gly Arg Ser Thr Ala Ala Ser Ser Ala Ala Ser	165	170	175
Ser Arg Ala Pro Ser Arg Asp Gly Ser Arg Gly Arg Arg Ser Gly Ser	180	185	190
Glu Asp Asp Leu Ile Ala Arg Ala Ala Lys Ile Ile Gln Asp Gln Gln	195	200	205
Lys Lys Gly Ser Arg Ile Thr Lys Val Lys Ala Asp Glu Met Ala His	210	215	220
Arg Arg Tyr Cys Lys Arg Thr Ile Pro Pro Gly Tyr Lys Val Asp Gln	225	230	235
Val Phe Gly Pro Arg Thr Lys Gly Lys Glu Gly Asn Phe Gly Asp Asp	245	250	255
Lys Met Asn Glu Glu Gly Ile Lys Asp Gly Arg Val Thr Ala Met Leu	260	265	270
Asn Leu Val Pro Ser Ser His Ala Cys Leu Phe Gly Ser Arg Val Thr	275	280	285
Pro Lys Leu Gln Pro Asp Gly Leu His Leu Lys Phe Glu Phe Thr Thr	290	295	300
Val Val Pro Arg Asp Asp Pro Gln Phe Asp Asn Tyr Val Lys Ile Cys	305	310	315
Asp Gln Cys Val Asp Gly Val Gly Thr Arg Pro Lys Asp Asp Glu Pro	325	330	335
Arg Pro Lys Ser Arg Ser Ser Ser Arg Pro Ala Thr Arg Thr Ser Ser	340	345	350
Pro Ala Pro Arg Gln Gln Arg Pro Lys Lys Glu Lys Lys Pro Lys Lys	355	360	365
Gln Asp Asp Glu Val Asp Lys Ala Leu Thr Ser Asn Glu Glu Arg Asn	370	375	380
Asn Ala Gln Leu Glu Phe Asp Glu Glu Pro Lys Val Ile Asn Trp Gly	385	390	395
Asp Ala Ala Leu Gly Glu Asn Glu Leu Gly Gly Gly	405	410	

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<210> SEQ ID NO 6
<211> LENGTH: 1257
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: synthetic

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gccgctgacc tgaattgggg tgatagccaa gatggtatag tgtgggttgc tgcaaaggt      360
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<210> SEQ ID NO 7
<211> LENGTH: 1098
<212> TYPE: PRT
<213> ORGANISM: Infectious Bronchitis Virus

<400> SEQUENCE: 7
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Ser Ala Asn Leu Tyr Asp Asn Glu Ser Phe Val Tyr Tyr Tyr Gln Ser
 20            25            30

Ala Phe Arg Pro Gly His Gly Trp His Leu His Gly Gly Ala Tyr Ala
 35            40            45

Val Val Asn Val Ser Ser Glu Asn Asn Asn Ala Gly Thr Ala Pro Ser
 50            55            60

Cys Thr Ala Gly Ala Ile Gly Tyr Ser Lys Asn Phe Ser Ala Ala Ser
 65            70            75            80

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Val	Ala	Met	Thr	Ala	Pro	Leu	Ser	Gly	Met	Ser	Trp	Ser	Ala	Ser	Ser	85	90	95
Phe	Cys	Thr	Ala	His	Cys	Asn	Phe	Thr	Ser	Tyr	Ile	Val	Phe	Val	Thr	100	105	110
His	Cys	Phe	Lys	Ser	Gly	Ser	Asn	Ser	Cys	Pro	Leu	Thr	Gly	Leu	Ile	115	120	125
Pro	Ser	Gly	Tyr	Ile	Arg	Ile	Ala	Ala	Met	Lys	His	Gly	Ser	Ala	Thr	130	135	140
Pro	Gly	His	Leu	Phe	Tyr	Asn	Leu	Thr	Val	Ser	Val	Thr	Lys	Tyr	Pro	145	150	155
Lys	Phe	Arg	Ser	Leu	Gln	Cys	Val	Asn	Asn	His	Thr	Ser	Val	Tyr	Leu	165	170	175
Asn	Gly	Asp	Leu	Val	Phe	Thr	Ser	Asn	Tyr	Thr	Glu	Asp	Val	Val	Ala	180	185	190
Ala	Gly	Val	His	Phe	Lys	Ser	Gly	Gly	Pro	Ile	Thr	Tyr	Lys	Val	Met	195	200	205
Arg	Glu	Val	Lys	Ala	Leu	Ala	Tyr	Phe	Val	Asn	Gly	Thr	Ala	His	Asp	210	215	220
Val	Ile	Leu	Cys	Asp	Asp	Thr	Pro	Arg	Gly	Leu	Leu	Ala	Cys	Gln	Tyr	225	230	235
Asn	Thr	Gly	Asn	Phe	Ser	Asp	Gly	Phe	Tyr	Pro	Phe	Thr	Asn	Thr	Ser	245	250	255
Ile	Val	Lys	Asp	Lys	Phe	Ile	Val	Tyr	Arg	Glu	Ser	Ser	Val	Asn	Thr	260	265	270
Thr	Leu	Thr	Leu	Thr	Asn	Phe	Thr	Phe	Ser	Asn	Glu	Ser	Gly	Ala	Pro	275	280	285
Pro	Asn	Thr	Gly	Gly	Val	Asp	Ser	Phe	Ile	Leu	Tyr	Gln	Thr	Gln	Thr	290	295	300
Ala	Gln	Ser	Gly	Tyr	Tyr	Asn	Phe	Asn	Phe	Ser	Phe	Leu	Ser	Ser	Phe	305	310	315
Val	Tyr	Arg	Glu	Ser	Asn	Tyr	Met	Tyr	Gly	Ser	Tyr	His	Pro	Arg	Cys	325	330	335
Ser	Phe	Arg	Pro	Glu	Thr	Leu	Asn	Gly	Leu	Trp	Phe	Asn	Ser	Leu	Ser	340	345	350
Val	Ser	Leu	Thr	Tyr	Gly	Pro	Ile	Gln	Gly	Gly	Cys	Lys	Gln	Ser	Val	355	360	365
Phe	Asn	Gly	Lys	Ala	Thr	Cys	Cys	Tyr	Ala	Tyr	Ser	Tyr	Gly	Gly	Pro	370	375	380
Arg	Ala	Cys	Lys	Gly	Val	Tyr	Arg	Gly	Glu	Leu	Thr	Gln	His	Phe	Glu	385	390	395
Cys	Gly	Leu	Leu	Val	Tyr	Val	Thr	Lys	Ser	Asp	Gly	Ser	Arg	Ile	Gln	405	410	415
Thr	Ala	Thr	Gln	Pro	Pro	Val	Leu	Thr	Gln	Asn	Phe	Tyr	Asn	Asn	Ile	420	425	430
Thr	Leu	Gly	Lys	Cys	Val	Asp	Tyr	Asn	Val	Tyr	Gly	Arg	Thr	Gly	Gln	435	440	445
Gly	Phe	Ile	Thr	Asn	Val	Thr	Asp	Leu	Ala	Thr	Ser	His	Asn	Tyr	Leu	450	455	460
Ala	Asp	Gly	Gly	Leu	Ala	Ile	Leu	Asp	Thr	Ser	Gly	Ala	Ile	Asp	Ile	465	470	475
Phe	Val	Val	Gln	Gly	Glu	Tyr	Gly	Pro	Asn	Tyr	Tyr	Lys	Val	Asn	Leu	480		

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485																490						495					
Cys	Glu	Asp	Val	Asn	Gln	Gln	Phe	Val	Val	Ser	Gly	Gly	Lys	Leu	Val												
			500					505					510														
Gly	Ile	Leu	Thr	Ser	Arg	Asn	Glu	Thr	Gly	Ser	Gln	Pro	Leu	Glu	Asn												
		515					520					525															
Gln	Phe	Tyr	Ile	Lys	Ile	Thr	Asn	Gly	Thr	His	Arg	Ser	Arg	Arg	Ser												
	530					535					540																
Val	Asn	Glu	Asn	Val	Thr	Asn	Cys	Pro	Tyr	Val	Ser	Tyr	Gly	Lys	Phe												
545					550					555					560												
Cys	Ile	Lys	Pro	Asp	Gly	Ser	Val	Ser	Pro	Ile	Val	Pro	Lys	Glu	Leu												
				565					570					575													
Glu	Gln	Phe	Val	Ala	Pro	Leu	Leu	Asn	Val	Thr	Glu	Asn	Val	Leu	Ile												
			580					585					590														
Pro	Asn	Ser	Phe	Asn	Leu	Thr	Val	Thr	Asp	Glu	Tyr	Ile	Gln	Thr	His												
		595					600					605															
Met	Asp	Lys	Ile	Gln	Ile	Asn	Cys	Leu	Gln	Tyr	Val	Cys	Gly	Asn	Ser												
610						615					620																
Leu	Ala	Cys	Arg	Lys	Leu	Phe	Gln	Gln	Tyr	Gly	Pro	Val	Cys	Asp	Asn												
625					630					635					640												
Ile	Leu	Ser	Val	Val	Asn	Ser	Val	Gly	Gln	Lys	Glu	Asp	Met	Glu	Leu												
				645					650					655													
Leu	Ser	Phe	Tyr	Ser	Ser	Thr	Lys	Pro	Ser	Gly	Phe	Asn	Thr	Pro	Val												
		660						665					670														
Phe	Ser	Asn	Leu	Ser	Thr	Gly	Glu	Phe	Asn	Ile	Ser	Leu	Leu	Leu	Thr												
		675					680					685															
Thr	Pro	Ser	Ser	Pro	Arg	Gly	Arg	Ser	Phe	Ile	Glu	Asp	Leu	Leu	Phe												
	690					695					700																
Thr	Ser	Val	Glu	Ser	Val	Gly	Leu	Pro	Thr	Asp	Glu	Ala	Tyr	Lys	Lys												
705					710					715					720												
Cys	Thr	Ala	Gly	Pro	Leu	Gly	Phe	Leu	Lys	Asp	Leu	Ala	Cys	Ala	Arg												
				725					730					735													
Glu	Tyr	Asn	Gly	Leu	Leu	Val	Leu	Pro	Pro	Ile	Ile	Thr	Ala	Glu	Met												
		740						745					750														
Gln	Thr	Leu	Tyr	Thr	Ser	Ser	Leu	Val	Val	Ser	Met	Ala	Phe	Gly	Gly												
		755					760					765															
Ile	Thr	Ser	Ala	Gly	Ala	Ile	Pro	Phe	Ala	Thr	Gln	Leu	Gln	Ala	Arg												
	770					775					780																
Ile	Asn	His	Leu	Gly	Ile	Thr	Gln	Ser	Leu	Leu	Leu	Lys	Asn	Gln	Glu												
785					790					795					800												
Lys	Ile	Ala	Ala	Ser	Phe	Asn	Lys	Ala	Ile	Gly	His	Met	Gln	Glu	Gly												
			805						810					815													
Phe	Arg	Ser	Thr	Ser	Leu	Ala	Leu	Gln	Gln	Ile	Gln	Asp	Val	Val	Asn												
			820					825					830														
Lys	Gln	Ser	Ala	Ile	Leu	Thr	Glu	Thr	Met	Leu	Ala	Leu	Asn	Lys	Asn												
		835						840					845														
Phe	Gly	Ala	Ile	Ser	Ser	Val	Ile	Gln	Asp	Ile	Tyr	Gln	Gln	Leu	Asp												
	850					855					860																
Asp	Ile	Gln	Ala	Asp	Ala	Gln	Val	Asp	Arg	Leu	Ile	Thr	Gly	Arg	Leu												
865					870					875					880												
Ser	Ser	Leu	Ser	Val	Leu	Ala	Ser	Ala	Lys	Gln	Ser	Glu	Tyr	Ile	Arg												
				885					890					895													

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Val Ser Gln Gln Arg Glu Leu Ala Thr Gln Lys Ile Asn Glu Cys Val
900 905 910

Lys Ser Gln Ser Ile Arg Tyr Ser Phe Cys Gly Asn Gly Arg His Val
915 920 925

Leu Thr Ile Pro Gln Asn Ala Pro Asn Gly Ile Val Phe Ile His Phe
930 935 940

Thr Tyr Thr Pro Glu Ser Phe Ile Asn Val Thr Ala Val Val Gly Phe
945 950 955 960

Cys Val Ser Pro Ala Asn Ala Ser Gln Tyr Ala Ile Val Pro Ala Asn
965 970 975

Gly Arg Gly Ile Phe Ile Gln Val Asn Gly Ser Tyr Tyr Ile Thr Ala
980 985 990

Arg Asp Met Tyr Met Pro Arg Asp Ile Thr Ala Gly Asp Ile Val Thr
995 1000 1005

Leu Thr Ser Cys Gln Ala Asn Tyr Val Ser Val Asn Lys Thr Val
1010 1015 1020

Ile Thr Thr Phe Val Asp Asn Asp Asp Phe Asp Phe Asp Asp Glu
1025 1030 1035

Leu Ser Lys Trp Trp Asn Glu Thr Lys His Glu Leu Pro Asp Phe
1040 1045 1050

Asp Gln Phe Asn Tyr Thr Ile Pro Val Leu Asn Ile Thr Tyr Asp
1055 1060 1065

Ile Asp Lys Ile Glu Glu Val Ile Lys Gly Leu Asn Asp Ser Leu
1070 1075 1080

Ile Asp Leu Glu Thr Leu Ser Ile Leu Lys Thr Tyr Ile Lys Trp
1085 1090 1095

<210> SEQ ID NO 8

<211> LENGTH: 3296

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: synthetic

<400> SEQUENCE: 8

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tatgacaacg aatcttttgt gtattactac cagagtgcct ttaggccagg acatggttgg	120
catttacatg gaggtgctta tgcagtagtt aatgtgtcta gtgaaaataa taatgcaggt	180
actgccccaa gttgcactgc tgggtgctatt ggctacagta agaatttcag tgcggcctca	240
gtagccatga ctgcaccact aagtggatg tcatggctcg cctcatcttt ttgtacagct	300
cactgtaatt ttacttctta tatagtgttt gttacacatt gttttaagag cggatctaatt	360
agttgtcctt tgacaggtct tattccaagc gggttatattc gtattgctgc tatgaaacat	420
ggaagtgcta cgctgggtca cttattttat aacttaacag tttctgtgac taaatatcct	480
aagtttagat cgctacaatg tgtaataaat catacttctg tatattttaa tggtgacctt	540
gttttcacat ctaactatac tgaagatggt gtagctgcag gtgtccattt taaaagtggt	600
ggacctataa cttataaagt tatgagagag gttaaagcct tggcttattt tgccaatggt	660
actgcacatg atgtcattct atgtgatgac acacctagag gtttggttagc atgccaatat	720
aatactggca atttttcaga tggcttctat ccttttacta atactagtat tgttaaggat	780

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aagtttatgtg	tttatcgtga	aagtagtgtc	aatactactt	tgacattaac	taatttcacg	840
tttagtaatg	aaagtgggtg	ccctccta	acaggtgggtg	ttgacagttt	tattttatac	900
cagacacaaa	cagctcagag	tggttattat	aattttaatt	tttcatttct	gagtagtttt	960
gtttataggg	aaagtaatta	tatgtatgga	tcttaccatc	cacgttgtag	ttttagacct	1020
gaaaccctta	atggtttggtg	gtttaattcc	ctttctgttt	cattaacata	cgggccatt	1080
caagtggtt	gtaagcaatc	tgtattta	ggtaagcaa	cttggtgtta	tgcttattca	1140
tacggaggac	ctcgtgcttg	taaagtggtc	tatagagggtg	agctaacaca	gcattttgaa	1200
tgtggtttgt	tagtttatgt	tactaagagc	gatggctccc	gtatacaaac	tgcaacacaa	1260
ccacctgtat	taacccaaaa	tttttataat	aacatcactt	taggtaagtg	tggtgattat	1320
aatgtttatg	gtagaactgg	acaaggtttt	attactaatg	taactgattt	agctacttct	1380
cataattact	tagcggatgg	aggattagct	attttagata	catctgggtc	catagacatc	1440
ttcgttgtag	aagtggaata	tgccctaac	tactataagg	ttaatctatg	tgaagatggt	1500
aaccaacagt	ttgtagtttc	tggtggtaaa	ttagtaggta	ttctcacttc	acgtaatgaa	1560
actggttctc	agcctcttga	aaaccagttt	tacattaaga	tcactaatgg	aacacatcgt	1620
tctagacgtt	ctgttaatga	aaatgttacg	aattgccctt	atgttagtta	tggaagttt	1680
tgtataaaac	ctgatgggtc	agtttctcct	atagtaccaa	aagaacttga	acagtttggtg	1740
gcacctttac	ttaatgttac	tgaaaatgtg	ctcataccta	acagttttaa	cttaactggt	1800
acagatgagt	acatacaaac	gcataatgat	aagatccaaa	ttaattgtct	gcagtatggt	1860
tgtggcaact	ctttggcttg	tagaaagctg	tttcaacaat	atgggcctgt	ttgtgacaa	1920
atattgtctg	tagtaaatag	tgttggtcaa	aaagaagata	tggaactttt	aagcttctat	1980
tcttctacta	aacctctgg	ttttaataca	ccagttttta	gtaatcttag	cactggtgag	2040
tttaatatct	ctcttttggt	aacaaccct	agtagtccta	gagggcgttc	ttttattgaa	2100
gatcttttat	ttacaagtgt	tgaatctgtt	ggattaccaa	cagatgaagc	ttataaaaag	2160
tgcactgcag	gacctttagg	ctttcttaaa	gaccttgc	gtgctcgtga	atataatggt	2220
ttgcttggtg	tgctcctat	tataacagca	gaaatgcaaa	ctttgtatac	tagttctcta	2280
gtagtttcta	tggttttggtg	tggtattact	tcagctgggtg	ctataccttt	tgccacacaa	2340
ctgcaggcta	gaattaatca	cttgggtatt	accagtcac	ttttgttgaa	gaatcaagaa	2400
aaaattgctg	cttcctttta	taaggccatt	ggcatatgc	aggaaggttt	taggagtaca	2460
tctctagcat	tacaacaaat	tcaagatggt	gttaataagc	agagtgcctat	tcttactgag	2520
actatggttag	cacttaataa	aaattttggt	gctatttctt	ctgtgattca	agacatttac	2580
cagcaacttg	atgacataca	agcagatgct	caagtggatc	gactcataac	tggtagattg	2640
tcacactttt	ctgtcttagc	atctgctaag	cagtcggagt	acattagagt	gtcacaacag	2700
cgtgagtttag	ctactcagaa	aattaatgag	tgtgttaaat	cacagtctat	taggtattcc	2760
ttttgtggta	atggacgaca	tgttttaacc	ataccacaaa	atgcccctaa	tggtatagtg	2820
tttatacact	ttacttatac	accagagagc	tttattaatg	ttactgcagt	agtaggtttt	2880
tgtgtaagtc	ctgctaagtc	tagtcagtat	gcaatagtgc	ccgctaattg	taggggtatt	2940
tttatacaag	ttaatggtag	ttactacatc	actgcacgtg	atatgtatat	gccaagagat	3000
attactgcag	gagatatagt	tacgcttact	tctgtgcaag	caaattatgt	aagtgtaaac	3060

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aagaccgtca ttactacatt ttagacaaat gatgattttg attttgatga cgagttatca 3120
aaatgggtgga atgaaactaa gcacgagttg ccagactttg accagtttaa ttacaccatt 3180
cccgtttttaa atataactta tgatattgac aagattgagg aagttattaa gggacttaat 3240
gattccttga ttgacctoga aacattgtca attctcaaaa cttatattaa gtggcc 3296

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<210> SEQ ID NO 9

<211> LENGTH: 422

<212> TYPE: PRT

<213> ORGANISM: SARS-CoV-2

<400> SEQUENCE: 9

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Met Ser Asp Asn Gly Pro Gln Asn Gln Arg Asn Ala Pro Arg Ile Thr
1      5      10      15
Phe Gly Gly Pro Ser Asp Ser Thr Gly Ser Asn Gln Asn Gly Glu Arg
20     25     30
Ser Gly Ala Arg Ser Lys Gln Arg Arg Pro Gln Gly Leu Pro Asn Asn
35     40     45
Thr Ala Ser Trp Phe Thr Ala Leu Thr Gln His Gly Lys Glu Asp Leu
50     55     60
Lys Phe Pro Arg Gly Gln Gly Val Pro Ile Asn Thr Asn Ser Ser Pro
65     70     75     80
Asp Asp Gln Ile Gly Tyr Tyr Arg Arg Ala Thr Arg Arg Ile Arg Gly
85     90     95
Gly Asp Gly Lys Met Lys Asp Leu Ser Pro Arg Trp Tyr Phe Tyr Tyr
100    105    110
Leu Gly Thr Gly Pro Glu Ala Gly Leu Pro Tyr Gly Ala Asn Lys Asp
115    120    125
Gly Ile Ile Trp Val Ala Thr Glu Gly Ala Leu Asn Thr Pro Lys Asp
130    135    140
His Ile Gly Thr Arg Asn Pro Ala Asn Asn Ala Ala Ile Val Leu Gln
145    150    155    160
Leu Pro Gln Gly Thr Thr Leu Pro Lys Gly Phe Tyr Ala Glu Gly Ser
165    170    175
Arg Gly Gly Ser Gln Ala Ser Ser Arg Ser Ser Ser Arg Ser Arg Asn
180    185    190
Ser Ser Arg Asn Ser Thr Pro Gly Ser Ser Arg Gly Thr Ser Pro Ala
195    200    205
Arg Met Ala Gly Asn Gly Gly Asp Ala Ala Leu Ala Leu Leu Leu Leu
210    215    220
Asp Arg Leu Asn Gln Leu Glu Ser Lys Met Ser Gly Lys Gly Gln Gln
225    230    235    240
Gln Gln Gly Gln Thr Val Thr Lys Lys Ser Ala Ala Glu Ala Ser Lys
245    250    255
Lys Pro Arg Gln Lys Arg Thr Ala Thr Lys Ala Tyr Asn Val Thr Gln
260    265    270
Ala Phe Gly Arg Arg Gly Pro Glu Gln Thr Gln Gly Asn Phe Gly Asp
275    280    285
Gln Glu Leu Ile Arg Gln Gly Thr Asp Tyr Lys His Trp Pro Gln Ile
290    295    300
Ala Gln Phe Ala Pro Ser Ala Ser Ala Phe Phe Gly Met Ser Arg Ile
305    310    315    320

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Gly	Met	Glu	Val	Thr	Pro	Ser	Gly	Thr	Trp	Leu	Thr	Tyr	Thr	Gly	Ala
				325					330					335	
Ile	Lys	Leu	Asp	Asp	Lys	Asp	Pro	Asn	Phe	Lys	Asp	Gln	Val	Ile	Leu
		340						345					350		
Leu	Asn	Lys	His	Ile	Asp	Ala	Tyr	Lys	Thr	Phe	Pro	Pro	Thr	Glu	Pro
		355						360					365		
Lys	Lys	Asp	Lys	Lys	Lys	Lys	Ala	Asp	Glu	Thr	Gln	Ala	Leu	Pro	Gln
		370					375				380				
Arg	Gln	Lys	Lys	Gln	Gln	Thr	Val	Thr	Leu	Leu	Pro	Ala	Ala	Asp	Leu
	385				390					395					400
Asp	Asp	Phe	Ser	Lys	Gln	Leu	Gln	Gln	Ser	Met	Ser	Ser	Ala	Asp	Ser
				405					410					415	
Thr	Gln	Ala	Gly	Gly	Gly										
				420											

<210> SEQ ID NO 10
 <211> LENGTH: 1287
 <212> TYPE: DNA
 <213> ORGANISM: Artificial Sequence
 <220> FEATURE:
 <223> OTHER INFORMATION: synthetic

<400> SEQUENCE: 10

atgagcgata acggacccca gaaccaacgt aacgcccctc gcattacttt tggcgggcct	60
tccgactcta caggttctaa tcaaaacggg gagcgaagcg gggcacgcag caagcaacgc	120
aggcctcaag gtctgccaaa caacactgct tcttggttca ctgctctcac tcaacatggc	180
aaagaggatc tgaaattccc cggaggacaa ggggtaccga tcaatactaa ctctctcccc	240
gatgatcaaa ttggatacta caggcgcgcc acccggagaa ttcgcggcgg agacgggaaa	300
atgaaggacc ttagtccaag atgggtacttt tattacttgg ggaccggacc ggaagctggc	360
ttgcctatg gtgccataa ggacggaatt atttggttgg cgacagaggg cgctctgaat	420
actcccaagg accacatagg tactcggaat cctgctaata atgcgcccat cgtgttgacg	480
ctcccccagg gcaccaccct acccaagggc ttttatgccg aggggtcccc cggcgggagc	540
caggcttcat ccaggagtgc tagtcgctct cgcaactcat cccgcaactc caccctggc	600
tctagtaggg gtactagtcc cgcccgaatg gccggcaatg gcggagacgc tgcctagcgc	660
ctgttgttgc tggatagact caatcagctc gaaagtaaga tgtccggcaa aggacaacag	720
cagcaaggac aaacggttac caagaaaagc gcagcagagg caagtaagaa acccaggcaa	780
aagcgcaactg ccaccaaaagc ttacaacgtg acacaggcct ttggtcgcag aggaccgaa	840
caaacacaag gcaatttttg agatcaggaa cttataaggc aaggaacaga ctataagcat	900
tggcccccagg ttgcacaatt tgcccctacc gcttctgcac tcttcgggat gagtaggatt	960
ggaatggaag ttaccccag tggcacctgg ctcacatata caggcgctat caagcttgat	1020
gataaggatc cgaattttta ggaccagtg atcctgttga acaaacatat agacgcttat	1080
aaaaccttcc ccccgactga acctaagaaa gacaagaaga agaaagctga tgaaactcaa	1140
gcgctgccgc aaagacaaaa gaaacaacaa accgttacgc tcctgccagc agctgacctg	1200
gacgatttta gtaaacaaact ccaacaaagc atgtctagcg ccgatagtac tcaggctggc	1260
ggcgggcacc accatcatca tcaactga	1287

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<210> SEQ ID NO 11
<211> LENGTH: 1215
<212> TYPE: PRT
<213> ORGANISM: SARS-CoV-2

<400> SEQUENCE: 11
Met Phe Val Phe Leu Val Leu Leu Pro Leu Val Ser Ser Gln Cys Val
1           5           10           15
Asn Leu Thr Thr Arg Thr Gln Leu Pro Pro Ala Tyr Thr Asn Ser Phe
20           25           30
Thr Arg Gly Val Tyr Tyr Pro Asp Lys Val Phe Arg Ser Ser Val Leu
35           40           45
His Ser Thr Gln Asp Leu Phe Leu Pro Phe Phe Ser Asn Val Thr Trp
50           55           60
Phe His Ala Ile His Val Ser Gly Thr Asn Gly Thr Lys Arg Phe Asp
65           70           75           80
Asn Pro Val Leu Pro Phe Asn Asp Gly Val Tyr Phe Ala Ser Thr Glu
85           90           95
Lys Ser Asn Ile Ile Arg Gly Trp Ile Phe Gly Thr Thr Leu Asp Ser
100          105          110
Lys Thr Gln Ser Leu Leu Ile Val Asn Asn Ala Thr Asn Val Val Ile
115          120          125
Lys Val Cys Glu Phe Gln Phe Cys Asn Asp Pro Phe Leu Gly Val Tyr
130          135          140
Tyr His Lys Asn Asn Lys Ser Trp Met Glu Ser Glu Phe Arg Val Tyr
145          150          155          160
Ser Ser Ala Asn Asn Cys Thr Phe Glu Tyr Val Ser Gln Pro Phe Leu
165          170          175
Met Asp Leu Glu Gly Lys Gln Gly Asn Phe Lys Asn Leu Arg Glu Phe
180          185          190
Val Phe Lys Asn Ile Asp Gly Tyr Phe Lys Ile Tyr Ser Lys His Thr
195          200          205
Pro Ile Asn Leu Val Arg Asp Leu Pro Gln Gly Phe Ser Ala Leu Glu
210          215          220
Pro Leu Val Asp Leu Pro Ile Gly Ile Asn Ile Thr Arg Phe Gln Thr
225          230          235          240
Leu Leu Ala Leu His Arg Ser Tyr Leu Thr Pro Gly Asp Ser Ser Ser
245          250          255
Gly Trp Thr Ala Gly Ala Ala Ala Tyr Tyr Val Gly Tyr Leu Gln Pro
260          265          270
Arg Thr Phe Leu Leu Lys Tyr Asn Glu Asn Gly Thr Ile Thr Asp Ala
275          280          285
Val Asp Cys Ala Leu Asp Pro Leu Ser Glu Thr Lys Cys Thr Leu Lys
290          295          300
Ser Phe Thr Val Glu Lys Gly Ile Tyr Gln Thr Ser Asn Phe Arg Val
305          310          315          320
Gln Pro Thr Glu Ser Ile Val Arg Phe Pro Asn Ile Thr Asn Leu Cys
325          330          335
Pro Phe Gly Glu Val Phe Asn Ala Thr Arg Phe Ala Ser Val Tyr Ala
340          345          350
Trp Asn Arg Lys Arg Ile Ser Asn Cys Val Ala Asp Tyr Ser Val Leu
355          360          365

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Tyr	Asn	Ser	Ala	Ser	Phe	Ser	Thr	Phe	Lys	Cys	Tyr	Gly	Val	Ser	Pro
370						375					380				
Thr	Lys	Leu	Asn	Asp	Leu	Cys	Phe	Thr	Asn	Val	Tyr	Ala	Asp	Ser	Phe
385					390					395					400
Val	Ile	Arg	Gly	Asp	Glu	Val	Arg	Gln	Ile	Ala	Pro	Gly	Gln	Thr	Gly
				405					410					415	
Lys	Ile	Ala	Asp	Tyr	Asn	Tyr	Lys	Leu	Pro	Asp	Asp	Phe	Thr	Gly	Cys
			420					425					430		
Val	Ile	Ala	Trp	Asn	Ser	Asn	Asn	Leu	Asp	Ser	Lys	Val	Gly	Gly	Asn
		435					440					445			
Tyr	Asn	Tyr	Leu	Tyr	Arg	Leu	Phe	Arg	Lys	Ser	Asn	Leu	Lys	Pro	Phe
450						455					460				
Glu	Arg	Asp	Ile	Ser	Thr	Glu	Ile	Tyr	Gln	Ala	Gly	Ser	Thr	Pro	Cys
465					470					475					480
Asn	Gly	Val	Glu	Gly	Phe	Asn	Cys	Tyr	Phe	Pro	Leu	Gln	Ser	Tyr	Gly
				485					490					495	
Phe	Gln	Pro	Thr	Asn	Gly	Val	Gly	Tyr	Gln	Pro	Tyr	Arg	Val	Val	Val
			500					505					510		
Leu	Ser	Phe	Glu	Leu	Leu	His	Ala	Pro	Ala	Thr	Val	Cys	Gly	Pro	Lys
		515					520					525			
Lys	Ser	Thr	Asn	Leu	Val	Lys	Asn	Lys	Cys	Val	Asn	Phe	Asn	Phe	Asn
530						535					540				
Gly	Leu	Thr	Gly	Thr	Gly	Val	Leu	Thr	Glu	Ser	Asn	Lys	Lys	Phe	Leu
545					550					555					560
Pro	Phe	Gln	Gln	Phe	Gly	Arg	Asp	Ile	Ala	Asp	Thr	Thr	Asp	Ala	Val
				565					570					575	
Arg	Asp	Pro	Gln	Thr	Leu	Glu	Ile	Leu	Asp	Ile	Thr	Pro	Cys	Ser	Phe
			580					585					590		
Gly	Gly	Val	Ser	Val	Ile	Thr	Pro	Gly	Thr	Asn	Thr	Ser	Asn	Gln	Val
		595					600					605			
Ala	Val	Leu	Tyr	Gln	Asp	Val	Asn	Cys	Thr	Glu	Val	Pro	Val	Ala	Ile
610					615						620				
His	Ala	Asp	Gln	Leu	Thr	Pro	Thr	Trp	Arg	Val	Tyr	Ser	Thr	Gly	Ser
625				630						635					640
Asn	Val	Phe	Gln	Thr	Arg	Ala	Gly	Cys	Leu	Ile	Gly	Ala	Glu	His	Val
				645					650					655	
Asn	Asn	Ser	Tyr	Glu	Cys	Asp	Ile	Pro	Ile	Gly	Ala	Gly	Ile	Cys	Ala
			660					665					670		
Ser	Tyr	Gln	Thr	Gln	Thr	Asn	Ser	Pro	Arg	Arg	Ala	Arg	Ser	Val	Ala
		675					680					685			
Ser	Gln	Ser	Ile	Ile	Ala	Tyr	Thr	Met	Ser	Leu	Gly	Ala	Glu	Asn	Ser
690					695						700				
Val	Ala	Tyr	Ser	Asn	Asn	Ser	Ile	Ala	Ile	Pro	Thr	Asn	Phe	Thr	Ile
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We claim:

1. A composition comprising disaggregated spherical nanostructures comprising Quil-A and chitosan, wherein the Quil-A and chitosan are present at a ratio between 1:15 and 1:100.

2. The composition of claim 1, additionally comprising a payload molecule.

3. The composition of claim 2, wherein the payload molecule is selected from the group consisting of a DNA molecule, an RNA molecule, a polynucleotide, a protein, a polypeptide, a virus, a microbe, an attenuated virus, an attenuated microbe, a small molecule, an antibody, or a mixture thereof.

4. The composition of claim 2, wherein the payload molecule is negatively charged.

5. The composition of claim 2, wherein the payload is functionalized.

6. The composition of claim 2, wherein the payload molecule is an antigen specific for Infectious Bronchitis Virus (IBV), Severe Acute Respirator Syndrome Coronavirus 2 (SARS-CoV-2), *Mycobacterium avium* subspecies *paratuberculosis*, *Mycobacterium bovis*, *Mycobacterium tuberculosis*, or *Mycobacterium avium* subspecies *avium*.

7. The composition of claim 1, wherein the chitosan is functionalized by treatment with 5-formyl-2-furan sulfonic acid and sodium borohydride such that the chitosan surface is negatively charged.

8. The composition of claim 1, wherein the spherical nanostructures are between about 5 nm and about 100 nm in diameter in the absence of a payload molecule.

9. A vaccine formulation comprising an antigen, the composition of claim 1 as an adjuvant, and a pharmaceutically acceptable carrier.

10. A vaccine formulation comprising the composition of claim 6.

11. A method of forming a composition comprising Quil-A chitosan spherical nanostructures, comprising the steps of:

heating a first solution comprising Quil-A at about 55° C. for about 30 minutes;

heating a second solution comprising chitosan at about 55° C. for about 30 minutes;

mixing equal volumes of the first and second solution to form a combined solution;

vortex mixing the combined solution for about 30 seconds to form a combined, vortexed solution; and

incubating the combined vortexed solution whereby a composition comprising Quil-A chitosan spherical nanostructures is formed.

12. The method of claim 11, wherein the combined vortexed solution is incubated at room temperature for about 1 hour.

13. The method of claim 12, wherein the first solution additionally comprises a DNA antigen.

14. The method of claim 11, wherein the combined vortexed solution is incubated at about 37 C with shaking at about 110 rpm for about 1 hour.

15. The method of claim 14, wherein the first solution additionally comprises a protein antigen.

16. The method of claim 11, wherein the first solution comprises about 0.002% Quil-A and the second solution comprises about 0.04% chitosan.

17. The method of claim 11, wherein the first solution and the second solution each have a pH between 5.5 and 7.0.

18. A composition comprising Quil-A chitosan spherical nanostructures produced by the method of claim 16.

19. The composition of claim 18 additionally comprising an antigen payload molecule.

20. A method of immunizing a subject against an antigen comprising the step of administering to the subject a vaccine formulation comprising the composition of claim 19.

21. The method of claim 20, wherein the subject is selected from the group consisting of a human, a mouse, a rat, a cow, a horse, a pig, a goat, a sheep, a cat, a dog, or a bird.

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