(19) DANMARK

(10) **DK/EP 2709657 T3**

Patent- og Varemærkestyrelsen

(12) # Oversættelse af europæisk patentskrift

(51) Int.Cl.: ***A 61 K 39/00 (2006.01)***

(45) Oversættelsen bekendtgjort den: **2019-01-14**

(80) Dato for Den Europæiske Patentmyndigheds bekendtgørelse om meddelelse af patentet: **2018-10-24**

(86) Europæisk ansøgning nr.: **12786638.2**

(86) Europæisk indleveringsdag: **2012-05-17**

(87) Den europæiske ansøgnings publiceringsdag: **2014-03-26**

(86) International ansøgning nr.: **US2012038457**

(87) Internationalt publikationsnr.: **WO2012158978**

(30) Prioritet: **2011-05-17 US 201161487206 P**

(84) Designerede stater: **AL AT BE BG CH CY CZ DE DK EE ES FI FR GB GR HR HU IE IS IT LI LT LU LV MC MK MT NL NO PL PT RO RS SE SI SK SM TR**

(73) Patenthaver: **Soligenix, Inc., 29 Emmons Drive, Suite C-10, Princeton, NJ 08540, USA**
The Regents of The University of Colorado, A Body Corporate, 1800 Grant Street, 8th Floor, Denver, CO 80203, USA

(72) Opfinder: **HASSETT, Kimberly, 1800 Grant Street, 8th Floor, Denver,CO 80203, USA**
NANDI, Pradyot, 1800 Grant Street, 8th Floor, Denver,CO 80203, USA
BREY, Robert, 29 Emmons Drive, Suite C-10, Princeton,NJ 08540, USA
CARPENTER, John, 1800 Grant Street, 8th Floor, Denver,CO 80203, USA
RANDOLPH, Theodore, 1800 Grant Street, 8th Floor, Denver,CO 80203, USA

(74) Fuldmægtig i Danmark: **Zacco Denmark A/S, Arne Jacobsens Allé 15, 2300 København S, Danmark**

(54) Benævnelse: **Termostabile vaccine sammensætninger og fremgangsmåder til fremstilling deraf**

(56) Fremdragne publikationer:

WO-A2-00/53215
WO-A2-2008/118691
US-A1- 2010 158 951
US-B2- 7 037 510
US-B2- 7 097 965
HASSETT KIMBERLY J ET AL: "Stabilization of a recombinant ricin toxin A subunit vaccine through lyophilization", EUROPEAN JOURNAL OF PHARMACEUTICS AND BIOPHARMACEUTICS, vol. 85, no. 2, 2013, pages 279-286, XP028737332, ISSN: 0939-6411, DOI: 10.1016/J.EJPB.2013.03.029

DESCRIPTION

FIELD OF THE INVENTION

[0001] The present invention relates generally to the field of dried vaccine compositions. More specifically, to methods of producing dried vaccine compositions bound to adjuvant and containing immunostimulatory molecules.

BACKGROUND OF THE INVENTION

[0002] Vaccines containing recombinant proteins benefit from or absolutely require an adjuvant to elicit an immune response. (Callahan et al., 1991, The importance of surface charge in the optimization of antigen-adjuvant interactions, Pharm. Res. 8(7):851-858; Singh and O'Hagan 1999, Advances in vaccine adjuvants, Nat Biotechnol 17(11): 1075-81; and O'Hagan et al., 2001, Recent developments in adjuvants for vaccines against infectious diseases, Biomol Eng 18(3): 69-85). Aluminum-salt adjuvants are currently the most widely used adjuvants for general use in humans because of the extensive history of safe use in vaccines administered to children and adults. The only adjuvants currently appearing in FDA-approved vaccines are the aluminum salt adjuvants, aluminum hydroxide and aluminum phosphate. Aluminum-salt adjuvants enhance the immunogenicity of vaccines and cause significant improvements in the outcomes of vaccination by reducing the dose level of protein in vaccine, elevating the titers of protective antibodies, and reducing the need for annual vaccination after a primary series of vaccination has been completed. Nonetheless, there are significant limitations in the use of aluminum-salt adjuvants in many subunit vaccines based on recombinant proteins, peptides, and chemically synthesized vaccines. These limitations include the general aspects of vaccine storage and stability, since vaccine containing aluminum adjuvants can be stored only within narrow temperature ranges, and cannot be frozen. Further limitations include the generally accepted view that aluminum adjuvants are relatively weak, do not foster the development of cellular immunity, and may favor the development of antibodies that are non-neutralizing in cases where neutralizing antibodies are necessary to block viral infections or impede the activity of biological toxins.

[0003] In the case of aluminum adjuvants, it has been suggested that to provide adequate immunogenicity, antigens must be adsorbed on the surface of the adjuvant. (Gupta et al., 1995, Adjuvant Properties of Aluminum and Calcium Compounds. Pharmaceutical Biotechnology. 6: 229-248; and White and Hem, 2000, Characterization of aluminium-containing adjuvants, Dev Biol (Basel) 103: 217-28). This adsorption is typically facilitated through electrostatic interactions between the antigen and adjuvant, and the formulation pH is usually chosen so that the antigen and adjuvant are oppositely charged (Callahan et al. 1991). The surface charge on the adjuvant also can be modified by surface exchange reactions with buffer salts such as phosphate, succinate, and citrate (Hem and White, 1984, Characterization of aluminum hydroxide for use as an adjuvant in parenteral vaccines. J Parenter Sci Technol, 38(1): p. 2-10; Chang et al., 1997, Role of the electrostatic attractive force in the adsorption of proteins by aluminum hydroxide adjuvant. PDA J Pharm Sci Technol, 51(1): p. 25-9; and Rinella et al., 1996, Treatment of aluminium hydroxide adjuvant to optimize the adsorption of basic proteins. Vaccine, 14(4): p. 298-300.) The mechanisms of action of aluminum-salt adjuvants are poorly understood, but likely due to several different mechanisms. (Lindblad 2004. "Aluminium compounds for use in vaccines" Immunol. Cell. Biol. 82(5):497-505; Gupta and Siber, 1995, Adjuvants for Human Vaccines--Current Status, Problems and Future-Prospects. Vaccine 13(14):1263-1276; Gupta and Rost, 2000, Aluminum Compounds as Vaccine Adjuvants, In O'Hagan D, editor Vaccine Adjuvants: Preparation Methods and Research Protocols, ed., Totowa, N.J.: Humana Press Inc. p 65-89; Cox and Coulter, 1997, Adjuvants--a classification and review of their modes of action, Vaccine 15(3):248-256). Common proposed mechanisms are that the adjuvant acts as a depot at the site of injection, wherein the antigen is slowly released after administration. (Cox and Coulter, 1997). Another proposed mechanism is that the adjuvant aids in delivery of the antigen to antigen-presenting cells (Lindblad 2004). A further proposed mechanism is that adjuvant serves as an immunostimulator and elicits Th2 cytokines (Grun and Maurer 1989, Different T helper cell subsets elicited in mice utilizing two different adjuvant vehicles: the role of endogenous interleukin 1 in proliferative responses. Cell Immunol 121(1):134-145). Yet another proposed mechanism is that adjuvant destabilizes protein antigens on the surface of the adjuvant making them more susceptible to proteolytic degradation (Jones et al., 2005, Effects of adsorption to aluminum salt adjuvants on the structure and stability of model protein antigens. J Biol Chem 280(14):13406-13414; and That et al., 2004. "Antigen stability controls antigen presentation" J. Biol. Chem. 279(48):50257-50266).

[0004] Although the mechanism of action is not fully understood, it is likely that surface area, surface charge, and morphology of the adjuvant are important factors dictating the immune response to antigens adsorbed onto these adjuvants (Hem and White 1984). It is generally theorized that the smaller the particle size of the vaccine adjuvant, the more immunogenic the vaccine preparation, especially when particle size is approximately 1 micron, a size best suited for uptake into professional antigen presenting cells (Maa et al., 2003. Stabilization of alum-adjuvanted vaccine dry powder formulations: mechanism and application. J Pharm Sci 92(2):319-332, Diminsky et al., 1999. Physical, chemical and immunological stability of CHO-derived hepatitis B surface antigen (HBsAg) particles. Vaccine 18(1-2):3-17).

[0005] Lyophilization (freeze drying) is a process frequently utilized to improve long term stability of various protein preparations. However, when vaccines formulated with aluminum-salt adjuvants are processed in an attempt to improve stability through freezing and lyophilization, a loss of potency occurs, where potency is a summation of the quality of the vaccine measurable by a series of tests that can include immunogenicity in animals, chemical degradation of protein antigen, denaturation of protein antigen, or loss of substituent immunogenic epitopes. Loss of potency is associated with loss of efficacy in humans. Previous studies have suggested that a freeze-dried vaccine product containing adjuvant cannot be produced due to aggregation of the adjuvant particles. (Diminsky et al., 1999; Maa et al., 2003). A number of theories have been set forth to explain possible mechanisms responsible for the loss of potency following lyophilization of vaccines formulated with aluminum-salt adjuvants. Particle aggregation may account for significant losses. For example, the aggregation of aluminum hydroxycarbonate and magnesium hydroxide gels after freezing and thawing has been attributed to ice crystal formation which forces particles together, resulting in irreversible aggregation. (Zapata et al., 1984, Mechanism of freeze-thaw instability of aluminum hydroxycarbonate and magnesium hydroxide gels. J Pharm Sci 73(1):3-8). This explanation has been proposed by Maa et al., 2003 suggesting further that faster cooling rates result in a greater rate of ice nucleation and the formation of smaller ice crystals, which would not force aluminum particles into an aggregate. Particle aggregation can thus account for losses of potency, but other factors, such as loss of protein configuration (tertiary structure), loss of protein secondary structure, and modifications of primary structure through deamidation or oxidation of amino acid side chains.

[0006] The capacity of particles to increase allergic sensitization is predicted by particle number and surface area, not by particle mass. Moorefield et al. showed that the degree of antigen internalization of adjuvant particles is inversely related to the particle size of the adjuvant aggregates (Moorefield et al., 2005. "Role of aluminum-containing adjuvants in antigen internalization by dendritic cells in vitro" Vaccine 23(13):1588-1595). Nygaard et al. showed that the particle diameter, and thus surface area and number of particles, and not mass or volume, is the dominant property in the immunological response of polystyrene particles in mice (Nygaard et al., 2004). While it is likely that the particle size is an important characteristic parameter for immunogenicity, there has yet to be a comprehensive study examining the particle size distribution (PSD) as a function of formulation and cooling rates along with other physical properties of the products produced.

[0007] There is some consensus view that the more effective vaccines with aluminum adjuvants are ones in which antigen is bound to the aluminum surface, rather than free in solution (Lindblad, 2004, Aluminium adjuvants--in retrospect and prospect, Vaccine, 22:3658-68). For reproducibility of formulations and stability, it is desirable to define conditions for optimal binding of antigen to crystal surfaces, and conditions in which antigen does not desorb over time or under elevated stress conditions. To construct aluminum vaccines, it is necessary to carry out studies to optimize binding and desorption. Aluminum adjuvants have a point of zero charge (PZC) at a certain solution pH, but are charged at pHs above or below this value (White and Hem, 2000, Characterization of aluminium-containing adjuvants, Dev Biol (Basel), 103:217-28). Selecting an optimal formulation pH is further complicated for a recombinant protein vaccine for which binding to aluminum salt adjuvants is generally required to obtain the desired immune response (McInerney, Brennan et al., 1999, Analysis of the ability of five adjuvants to enhance immune responses to a chimeric plant virus displaying an HIV-1 peptide, Vaccine, 17:1359-68). To facilitate protein binding to adjuvant, a solution pH is selected in which the protein and adjuvant have opposite charges. However, a solution pH that provides optimal protein stability, may not allow for appropriate binding of the vaccine to adjuvants. In such a scenario, a vaccine protein may have to be prepared at pH that is suboptimal for stability and lyophilized with appropriate stabilizing excipients to minimize degradation during long-term storage.

[0008] Lyophilization of proteins to stabilize structure and activity for storage and reconstitution has been commonly applied to recombinant protein therapeutic proteins. This has been usually accomplished by freeze drying in the presence of disaccharides such as trehalose and other excipients that promote a glass state during process and storage. Proteins can be stored for long term as long as the product is stored below its glass transition temperature (T_g) above which the material transitions into a rubbery state. Excipients are thought to stabilize protein in the amorphous state through interactions of the stabilizer with specific sites substituting for water during drying and by simultaneously suppressing translational and rotational motions of the protein molecule (α-relaxations) or portions of the molecule (β-relaxations). Drying technology has been less frequently applied to long term storage of vaccines, especially in the case of vaccines adsorbed to aluminum phosphate or aluminum hydroxide adjuvants. Very little data is available on the storage of dried vaccines under elevated temperature conditions, as most of the attempts to generate dried vaccines have been to obtain inhalable powders or preparations able to survive moderate excursions in temperature. For example, because the yellow fever vaccine is use primarily in tropical climates, lyophilization in the presence of stabilizers (lactose, sorbitol) has been used to preserve viability of the live virus vaccine (Monath, 1996, Stability of yellow fever vaccine, Dev Biol Stand, 87:219-25). Without excipients during lyophilization and storage, activity is rapidly lost above -20°C, but the stabilized vaccine can withstand more than two weeks at 37°C. A lyophilized dried vaccine for the cattle disease rinderpest has also been developed and can be employed for up to a month after leaving the cold chain in African field conditions (House and Mariner, 1996, Stabilization of rinderpest vaccine by modification of the lyophilization process, Dev Biol Stand, 87:235-44). Similar attempts to use variations on process and drying have been recently applied to measles vaccine development (Burger, Cape et al., 2008, Stabilizing formulations for inhalable powders of live-attenuated measles virus vaccine, J Aerosol Med Pulm Drug Deliv, 21:25-34; Burger, Cape et al., 2008, Stabilizing Formulations for Inhalable Powders of Live-Attenuated Measles Virus Vaccine, J Aerosol Med) and for dried vaccine powders for influenza where it is likely very important to devise conditions that permit retention of the structure of the immunogen (Amorij, Meulenaar et al., 2007, Rational design of an influenza subunit vaccine powder with sugar glass technology: preventing conformational changes of haemagglutinin during freezing and freeze-drying, Vaccine, 25:6447-57; Amorij, Huckriede et al., 2008, Development of Stable Influenza Vaccine Powder Formulations: Challenges and Possibilities, Pharm Res). As a stabilizer, small amounts of formaldehyde are occasionally added to vaccines, including the current AVA anthrax vaccine (Biothrax®), and may act by cross-linking proteins forming more immunogenic protein aggregates on the surface of aluminum crystals (Little, Ivins et al., 2007, Effect of aluminum hydroxide adjuvant and formaldehyde in the formulation of rPA anthrax vaccine, Vaccine, 25:2771-7). Formaldehyde had been used historically as the stabilizer of choice in the older vaccines derived from culture supernatants, such as tetanus toxoid, botulinum toxoids, and others. The current AVA vaccine is labeled for 3 year stability, where stability is a function of a number of biochemical evaluations and potency. Although a moderate amount of stability can be achieved with liquid suspension vaccines, it is not likely that all stability parameters can be met for longer storage periods that are required for vaccines to be stockpiled and distributed.

[0009] Successful drying of therapeutic proteins, while retaining structure and function, is dependent on the knowledge of the degradation pathways that occur in solution, which can be retarded or eliminated by appropriate drying and excipients for stabilization. For vaccines, function is largely determined by immunogenicity and protection studies, rather than enzymatic activity. In the case of protein immunogens that are adsorbed to aluminum adjuvants crystals, the measurement of function and other parameters *in vitro* is correspondingly more difficult, since protein may be sequestered and difficult to remove for analysis. Thus, function can only be tested by immunogenicity and protection studies. The tertiary conformation of proteins can obviously affect enzymatic functions, if present, but also can affect immunogenicity of B cell epitopes dependent on conformation. Linear B and T cell epitopes contained therein can be also affected by oxidation (of methionine and cysteine residues) and deamidation (especially of asparagine residues). pH is one of the most critical formulation variables governing stability of therapeutic proteins. (Carpenter, Chang et al., 2002, Rational design of stable lyophilized protein formulations: theory and practice, Pharm Biotechnol, 13:109-33; Chi, Krishnan et al., 2003, Physical stability of proteins in aqueous solution: mechanism and driving forces in nonnative protein aggregation, Pharm Res, 20:1325-36) By affecting the conformational and colloidal stability of proteins in solution, pH can greatly modulate their aggregation rates (Chi, Krishnan et al., 2003, Physical stability of proteins in aqueous solution: mechanism and driving forces in nonnative protein aggregation, Pharm Res, 20:1325-36). In addition, rates of deamidation are strongly dependent on pH (Manning, Patel et al., 1989, Stability of protein pharmaceuticals, Pharm Res, 6:903-18). There can be different optimal pH values for physical and chemical stability for a given protein (Kolvenbach, Narhi et al., 1997, Granulocyte-colony stimulating factor maintains a thermally stable, compact, partially folded structure at pH2, J Pept Res, 50:310-8). For example, physical stability may be optimal at a pH where deamidation is unacceptably rapid (Chang, Reeder et al., 1996, Development of a stable freeze-dried formulation of recombinant human interleukin-1 receptor antagonist, Pharm Res, 13:243-9). In such cases, development of a lyophilized formulation where the rates of these reactions are minimized may provide a viable strategy to obtain a stable product. The few published studies examining effects of pre-lyophilization solution pH on the stability of therapeutic proteins during lyophilization and storage in dried formulations demonstrated the importance of this parameter (Prestrelski, Pikal et al., 1995, Optimization of lyophilization conditions for recombinant human interleukin-2 by dried-state conformational analysis using Fourier-transform infrared spectroscopy, Pharm Res, 12:1250-9; Chang, Reeder et al., 1996, Development of a stable freeze-dried formulation of recombinant human interleukin-1 receptor antagonist, Pharm Res, 13:243-9; Katayama, Kirchhoff et al., 2004, Retrospective statistical analysis of lyophilized protein formulations of progenipoietin using PLS: determination of the critical parameters for long-term storage stability, J Pharm Sci, 93:2609-23). These studies demonstrated the difficulty in identifying a pre-lyophilization solution pH that confers adequate physical and chemical stability to the proteins studied during lyophilization and storage. However, degradation of proteins could be minimized if sufficient amounts of stabilizing excipients are included in the formulation. For example, when recombinant human interleukin-1-receptor antagonist (rhIL-1ra) was formulated prior to lyophilization in a solution containing suboptimal sucrose at levels less than 0.3 mass ratio of sucrose/protein and at pH less than 6.5, severe protein aggregation occurred after lyophilization, during storage and reconstitution (Chang, Reeder et al., 1996, Development of a stable freeze-dried formulation of recombinant human interleukin-1 receptor antagonist, Pharm Res, 13:243-9). Protein aggregation was minimized following lyophilization from a solution at pH greater than 6, although, deamidation occurred at an unacceptably high rate. Following lyophilization from a solution containing amounts of sucrose greater than 0.3 sucrose/protein mass ratio at pH 6.5, both destabilization pathways could be inhibited. In another example, interleukin-2 (IL-2) had significantly greater structural perturbation during freeze-drying at pH 7, which resulted in higher levels of aggregation after storage and rehydration than samples lyophilized from solutions at pH 5 (Prestrelski, Pikal et al., 1995, Optimization of lyophilization conditions for recombinant human interleukin-2 by dried-state conformational analysis using Fourier-transform infrared spectroscopy, Pharm Res, 12:1250-9). The addition of sucrose to the pre-lyophilization solution formulation at pH 7 improved the stability of IL-2 during storage following lyophilization. More recently, this approach to pre-formulation has been taken with anthrax rPA to create a dried powder vaccine candidate for nasal administration (Jiang, Joshi et al., 2006, Anthrax vaccine powder formulations for nasal mucosal delivery, J Pharm Sci, 95:80-96). In this case, conditions for optimizing pH and excipient stabilizers were established for rPA in solution prior to lyophilization. As trehalose was one of the excipients determined to stabilize soluble rPA to thermal stress, there was evidence of at least 30 days stability at 40°C for the dried trehalose-containing vaccines in terms of the total content of rPA in comparison to liquid samples in which rPA quickly disappeared. In an effort to obtain a dried powder composition for epidermal delivery using a gas-driven injection device, it was found that rapid freezing of aluminum-adsorbed hepatitis B vaccine (HBsAg) in the presence of a mixture of mannitol, glycine, and dextran (not more than ~6% w/v of total excipients) resulted in vaccines that retained particle size and relative immunogenicity in mice after a rapid freezing (spray freeze drying) that involved injection of the sprayed vaccine into liquid nitrogen prior to drying (Maa, Zhao et al., 2003, Stabilization of alum-adjuvanted vaccine dry powder formulations: mechanism and application, J Pharm Sci, 92:319-32). The behavior of the spray-freeze dried vaccines under thermal stress conditions was not determined, although normally lyophilized vaccine aggregated after processing and was minimally immunogenic. Diminished immunogenicity was associated with aluminum particle aggregation after reconstitution.

[0010] More lately, Roser et al. have suggested lyophilization methods that will prevent aggregation of aluminum particles. Roser et al., U.S. Pat. No. 6,890,512 disclose a method of preventing gross aggregation during dehydration and rehydration of particulates in suspension by adding to a particulate suspension of aluminum hydroxide in excess of 15% (w/v) of trehalose. Trehalose, alpha.-D-glucopyranosyl-alpha-D-glucopyranoside, is a naturally occurring disaccharide responsible for protection of plant cells from desiccation. Trehalose has been shown to prevent denaturation of proteins during desiccation by forming sugar glasses that immobilize protein structure. However, Roser et al., while disclosing prevention of gross particle aggregation, do not disclose the importance of freezing rate of a particulate suspension or other factors critical to control and maintain pre-lyophilization particle size and protein structure in an aluminum-salts containing vaccine in the presence of trehalose.

Maintenance of particle size is a critical parameter in controlling the degree of adsorption of protein immunogens to the surface of aluminum particles, and is influenced by several factors during lyophilization cycle in addition to the content of trehalose or other glassifying excipients. These factors influence the immunogenicity and generation of protective immune responses.

[0011] Aluminum-salt adjuvants provide a well explored means to augment the immunogenicity of protein or peptide subunit vaccines. However, a variety of exploratory formulations to enhance vaccines have been developed as more potent alternative to aluminum-salts adjuvants, but are not currently available in FDA-licensed human vaccines. Formulations designed to enhance immune responses include a variety of compositions based on water-in-oil emulsions, oil-in-water emulsions, self-assembling macrostructures, cytokines, saponins, toll-like receptor agonists (TLR-4, TLR-5, and TLR-9), immunostimulatory double stranded RNA species, unmethylated DNA oligonucleotides, and polymeric microparticles and nanostructures. Many of these compositions are directed towards improving the immunogenicity of injected vaccines, and some variations can be applied to altering routes of delivery for intranasal or oral vaccination. As an example of one class of immunostimulatory molecules that can be used to enhance vaccine immunogenicity, bacterial DNA, but not vertebrate DNA, can be used because of direct immunostimulatory effects that activate lymphocytes. This is due to unmethylated CpG dinucleotides are present at the expected frequency in bacterial DNA but are under-represented and methylated in vertebrate DNA (Krieg et al., 1995). Activation may also be triggered by addition of synthetic oligodeoxynucleotides (ODN) that contain an unmethylated CpG dinucleotide in a particular sequence context. CpG DNA induces proliferation of almost all (>95%) B cells and increases immunoglobulin (Ig) secretion. This B cell activation by CpG DNA is T cell independent and antigen non-specific. However, B cell activation by low concentrations of CpG DNA has strong synergy with signals delivered through the B cell antigen receptor for both B cell proliferation and Ig secretion (Krieg et al., 1995). This strong synergy between the B cell signaling pathways triggered through the B cell antigen receptor and by CpG DNA promotes antigen specific immune responses. In addition to its direct effects on B cells, CpG DNA also directly activates monocytes, macrophages, and dendritic cells to secrete a variety of cytokines, including high levels of IL-12 (Klinman et al., 1996; Halpern et al., 1996; Cowdery et al., 1996). These cytokines stimulate natural killer (NK) cells to secrete gamma-interferon (IFN-.gamma.-) and have increased lytic activity (Klinman et al., 1996, supra; Cowdery et al., 1996, supra; Yamamoto et al., 1992; Ballas et al., 1996). Overall, CpG DNA induces a Th1 like pattern of cytokine production dominated by IL-12 and IFN-gamma with little secretion of Th2 cytokines (Klinman et al., 1996). Other molecules stimulate toll like receptors. One example is flagellin, the protein subunit comprising numerous bacterial flagella. Flagellin is a TLR-5 ligand and triggers at least one of the biological functions of antigen presenting cells upon such binding. Flagella are found on the surface of rod and spiral shaped bacteria, including members of the genera Escherichia, Salmonella, Proteus, Pseudomonas, Bacillus, Campylobacter, Vibrio, Treponema, Legionella, Clostridia, and Caulobacter. The conserved regions of flagellins are important for TLR5 binding, while the polymorphic central region can be deleted without affecting binding to TLR5. Flagellin sequences from numerous bacteria are available in the art, such as Genbank accession numbers D13689, YP.sub.--275549, YP.sub.-275550, AAU18718, AAU18717, ZP.sub.--00743095, EAO52626, YP.sub.--315348, AAT28337, AAT28336, AAT28335, AAT28334, AAT28333, AAZ36356, AAZ33167, AAZ94424, AAZ91670, NP.sub.--414908, BAD18052, and BAD18051. As a third example of purified adjuvant immune stimulants, non toxic chemically synthesized or enzymatically modified derivatives of gram negative lipopolysaccharides are potent adjuvants and act by stimulating lymphocytes through TLR-4 binding and activation. For example, monophosphoryl lipid A (MPL) is a derivative of the lipid A component of lipopolysaccharide and is a potent activator of pro-inflammatory cytokines. Although native lipid A and its parent LPS have powerful pyrogenic properties and in humans induce febrile responses (Greisman and Homick, J Immunol, 109:1210-1215 (1972); Greisman and Homick, J Infect Dis, 128:257-263 (1973); Abemathy and Spink, J Clin Invest, 37:219-225 (1958); Rietschel et al, supra; and Raetz, supra (1993)), MPL and its chemically synthesized analogues are not toxic but induce a compendium of host proinflammatory cytokines including IL-1, IL-6, and TNF-alpha.

[0012] In addition, to enhance the immune response to subunits adsorbed to aluminum salts, it is likely that co-adjuvants will be required in order to generate effective antibody responses in humans after one or two doses. A number of adjuvant compounds that are compatible with aluminum salts have been evaluated as adjuvants in recent years. Primarily these adjuvants include Monophosphoryl Lipid A (MPL) and QS-21, and CpG sequences. Recent data with anthrax vaccine indicates in human studies that AVA, an AlOH adsorbed vaccine, can be significantly enhanced by adding CpG 7909 to the adjuvant formulations in non-human primates and humans, in terms of total anti-rPA antibodies and anthrax toxin neutralizing antibodies, although no data describe the long term thermal stability of CpG-containing vaccines (Klinman, 2006, CpG oligonucleotides accelerate and boost the immune response elicited by AVA, the licensed anthrax vaccine, Expert Rev Vaccines, 5:365-9). MPL and QS-21 have been also used with aluminum salts as well as in proprietary oil emulsion formulations being developed by Glaxo Smith Kline Biologics. QS-21 has been evaluated in AlOH vaccines in humans and animal models with good evidence of tolerability and systemic safety. QS-21 is thought to bind to aluminum salts through ionic and hydrophobic interactions, as well as some part of it remaining in solution (in aqueous vaccines) in a micellar form. QS-21 is a saponin purified from tree bark with broad adjuvant effects to induce both antibody and cell mediated immunity. Though the mechanism is not understood, dose levels effective in conjunciton with human vaccines have been evaluated. QS-21 with aluminum has been evaluated in clinical studies and independent safety studies of QS-21 formulated with antigens have been studied. QS-21 has been associated with stinging at the site of injection (that resolves), with very little evidence of systemic side effects (Waite, Jacobson et al., 2001, Three double-blind, randomized trials evaluating the safety and tolerance of different formulations of the saponin adjuvant QS-21, Vaccine, 19:3957-67). Several studies in humans have shown that QS-21 enhances responses to antigens that are adsorbed to aluminum. These include several trials in malaria vaccine candidates (Nardin, Oliveira et al., 2000, Synthetic malaria peptide vaccine elicits high levels of antibodies in vaccinees of defined HLA genotypes, J Infect Dis, 182:1486-96; Kashala, Amador et al., 2002, Safety, tolerability and immunogenicity of new formulations of the Plasmodium falciparum malaria peptide vaccine SPf66 combined with the immunological adjuvant QS-21, Vaccine, 20:2263-77), HIV gp120 (Evans, McElrath et al., 2001, QS-21 promotes an adjuvant effect allowing for reduced antigen dose during HIV-1 envelope subunit immunization in humans, Vaccine, 19:2080-91) and more recently Rhesus macaque trials of Dengue virus subunits in which neutralizing titers and protection were enhanced by QS-21 (Putnak, Coller et al., 2005, An evaluation of dengue type-2 inactivated, recombinant subunit, and live-attenuated vaccine candidates in the rhesus macaque model, Vaccine, 23:4442-52). The solution stability of QS-21 has been well studied under long term stability studies, and has shown that adjuvant active QS-21 (which actually consists of two isomeric forms) is highly stable in slightly acidic buffers for over 4 years, whereas less than 10 days at 40°C (Kensil and Kammer, 1998, QS-21: a water-soluble triterpene glycoside adjuvant, Expert Opin Investig Drugs, 7:1475-82). QS-21 is stored as a dried powder and in that form is stable indefinitely.

[0013] Ricin toxin is a 64 kDa protein produced by castor beans (Ricinus communis) (Doan LG. Ricin: mechanism of toxicity, clinical manifestations, and vaccine development. A review. Journal of Toxicology - Clinical Toxicology 2004;42(2):201-8; Audi J, Belson M, Patel M, Schier J, Osterloh J. Ricin poisoning: a comprehensive review. JAMA 2005;294(18):2342-51). The holotoxin consists of two polypeptide chains (A and B) joined by a disulfide bond. The A chain (RTA) is a ribosome inactivating protein (RIP) that inhibits protein synthesis in mammalian cells. The B chain (RTB) is a lectin that binds to galactose residues on the surface of cells. Once internalized by a cell, RTA translocates into the cytosol where it enzymatically inactivates 60S ribosomes (Smallshaw, JE and Vitetta, ES, A lyophilized formulation of RiVax, a recombinant ricin subunit vaccine, retains immunogenicity. Vaccine 2010 March 11; 28(12): 2428-2435). A single molecule of RTA in the cytoplasm of a cell completely inhibits protein synthesis. The reported estimated lethal dose of ricin in humans is 1-25 μg/kg when inhaled, injected, or ingested (Audi et al). Because of its wide availability and extraordinary toxicity, ricin represents a potential agent for use in bioterrorism and is therefore classified by the Centers for Disease Control, Atlanta GA (CDC) as a level B biothreat. Ricin intoxication can be prevented in experimental animals by vaccination with toxoid or deglycosylated ricin A chain (dgRTA), or by passive immunization with anti-ricin antibodies. However the toxoid is considered to be too toxic for routine use in humans and dgRTA is difficult and expensive to produce, and also retains both active sites and could induce toxic side effects in humans. Passive immunization with anti-ricin antibodies is only effective if the ricin dose is relatively low and the antibody is administered within a few hours after exposure (Hewetson JF, Rivera VR, Creasia DA, Lemley PV, Rippy MK, Poli MA. Protection of mice from inhaled ricin by vaccination with ricin or by passive treatment with heterologous antibody. Vaccine 1993;11(7):743-6).

[0014] In order to avoid these limitations, a recombinant RTA vaccine (RiVax) was developed (Smallshaw et al.). RiVax incorporated two point mutations, Y80A and V76M, to greatly reduce or eliminate both of its known toxicities, i.e. ribotoxicity and vascular leak-inducing ability. In the absence of adjuvant, RiVax is non-toxic and immunogenic in mice, rabbits and humans (Smallshaw JE, Firan A, Fulmer JR, Ruback SL, Ghetie V, Vitetta ES. A novel recombinant vaccine which protects mice against ricin intoxication. Vaccine 2002;20(27-28):3422-7). Such a model has been showed to yield positive results without much of the toxicity implicated with other vaccine models.

[0015] Ricin is currently listed by NIAID and the Centers for Disease Control and Prevention (CDC) as a level B Biothreat agent (Rotz, Khan et al., 2002, Public health assessment of potential biological terrorism agents, Emerging Infectious Diseases, 8:225-30.). The vaccine candidate is based on a recombinant subunit vaccine against ricin toxin obtained by genetic inactivation of residues in the ricin toxin A chain (RTA) that are involved in well characterized activities of the molecule. This modified molecule is immunogenic in mice, rabbits, and humans and induces antibodies that neutralize the toxin or are involved with clearance of the toxin systemically or mucosally in each species. In the case of smallpox or anthrax, terrorist induced epidemics could be controlled by mass vaccination, selective vaccination, or ring vaccination after evidence of an outbreak (Halloran, Longini et al., 2002, Containing bioterrorist smallpox, Science, 298:1428-32; Kaplan, Craft et al., 2002, Emergency response to a smallpox attack: the case for mass vaccination, Proc Natl Acad Sci U S A, 99:10935-40; Bozzette, Boer et al., 2003, A model for a smallpox-vaccination policy, N Engl J Med, 348:416-25; Kretzschmar, van den Hof et al., 2004, Ring vaccination and smallpox control, Emerg Infect Dis, 10:832-41). On the other hand, vaccination against bioterrorist exposure to biological toxins, because the agents do not replicate, is more likely to be used in select populations, such as the military or first responders, rather than mass vaccination. The isolated A and B subunit of ricin can be produced in *E. coli* and other recombinant hosts. The B chain is also a candidate for inclusion in a vaccine, but is considered less immunogenic and less protective than the A chain (Maddaloni, Cooke et al., 2004, Immunological characteristics associated with the protective efficacy of antibodies to ricin, Journal of Immunology, 172:6221-8). Although ricin A chain is at least 1000-fold less toxic than the native ricin, it still retains enzymatic activity that may result in toxicity when used as a vaccine (Thorpe, Detre et al., 1985, Modification of the carbohydrate in ricin with metaperiodate-cyanoborohydride mixtures. Effects on toxicity and in vivo distribution, European Journal of Biochemistry, 147:197-206.). Several key amino acid residues in ricin A chain, Y80, Y123, E177, R180, N209, and W211, constitute its enzymatically-active site (Olson, 1997, Ricin A-chain structural determinant for binding substrate analogues: a molecular dynamics simulation analysis, Proteins, 27:80-95.; Lebeda and Olson, 1999, Prediction of a conserved, neutralizing epitope in ribosome-inactivating proteins, International Journal of Biological Macromolecules, 24:19-26). Mutations in some of these amino acid residues have yielded ricin A chains with negligible toxicity as determined by the inhibition of protein synthesis (Kim and Robertus, 1992, Analysis of several key active site residues of ricin A chain by mutagenesis and X-ray crystallography, Protein Engineering, 5:775-9.). An additional site and residues involved in the binding of RTA to endothelial cells have also been identified, which occur extracellularly and do not require toxin entry into host cells (Baluna and Vitetta, 1999, An in vivo model to study immunotoxin-induced vascular leak in human tissue, J Immunother, 22:41-7; Baluna, Coleman et al., 2000, The effect of a monoclonal antibody coupled to ricin A chain-derived peptides on endothelial cells in vitro: insights into toxin-mediated vascular damage, Exp Cell Res, 258:417-24; Smallshaw, Ghetie et al., 2003, Genetic engineering of an immunotoxin to eliminate pulmonary vascular leak in mice, Nature Biotechnology, 21:387-91). The endothelial binding site on RTA is implicated in the damage to isolated HUVEC cells and to the induction of vascular leak syndrome (VLS), which has been determined to be a dose limiting toxicity in the use of RTA-containing immunotoxins (Smallshaw, Ghetie et al., 2003, Genetic engineering of an immunotoxin to eliminate pulmonary vascular leak in mice, Nature

Biotechnology, 21:387-91). The portion of RTA involved in both pulmonary vascular leak, vascular leak in human skin xenografts in SCID mice, and HUVEC cells appears to involve amino acid residues L74, D75, and V76 (Baluna, Rizo et al., 1999, Evidence for a structural motif in toxins and interleukin-2 that may be responsible for binding to endothelial cells and initiating vascular leak syndrome, Proceedings of the National Academy of Sciences of the United States of America, 96:3957-62.). Therefore, an A chain vaccine taking into account those toxic sites has been constructed and comprises a double mutant of ricin A chain. Tyrosine 80 is mutated to alanine in the enzymatic site and valine 76 is mutated to methionine in the vascular leak site to minimize any possible in endothelial cell damage. It is known now based on crystal structure data that this protein is identical in structure to native ricin A chain (RTA), indicating that the point mutations contained in the molecule do not disrupt any potential tertiary structure (or potential conformationally-dependent epitopes). When administered intramuscularly (i.m.) to mice in the absence of adjuvant, the mutant A chain elicited antibodies that recognized ricin and animals were protected from a 10 x LD_{50} dose of ricin (Smallshaw, Firan et al., 2002, A novel recombinant vaccine which protects mice against ricin intoxication, Vaccine, 20:3422-7.).

[0016] Native PA is the dominant immunogen in AVA (anthrax vaccine adsorbed) and target for protective immunity in pre-exposure and post exposure prophylaxis as a single component vaccine. Several aluminum adsorbed recombinant PA vaccines (2nd generation), based on expression of native PA in avirulent *B. anthracis,* have been advanced and tested in recent Phase I trials, and have been shown to be immunogenic in relationship to AVA (Gorse, Keitel et al., 2006, Immunogenicity and tolerance of ascending doses of a recombinant protective antigen (rPA102) anthrax vaccine: a randomized, double-blinded, controlled, multicenter trial, Vaccine, 24:5950-9; Campbell, Clement et al., 2007, Safety, reactogenicity and immunogenicity of a recombinant protective antigen anthrax vaccine given to healthy adults, Hum Vaccin, 3:205-11). The major correlates of immunity have been well characterized in rabbit aerosol spore challenge studies (total ELISA-reactive antibodies and toxin neutralizing activity (TNA)) (Little, Ivins et al., 2004, Defining a serological correlate of protection in rabbits for a recombinant anthrax vaccine, Vaccine, 22:422-30). It is well known that anthrax toxin is a tripartite toxin consisting of a set of three plasmid-encoded proteins expressed by *B. anthracis:* Protective Antigen (PA; 83 kDa), Lethal Factor (LF; 90 kDa) and Edema Factor (EF; 89 kDa). LF and EF are transported from the extracellular surface into the cytoplasm by the heptamerized PA where they act by enzymatically modifying molecular targets of mammalian cells. LF is a metalloprotease that cleaves and activates several mitogen-activated protein kinases (MAPK kinases) and EF is a calmodulin-dependent adenylate cyclase that causes a rapid increase in intracellular cAMP levels. (Young and Collier, 2007, Anthrax toxin: receptor binding, internalization, pore formation, and translocation, Annu Rev Biochem, 76:243-65). These proteins are nontoxic individually, but when administered together are a potent toxin, causing rapid cell death.

[0017] The AVA vaccine is still the only vaccine for anthrax and the major move to improve on it is based on several perceived shortcomings: requirement for a 6-dose regimen in order to achieve solid immunity and the perception that it is unsafe and reactogenic , and that the preparative processing of AVA is crude and lacks consistency. Although PA is the major immunogen in AVA, it is not clear whether the small amounts of LF and EF that may be present in some lots contribute to the vaccine's effectiveness. A major factor in loss of immunogenicity of anthrax rPA involves accelerated deamidation on the adjuvant surface. This could be prevented by the presence of small amounts of phosphate to lower the surface pH. There are other particular rPA vaccine candidates that are being developed. AVA or PA-based vaccines in general induce toxin neutralizing antibodies (Pitt, Little et al., 1999, In vitro correlate of immunity in an animal model of inhalational anthrax, J Appl Microbiol, 87:304; Reuveny, White et al., 2001, Search for correlates of protective immunity conferred by anthrax vaccine, Infect Immun, 69:2888-93; Little, Ivins et al., 2004, Defining a serological correlate of protection in rabbits for a recombinant anthrax vaccine, Vaccine, 22:422-30). The mechanism underlying the protective action of PA-based vaccines is attributable to anti-PA antibodies that protect the host from intoxication and thus allow the immune system to deal with the organism, though PA vaccine is not designed to limit the onset of infection.

[0018] There are 72 vaccines presently approved by FDA in the United States (U.S. Food and Drug Administration. Complete List of Vaccines Licensed for Immunization and Distribution in the US. FDA. 6/3/2010). Of these 72 vaccines, 36% contain an aluminum adjuvant and 30% are freeze dried. None of the 72 vaccines contain both an aluminum adjuvant and freeze dried component.

[0019] WO 2008/118691 A and WO 00/53215 A also disclose a lyophilized vaccine composition.

[0020] There is a need in the art to develop methods of producing thermostable, immunologically-active freeze dried vaccine preparations which incorporates recombinant antigens to promote rapid onset of protective immunity.

SUMMARY OF THE INVENTION

[0021] The disclosure provides a method of production of thermostable, freeze dried vaccine adjuvant-containing preparations. The disclosure further provides a method of production of thermostable, freeze dried vaccine preparations in which the vaccine antigens are recombinant antigens.

[0022] In one embodiment, the invention provides a method of preparing an immunologically-active adjuvant-bound dried vaccine composition, the method comprising: (a) providing aluminum hydroxide as aluminum salt adjuvant in a concentration of 1 mg/ml, at least one buffer system containing at least one volatile salt at 10 mM and at pH 6 selected from the group consisting of ammonium acetate, ammonium formate, ammonium carbonate, ammonium bicarbonate, at least one glass-forming agent consisting of trehalose in a weight-to-volume concentration of from about 5% to about 15%, and at least one antigen consisting of ricin toxin, and at least one TLR-4 agonists as an immunologically-active co-adjuvant selected from the group consisting of lipid A, lipid A derivatives, monophosphoryl lipid A; (b) combining (a) together to create a liquid vaccine formulation; (c) lyophilizing the liquid vaccine formulation in (b) where lyophilizing comprises steps of: i) freezing the liquid vaccine formulation to create a frozen vaccine formulation wherein the freezing step comprises one of tray freezing, shelf freezing, spray-freezing and shell-freezing and uses a pre-cooled tray to initiate the freezing step; and (d) drying the frozen vaccine formulation in (c) to create a dried vaccine composition, wherein the dried vaccine composition is capable of eliciting an immune response in a subject wherein the immune response developed by the subject may be humoral immunity and/or cell-mediated immunity specific to the antigen.

[0023] In another embodiment, the invention provides a vaccine composition, comprising: (a) aluminum hydroxide as aluminum salt adjuvant in a concentration of 1 mg/ml, and at least one TLR-4 agonists as an immunologically-active co-adjuvant selected from the group consisting of lipid A, lipid A derivatives, monophosphoryl lipid A; (b) at least one buffering agent, wherein the at least one buffering agent comprises a volatile salt at 10mM and at pH 6 selected from the group consisting of ammonium acetate, ammonium formate, ammonium carbonate, ammonium bicarbonate; (c) at least one glass forming agent consisting of trehalose in a weight-to-volume concentration of from about 5% to about 15%; and (d) at least one antigen consisting of ricin toxin, wherein the composition is lyophilized to create a dried vaccine composition and further wherein the dried vaccine composition is capable of eliciting an immune response in a subject.

BRIEF DESCRIPTION OF THE DRAWINGS

[0024]

Figure 1 depicts particle size distributions before and after freeze drying and reconstitutions based on % surface area.

Figure 2 depicts particle size distributions of histidine formulations based on % surface area before and after freeze drying.

Figure 3 depicts particle size distributions of arginine formulations based on % surface area before and after freeze drying.

Figure 4 depicts particle size distributions of glycine formulations based on % surface area before and after freeze drying.

Figure 5 shows Alhydrogel particles settling over time in 10 mM histidine buffer pH 6. (a) No settling; (b) After 30 minutes of settling; and (c) After 3 hours of settling.

Figure 6 depicts particle size distributions based on surface area after allowing particles to settle for varying amounts of time before freeze drying with -10°C pre-cooled shelves. Samples contained 1 mg/mL A1 in 10 mM histidine buffer at pH 6.

Figure 7 depicts particle size distributions based on surface area after allowing particles to settle for varying amounts of time before freeze drying with -10°C pre-cooled shelves. Samples contained 1 mg/mL Al and 8 w/v% trehalose in 10 mM histidine buffer at pH 6.

Figure 8 shows a comparison of mean particle size between formulations of 1 mg/mL Al in 10 mM histidine with and without trehalose while varying the settling time before freeze drying.

Figure 9 demonstrates the steps during lyophilization in which 1 ml vaccine samples contained in 3 ml glass vials were treated to varying freezing rates with an FTS System LyoStar Freeze Drying System before primary and secondary freezing. After freeze drying, vials were purged with nitrogen gas sealed and stored at -80°C before further analysis.

Figure 10 shows particle size distributions before and after freeze drying cycles under four conditions and increasing concentrations of trehalose. Faster rates of freezing before primary and secondary drying and higher concentrations of trehalose result in particle size distributions after freeze drying most similar to the initial particle distribution. From slowest to fastest: room temperature tray A (A), -10°C Pre-cooled Tray (B), Liquid Nitrogen dip (C), Liquid Nitrogen spray Freeze Drying (D). Formulation consisted of 1 mg/ml as Alhydrogel, 10 mM histidine, pH 6.0, with 0-12% trehalose.

Figure 11 shows the dependence of particle size distribution after freeze drying of room temperature freeze drying. Room temperature freeze drying was carried out as in Figure 10. With room temperature incubation on trays prior to the freezing cycle, particle size distribution shifted from < 1 micron to > than 20 microns, and the presence of 8% trehalose reduce the magnitude of the particle size shift.

Figure 12 shows SDS PAGE of RTA dissolved in 10 mM histidine, pH 6.0, 144 mM NaCl with 50% w/v glycerol in comparison to RTA dialyzed, concentrated and stored at -20 degree C. (Top panel - silver stain, bottom panel - Coomassie stain. The same set of samples was used to perform both studies).

Figure 13 shows adsorption of RTA to Alhydrogel prior to lyophilizaiont. The concentration of RTA was varied keeping the concentration of Al at 1mg/mL. At least 95% of the RTA protein was adsorbed to the surface of Alhydrogel at pH 6.0.

Figure 14 shows the results of vaccination of a liquid RTA vaccine adsorbed to Alhydrogel in which various dose of RTA were used to vaccinate groups of 8 Swiss Webster mice. When the vaccine was stored at 40°C for 1 month prior to vaccination, none of the animals exposed to ricin toxin survived and a significant loss of immunogenicity was observed. The vaccine stored at 4°C for one month induced total protection at the highest doses and partial protection in mice at lower doses.

Figure 15 shows rRTA antibody titers after one injection (week 3) and after two injections (week 5) for each vaccine after no incubation, 1 week and 1 month incubation at 40°C. Average titers are shown as the average of only the mice that responded with the standard deviation of those mice.

Figure 16 shows endpoint titer data from immunized mice. Endpoint Titers = reciprocal endpoint anti-RTA titers. Neutralizing IC50 Titers = the dilution of sera required to protect 50% of the cells in a well from ricin cytotoxicity Not shown here, but none of the sham immunized mice (#1-10 (except 5) had any anti-RTA titers in their sera. # 1 sham immunized mouse sera was also tested for neutralizing capacity in vitro, and did not protect cells.

Figure 17 shows total and neutralizing titers obtained from individual sera post 2nd vaccination of Swiss Webster mice with adsorbed liquid vaccine prior to lyophilization.

DETAILED DESCRIPTION OF THE INVENTION

Definitions

[0025] Trehalose dehydrate (high purity, low endotoxin) was obtained from Ferro Pfanstiehl (Cleveland, OH). Arginine, glycine, histidine, sodium citrate, and ammonium acetate were purchased from Sigma Chemical Company (St. Louis, MO). Alhydrogel™ 2.0% (aluminum hydroxide adjuvant), made by Brenntag Biosector, was purchased through E.M. Sergeant Pulp & Chemical Co, Inc (Clifton, NJ). 3-ml and 5-ml lyophilization vials and caps were obtained from West Pharmaceutical Services.

Sample Preparation

[0026] Aqueous solutions were prepared containing different concentrations of trehalose (0 - 15 w/v%). Unless otherwise noted, samples were prepared in 10 mM buffer (as indicated) at pH 6.0 and contained 1 mg/ml Al (as Alhydrogel™). Samples were processed as one-ml aliquots. With the exception of the adjuvant, all aqueous solutions were passed through a 0.2 μm filter prior to formulation.

Surface Charge Zeta Potential

[0027] Zeta potentials were measured for suspensions of aluminum hydroxide (Alhydrogel) in various formulations to probe electrostatic interactions. Formulations without antigen were then prepared to determine if aggregation of particles occurred during freeze drying. Alhydrogel at a concentration of 1 mg Al/mL was combined in 10mM buffer (glycine, arginine, histidine, ammonium acetate, sodium citrate) at pH 6 with the stabilizer trehalose ranging from 0-12w/v%. To determine if the rate of freezing affects particle aggregation, formulations were freeze dried using four methods of freezing: Room Temperature Tray Freezing, -10°C Pre-cooled Tray Freezing, Liquid Nitrogen Dip Freezing, and Liquid Nitrogen Spray Freezing before primary and secondary drying. Protein was also added to formulations to see its effect on particle size after freeze drying and reconstitution. Particle size distributions in the range of 0.04-2000µm were characterized by laser diffraction for each formulation.

Lyophilization

[0028] An FTS Systems Lyostar lyophilizer was used for the freeze-drying of samples. Samples were frozen at various cooling rates as follows from slowest to fastest: (i) Vials prepared at room temperature were placed on the lyophilizer trays and kept at room temperature for 1 hour prior to inaiting the (ii.) Frozen by placing the samples in lyophilizer, equilibrating 1 hr at a shelf temperature of -10°C, then cooling the shelves at 0.5 °C/min to -40 °C ("-10 pre-cooled tray-freezing"); (ii.) Frozen by immersion of bottom of vial into liquid N_2 (LN2 Dip Freeze Drying); and (iii.) Spray-freezing by dropping by ~20 µl droplets into liquid N_2. (LN2 spray freeze drying). Tray-frozen and liquid N_2-immersed samples were processed in 3-ml lyophilization vials, while the spray-frozen samples were processed in 5-ml lyophilization vials. Vials containing samples frozen using liquid N_2 were quickly transferred to the lyophilizer placed on lyophilizer shelves pre-cooled to -40 °C. Samples were spaced in the lyophilizer so that they were each separated from one another and were encircled with a row of vials containing water.

[0029] Primary drying of the samples was achieved by setting the shelf temperature to -20 °C and applying vacuum at 60 mTorr for 20 hours, and was followed by secondary drying, in which shelf temperatures were ramped from -20 °C to 0 °C at 0.2 °C/min, to 30 °C at 0.5 °C/min and finally held at 30 °C for 5 hours. Samples were sealed under vacuum and reconstituted with DI water prior to analysis. The variations of the freezing and drying cycles are depicted in (Figure 10).

Particle Size Distributions

[0030] Particle size distributions (PSD) were measured using a Beckman-Coulter LS230 laser diffraction particle size analyzer. Three one-ml samples were required for each run, and three replicates of each run were completed per formulation. Reported PSD's are surface area weighted and are composites of three runs.

Examples

I. -10°C Pre-Cooled Tray Freeze Drying with Varying Settling Time of Particles

[0031] 10 mM histidine buffer at pH 6.0, 1 mg/mL Al from Alhydrogel, and 0, 4, 8 or 12 w/v% trehalose was combined and rotated end over end for 30 minutes at 4°C. 1 mL of the solution was placed in each 3 mL glass freeze drying vial. The formulations were placed on -10°C pre-cooled shelves in the freeze drier and freeze dried as follows in the table below. Following freeze drying, the chamber was backfilled with dry nitrogen gas and the vials were sealed.

Table 1

Stage	**Time for Step**	**Initial Temp (°C)**	**Final Temp (°C)**	**Pressure**	**Rate**
Freezing	0.25 hours	-10	-10	Atmospheric	Constant temp - 10
	1 hours	-10	-40	Atmospheric	-0.5°C/min
	1 hour	-40	-40	Atmospheric	Constant temp - 40
Primary Drying	0.5 hours	-40	-40	60 mTorr	Constant temp - 40
	0.5 hour	-40	-20	60 mTorr	Increase temp
	20 hours	-20	-20	60 mTorr	Constant temp - 20
Secondary Drying	1 hour 40 min	-20	0	60 mTorr	0.2°C/min
	1 hour	0	30	60 mTorr	0.5°C/min
	5 hours	30	30	60 mTorr	Constant temp 30

Particle Size Analysis

[0032] Particle size analysis was done on the solutions before they were freeze dried as well as on the freeze dried samples reconstituted in 1 mL of DI water. Laser diffraction particle size analysis was done using a LS 230 instrument made by Beckman. For the analysis no sonication was done on the sample chamber. The model used for calculating particle size distributions used a solution refractive index of 1.33 and a sample refractive index of 1.57. Approximately 6 mL of sample was required to be added to filtered DI water in the analyzer before the reading was taken. For each run three ninety second averaged particle size distributions were taken. For each formulation three runs were taken.

Results

[0033] When formulations contained higher concentrations of trehalose (8-12 w/v%) the initial particle size distribution was able to be maintained as seen in Figure 1.

II. -10°C Pre-Cooled Tray Freeze Drying

[0034] 10 mM buffer, 1 mg/mL Al from alhydrogel, 10w/v% trehalose with and without 0.26 mg/mL rRTA was combined and rotated end over end for 30 minutes at 4°C. 1 mL of the solution was placed in each 3 mL glass freeze drying vial. The formulations were placed on -10°C pre-cooled shelves in the freeze drier and freeze dried as follows in the table below. Following freeze drying, the chamber was backfilled with dry nitrogen gas and the vials were sealed.

Table 2

Stage	Time for Step	Initial Temp (°C)	Final Temp (°C)	Pressure	Rate
Freezing	0.25 hours	-10	-10	Atmospheric	Const. temp 5
	1 hours	-10	-40	Atmospheric	-0.5°C/min
	1 hour	-40	-40	Atmospheric	Const. temp -40
Primary Drying	0.5 hours	-40	-40	60 mTorr	Const. temp -40
	0.5 hour	-40	-20	60 mTorr	Increase temp
	20 hours	-20	-20	60 mTorr	Const. temp -20
Secondary Drying	1 hour 40 min	-20	0	60 mTorr	0.2°C/min
	1 hour	0	30	60 mTorr	0.5°C/min
	5 hours	30	30	60 mTorr	Const. temp 30

Particle Size Analysis

[0035] Particle size analysis was done on the solutions before they were freeze dried as well as on the freeze dried samples reconstituted in 1 mL of DI water. Laser diffraction particle size analysis was done using a LS 230 instrument made by Beckman. For the analysis no sonication was done on the sample chamber. The model used for calculating particle size distributions used a solution refractive index of 1.33 and a sample refractive index of 1.57. Approximately 6-7 mL of sample was required to be added to filtered DI water in the analyzer before the reading was taken. For each run three ninety second averaged particle size distributions were taken. For each formulation three runs were taken.

Results

[0036] In arginine, histidine, and glycine buffers containing 10 w/v% trehalose, both with and without rRTA protein present, the particle size distribution was able to be maintained before freeze drying and after using -10°C pre-cooled shelves before freeze drying. Particle size distributions can be seen in Figures 1-3. Particle size distributions could possibly be maintain better with pre-cooled shelves before freeze drying than tray freeze drying because the adjuvant particles have less time to settle before the formulation freezes when pre-cooled shelves are used.

III. -10°C Pre-Cooled Tray Freeze Drying with Varying Settling Time of Particles at 3 Hour, 30 Minute and 0 Timepoints before Freeze Drying

[0037] 10 mM histidine buffer at pH 6, 1 mg/mL Al from alhydrogel, and 0 or 8 w/v% trehalose was combined and rotated end over end for 30 minutes at 4°C. 1 mL of the solution was placed in each 3 mL glass freeze drying vial. The vials were divided into three groups that were allowed to rest for 3 hours, 30 minutes and 0 minutes before being placed in the freeze drier. Once vials were filled they were allowed to sit at 4°C until it was time to be loaded in the freeze drier. The formulations were placed on -10°C pre-cooled shelves in the freeze drier and freeze dried as follows in the table below. Following freeze drying, the chamber was backfilled with dry nitrogen gas and the vials were sealed.

Table 3

Stage	Time for Step	Initial Temp (°C)	Final Temp (°C)	Pressure	Rate
Freezing	0.25 hours	-10	-10	Atmospheric	Constant temp 5
	1 hours	-10	-40	Atmospheric	-0.5°C/min
	1 hour	-40	-40	Atmospheric	Constant temp - 40
Primary Drying	0.5 hours	-40	-40	60 mTorr	Constant temp - 40
	0.5 hour	-40	-20	60 mTorr	Increase temp
	20 hours	-20	-20	60 mTorr	Constant temp - 20
Secondary Drying	1 hour 40 min	-20	0	60 mTorr	0.2°C/min
	1 hour	0	30	60 mTorr	0.5°C/min
	5 hours	30	30	60 mTorr	Constant temp

Stage	Time for Step	Initial Temp (°C)	Final Temp (°C)	Pressure	Rate
					30

Particle Size Analysis

[0038] Particle size analysis was done on the solutions before they were freeze dried as well as on the freeze dried samples reconstituted in 1 mL of DI water. Laser diffraction particle size analysis was done using a LS 230 instrument made by Beckman. For the analysis no sonication was done on the sample chamber. The model used for calculating particle size distributions used a solution refractive index of 1.33 and a sample refractive index of 1.57. Approximately 6 mL of sample was required to be added to filtered DI water in the analyzer before the reading was taken. For each run three ninety second averaged particle size distributions were taken. For each formulation two runs were taken.

Results

[0039] Before samples were placed in the freeze drier they were allowed to settle for 0 minutes, 30 minutes or 3 hours. In Figure 5, a vial containing 1 mg/mL Al from Alhydrogel in 10 mM histidine is shown at various time points during settling. Without settling, the formulation appears to be cloudy throughout the solution (Figure 5a). After 30 minutes of settling the majority of the alhydrogel particles appear to be close to the bottom of the vial with a slightly cloudy solution above (Figure 5b). After 3 hours of settling the alhydrogel particles have settled closer to the bottom of the vial and leave a clear solution above the alhydrogel layer (Figure 5c).

[0040] When the formulation contained alhydrogel and histidine without trehalose, the particle size distribution was shifted towards larger particles from the initial particle size distribution (Figure 6). Formulations that were allowed to settle for less time produced slightly smaller particles than those allowed to settle for longer periods of time.

[0041] When formulations contained 8 w/v% trehalose, the amount of time the samples were allowed to settle before being placed in the freeze drier effected the particle size distribution (Figure 7). When the formulation was not allowed to settle before being placed in the freeze drier, the particle size distribution was very similar to the initial particle size distribution before freeze drying. After 30 minutes of settling the particle size distribution starts to shift to larger particle sizes and at 3 hours of settling the particles are significantly larger than the initial particle size distribution.

[0042] When comparing the formulations with trehalose in comparison the ones without trehalose, trehalose presence in the formulation in maintains the particle size distribution after the freeze drying process. Although the initial mean particle size before freeze drying is the same with and without trehalose present in the formulation, the mean particle size after freeze drying is smaller when trehalose is present in the formulation at each amount of settling before freeze drying as can be seen in Figure 8. From these experiments we can also see the importance of not allowing the samples to settle before loading in the freeze drier if it is desired to maintain the initial particle size.

IV. Immunogenicty of ricin vaccine subunit in experimental animals.

[0043] As an example, a thermostable lyophilized ricin subunit vaccine was constructed and tested. Ricin A chain vaccine was used because it is subject to aggregation and denaturation in aqueous buffers and is prone to losses in structural integrity that affect immunogenicity and the induction of neutralizing antibodies involved in protection against ricin toxin exposure. A lyophilized ricin vaccine was prepared as follows. RTA dissolved in glycerol was dialyzed against 10 mM histidine buffer, pH 6.0 to remove glycerol (Figure 11). The liquid suspension vaccine was placed into vials and subjected to lyophilization as described in Figure 10 to compare precooled freeze drying at -10 degrees C with vaccine at room temperature prior to initiating of the primary freeze drying cycle at -40°C. The dried vaccines were stored either at refrigeration temperature (4-8°C) or at elevated temperature (40-60°C). Samples from the stored lyophilized vaccine were withdrawn periodically and tested for structural integrity by assessment of binding of a diagnostic monoclonal antibody termed R70 (Neal, O'Hara et al., 2010, A monoclonal immunoglobulin G antibody directed against an immunodominant linear epitope on the ricin A chain confers systemic and mucosal immunity to ricin, Infect Immun, 78:552-61). In addition, vaccines were subjected to additional biophysical tests including the determination of intrinsic fluorescence diagnostic of tertiary structure of protein bound to aluminum, determination of residual water, and immunogenicity/potency in mice. Immunogenicity was determined by injecting Swiss Webster mice as below and determining total antibodies against the vaccine by ELISA and determination of ricin neutralizing antibodies. Mice were exposed to ricin toxin at day 35 by injection of 10 x LD50 dose of toxin and lethality was determined in the exposed animals. In addition, peptide scans were performed in which serum from vaccinated and control mice were assessed for response to overlapping peptides encompassing the RTA molecule. This was done to determine the immunodominant regions and their preservation during high and low temperature storage conditions. When control liquid vaccine was used to vaccinate mice 3x by intramuscular injection, incubation of the vaccine at 40°C for one month resulted in loss of immunogenicity and the ability to induce protective immunity (Figure 14).

[0044] During the study, each Swiss Webster mouse was bled three times and injected with a vaccine formulation twice. Before the initial injection mice were bled and then on day 0 injected with a vaccine formulation. The initial bleeding was necessary so that each mouse could be its own baseline. 21 days later the mice were bled and injected with a booster vaccine formulation. 35 days after the initial injection the mice were bled one last time. Before bleeding procedures the mice were anesthetized using is isofluorane inhalant. Blood was drawn from the retro-orbital venous sinus of the mice. A drop of proparacaine was put on the eye from which blood was drawn and then blood was collected using 50μL capillary tubes. Approximately 100-200μL of blood was drawn during each bleeding.

Table 4

Group	Contents
Negative Control	Freeze dried Alhydrogel in histidine buffer
Negative Control	Freeze dried Alhydrogel in ammonium acetate buffer
Positive Control	Liquid formulation of rRTA and Alhydrogel
Experimental Group 1	Freeze dried (Room temp shelves) rRTA and Alhydrogel in histidine buffer
Experimental Group 2	Freeze dried (Room temp shelves) rRTA and Alhydrogel in ammonium acetate buffer
Experimental Group 3	Freeze dried (Pre-cooled shelves) rRTA and Alhydrogel in histidine buffer
Experimental Group 4	Freeze dried (Pre-cooled shelves) rRTA and Alhydrogel in ammonium acetate buffer

[0045] To create variations in the formulation particle size, different buffers such as histidine and ammonium acetate and the variation of freezing rate before freeze drying (such as room temperature shelves or pre-cooled shelves before freeze drying) were used. All samples contained the disaccharide trehalose up to 15% (w/V) and Alhydrogel is an aluminum hydroxide vaccine adjuvant used at 0.85 -1 mg/ml total aluminum.

V. Controlled lyophilization of adsorbed ricin vaccine. The central objective of this invention is to make subunit vaccines by employing controlled lyophilization of protein, aluminum adjuvant, and immunostimulant components for reconstitution with water at the point of use. Using aluminum adjuvant, it has not been feasible or possible up to this point to adequately combine these components together without loss of vaccine effectiveness on the one hand and gross clumping and inability to rehydrate adequately. A number of different conditions for precisely controlling points in the lyophilization cycle examining a spectrum of buffer conditions, salt conditions, and lyophilization cycle conditions and have reported that we had been able to define conditions for retaining gross integrity including protein structure pre and post lyophilization.

VI. Generation of Prototype Freeze Dried Vaccines. A series of freeze dried formulation was made according to the general lyophilization schemes presented in Table 1. Freeze dried formulations with RTA protein and placebo formulations without protein were created containing 1.0 mg Al/mL, 8 w/v% trehalose and 0.2 or 0 mg/mL rRTA in 10 mM histidine or ammonium acetate buffer pH 6, with either pre-cooling (PC) prior to lyophilization or room temperature incubation prior to lyophilization. Formulations were prepared by mixing with a stir bar at 4-8°C for 1 hour to allow protein to adsorb to Alhydrogel adjuvant. 1 mL of formulation was placed in a 3 mL glass vial and freeze dried as described in Table 4. Samples from each process condition were incubated at 40°C and withdrawn for analysis and vaccination studies at 1 week, one month (and continuing through month 6). Pre- and post-lyophilization samples were also obtained.

Table 5

Freeze Drying Cycle					
Stage	**Time for Step**	**Initial Temp (°C)**	**Final Temp (°C)**	**Pressure**	**Rate**
-10°C Pre-Cooled Tray Freezing	0.25 hour	-10	-10	Atmospheric	Constant temp - 10
	1 hour	-10	-40	Atmospheric	-0.5°C/min
	1 hour	-40	-40	Atmospheric	Constant temp - 40
Primary Drying	0.5 hours	-40	-40	60 mTorr	Constant temp - 40
	0.5 hour	-40	-20	60 mTorr	Increase temp
	20 hours	-20	-20	60 mTorr	Constant temp - 20
Secondary Drying	1 hour 40 min	-20	0	60 mTorr	0.2°C/min
	1 hour	0	30	60 mTorr	0.5°C/min
	5 hours	30	30	60 mTorr	Constant temp 30

VII. Particle Size Analysis of Reconstituted Dried Vaccines. Particle size analysis was done on the solutions before they were freeze dried as well as on the freeze dried samples reconstituted in 1 mL of deionized water. Laser diffraction particle size analysis was conducted using a LS 230 instrument made by Beckman. For the analysis no sonication was done on the sample chamber. The model used for calculating particle size distributions used a solution refractive index of 1.33 and a sample refractive index of 1.57. Approximately 6 mL of sample was required to be added to filtered DI water in the analyzer before the reading was taken. For each run three ninety second averaged particle size distributions were taken. For each formulation three runs were taken. The particle size distribution of the placebo stability study samples is being monitored over with using laser diffraction. The initial Time 0 liquid formulations all had similar particle size distributions and mean particle sizes based on surface area as can be seen in Table 5. When formulations were Tray Freeze Dried from Room Temperature, an increase in particle size was seen. When formulations were Tray Freeze Dried from -10°C Pre-Cooled Shelves, the particle size distribution stayed very similar to the initial particle size distribution.

Table 6

Mean particle size ± standard deviation based on surface area				
	Vaccine			
Time Point	**RT His**	**RT AA**	**PC His**	**PC AA**
Time 0 - Liquid	0.35 ± 0.01	0.34 ± 0.01	0.35 ± 0.01	0.35 ± 0.01
Time 0 - FD	9.43 ± 0.31	8.11 ± 0.74	0.38 ± 0.06	0.49 ± 0.05
Time 1 Week - FD	10.69 ± 0.41	8.96 ± 0.16	0.44 ± 0.09	0.42 ± 0.05
Time 1 Month - FD	10.31 ± 0.63	9.09 ± 0.12	0.46 ± 0.12	0.55 ± 0.07

VIII. Vaccination of Animals. Female Swiss Webster mice 5-6 weeks old were vaccinated with 50 µL of the indicated formulations containing 10 microgram of RTA protein subcutaneously on Day 0 and 20. Mice under anesthesia by isoflurane were bled through the retro orbital cavity collecting approximately 200µL of blood on Day 0, 20 and 34. In each group 10 mice were used. Mice were housed 5 per cage and were allowed food and water all the time. Serum was separated from blood by centrifugation at 10,000 rpm for 14 minutes at 4°C.

[0046] Total antibody to RTA in individual sera from vaccinated Swiss Webster mice was determined by ELISA and for determination of neutralizing antibodies (Figures 6 and 7). Nunc flat bottom MaxiSorb 96 well plates were coated with 50µL/well of stock protein diluted in PBS to 1µg rRTA/mL and incubated at 2-6°C overnight. Plates were washed 4 times with 300 µL/well of PBS with 0.05% Tween 20. Plates were blocked with 300 µL/well of PBS with 1% BSA and incubated at room temperature for 2 hours. Plates were washed as previously described. 40 µL of PBS with 1% BSA and 0.05% Tween 20 was added to each well. Serum was initially diluted in a dilution buffer of PBS with 1% BSA and 0.05% Tween 20. 70 µL of sample was added to the starting well and then a seven in-plate 2.33-fold dilution was created for each sample. The plate was then incubated for 2 hours at room temperature. Plates were washed again. 40 µL of HRP-conjugated donkey anti-mouse antibody diluted 10,000 times was added to each well and incubated for 2 hours at room temperature. Plates were washed again. TMB was added to each well at 40 µL and incubated for 30 minutes. Stop solution of 2N sulfuric acid was added at 40 µL to each well. The plate was read at 450 nm. Endpoint dilution analysis of individual serum samples from vaccinated mice is shown in Figure 15. The vaccines tested are abbreviated as follows:

RT His - Negative control (room temperature tray freeze dried in histidine with no protein)

RT AA - Negative control (room temperature tray freeze dried in ammonium acetate with no protein)

His + rRTA Liquid - Positive control (liquid formulation in histidine with protein)

RT His + rRTA - Experimental 1 (room temperature tray freeze dried in histidine with protein)

RT AA + rRTA - Experimental 2 (room temperature tray freeze dried in ammonium acetate with protein)

RPC His + rRTA - Experimental 3 (Pre-Cooled tray freeze dried in histidine with protein)

PC AA + rRTA - Experimental 4 (Pre-Cooled tray freeze dried in ammonium acetate with protein)

[0047] When vaccines were stored for one or one month at 40°C, there was no significant difference in the capacity of the vaccines to generate antibodies against RTA (by ELISA) after one injection of 10 microgram (week 3 titers) or 2 injections (week 5) in relationship to vaccine prepared without storage at 40°C (time 0 in Figure 15). At week three, 90-100% of mice in each experimental and positive control group responded and by week five all experimental and positive control responded (Table 3). More important, serum obtained from post 2 (week 5) contained antibodies that neutralized ricin (in vitro) where the titers and the proportion of mice with such titers were not obviously different from time 0 vaccines (Figure 16) or the liquid vaccines (Figure 17). Further, neutralizing titers decreased after storage of lyophilized RTA vaccine at 40°C for 1 month, in sera from mice that were given vaccine placed on a room temperature tray before freeze drying. In contrast, vaccines made by precooling prior to freeze drying had better total and neutralizing anti-RTA titers than those immunized with liquid vaccine.

Table 7

Number of mice responding to the vaccine after week 3 and week 5		
	# of with Antibody Titer	
Vaccine	**Week 3**	**Week 5**
Positive Control - Liquid His + rRTA	9/10	10/10
Time 0 - RT His + rRTA	10/10	10/10
Time 0 - RT AA + rRTA	10/10	10/10
Time 0 - PC His + rRTA	9/10	10/10
Time 0 - PC AA + rRTA	10/10	10/10
Time 1 Week - RT His + rRTA	10/10	10/10
Time 1 Week - RT AA + rRTA	9/9	10/10
Time 1 Week - PC His + rRTA	10/10	10/10
Time 1 Week - PC AA + rRTA	9/10	10/10
Time 1 Month - RT His + rRTA	9/10	10/10
Time 1 Month - RT AA + rRTA	10/10	10/10
Time 1 Month - PC His + rRTA	10/10	10/10
Time 1 Month - PC AA + rRTA	10/10	10/10

IX. Vaccination of animals with vaccines containing secondary co-adjuvants. A series of freeze dried formulation was made according to the general lyophilization schemes presented in Table 1. Freeze dried formulations with RTA protein and placebo formulations without protein were created containing 1.0 mg Al/mL, 8 w/v% trehalose and 0.2 or 0 mg/mL rRTA and 60 micrograms of TLR-4 agonist, a synthetic derivative of monophosphoryl Lipid A (MPL) termed PHAD, obtained from Avanti Polar Lipids (Alabaster, AL). Vaccines were made in several different manners. In method (1), RTA protein was adsorbed (bound) to aluminum hydroxide in 10 mM histidine or ammonium acetate buffer pH 6 in the presence of 8% trehalose, followed by addition of PHAD agonist to the aqueous suspension. For this method, RTA stored in stabilizer buffer consisting of 10 mM histidine, pH6.0, and 144 mM NaCl, was subjected to dialysis into glycerol- and salt-free buffer prior to adsorption to aluminum adjuvant. In method (2), RTA stored in stabilizing glycerol buffer was diluted 10 fold in 10 mM histidine, pH 6.0, 144 mM NaCl prior to the addition of aluminum to the diluted stabilizing buffer. For this method adsorption was allowed to occur at 4C° for more than 5 hours so that greater than 95% of the RTA became bound to aluminum gel particles. Subsequently, the aluminum particles were allowed to settle to the bottom of the adsorption vessel or the mixture was subjected to centrifugation to separate the particles from the aqueous buffer. To this Aluminum mixture was added a buffer system (ammonium acetate or histidine) containing 8% trehalose. In this manner the isotonicity of the system could be maintained. For method 1 and method 2, subsequent lyophilization proceeded with either pre-cooling (PC) prior to lyophilization or room temperature incubation prior to lyophilization.

Samples from each process condition were incubated at 4°C and 40°C and withdrawn for analysis and vaccination studies at 1 week, one month, 2 months, 3 months, 6 months, 9 months, 12 months, 18 months, and 24 months. For potency analysis, Swiss Webster mice were vaccinated with a concentration range that kept the ajduvant components (aluminum and PHAD) constant while varying the dose of RTA immunogen. For control studies, mice were vaccinated with vaccine that did not contain co-adjuvant PHAD, using the same dose range as the PHAD containing lyophilized vaccines. Two vaccination protocols were used.

One set of mice were vaccinated with one dose of vaccine on study day 1, and another set of mice was vaccinated with vaccine on study days 1 and 21. Serum was obtained from animals at the time of each vaccination and two weeks thereafter. For final analysis, mice were exposed to 10 x LD 50 of ricin toxin on day 35 and survivors were recorded. The animals that were vaccinated with the PHAD-containing dried reconstituted vaccine samples demonstrated a significant shift of the dose response curve toward lower doses of RTA immunogen for the serological endpoints (total RTA reactive antibodies and ricin neutralizing antibodies) and also demonstrated protective immunity at the lower dose range when subjected to ricin exposure in comparison to the vaccine without the co-adjuvant. Equally significant, the vaccine samples that were incubated at the higher temperature also demonstrated enhanced immune response, indicating that all of the components of the vaccine were stabilized. Furthermore, the PHAD vaccines induced a broader immune response reflected by a higher titer of neutralizing antibodies and broader response to neutralizing epitopes.

X. Glass transition temperature. Glass transition temperature (Tg) is an indicator of stability of the vaccine product. Below or near the Tg the vaccine behaves as a glass and all components of the vaccines are stabilized within the glass. Above the Tg, the sample becomes is less stable, and the components within the matrix also become less stable. The Tg is measured by differential scanning calorimetry in the following manner. The Tg of the sample is determined by subjecting the sample to a controlled temperature program from 0° C to 150° C. at a rate of 10° C/min. The heat flow to and from the sample is measured and expressed as a shift in the baseline. The Tg is expressed as the temperature at the midpoint of this baseline shift.

[0048] Lyophilized RTA vaccines subjected to DSC analysis demonstrate a high glass transition temperature in excess of 100°C and lower than 0.5% water content (Karl Fischer analysis).

REFERENCES CITED IN THE DESCRIPTION

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Patentkrav

1. Fremgangsmåde til fremstilling af en immunologisk aktiv adjuvansbundet tørret vaccinesammensætning, hvilken fremgangsmåde omfatter:
(a) at tilvejebringe aluminiumhydroxid som aluminiumsaltadjuvans i en koncentration på 1 mg/ml, mindst et buffersystem indeholdende mindst et flygtigt salt ved 10 mM og ved pH 6 udvalgt fra gruppen bestående af ammoniumacetat, ammoniumformat, ammoniumcarbonat, ammoniumbicarbonat, mindst et glasdannende middel bestående af trehalose i en vægt-til-volumen-koncentration på fra ca. 5 % til ca. 15 % og mindst et antigen bestående af ricintoksin og mindst en TLR-4 agonist som en immunologisk aktiv co-adjuvans udvalgt fra gruppen bestående af lipid A, lipid A-derivater, monophosphoryl lipid A;
(b) at kombinere (a) sammen til dannelse af en flydende vaccineformulering;
(c) at lyofilisere den flydende vaccineformulering i (b), hvor lyofilisering omfatter trinnene:
i) at fryse den flydende vaccineformulering til dannelse af en frossen vaccineformulering, hvor frysetrinnet omfatter et blandt bakkefrysning, hyldefrysning, spray-frysning og skalfrysning og anvender en forafkølet bakke til påbegyndelse af frysetrinnet; og
(d) at tørre den frosne vaccineformulering i (c) til dannelse af en tørret vaccinesammensætning, hvor den tørrede vaccinesammensætning kan fremkalde en immunreaktion hos et individ, hvor immunreaktionen udviklet hos individet kan være humoral immunitet og/eller celle-medieret immunitet, som er specifik for antigenet.

2. Fremgangsmåde ifølge krav 1, hvor den tørrede vaccinesammensætning rekonstitueres med et vandigt fortyndingsmiddel til dannelse af en rekonstitueret vaccinesammensætning.

3. Fremgangsmåde ifølge krav 1, hvor vaccinesammensætningen indeholder en gennemsnitlig partikeldiameter på mindre end 100 mikrometer.

4. Fremgangsmåde ifølge krav 1, hvor den flydende vaccineformulering først fremstilles som en hypertonisk blanding før frysning og dernæst justeres til isotoniske niveauer efter fortynding af den tørrede vaccinesammensætning med et vandigt fortyndingsmiddel.

5. Vaccinesammensætning omfattende:
(a) aluminiumhydroxid som aluminiumsaltadjuvans i en koncentration på 1 mg/ml og mindst en TLR-4 agonist som en immunologisk aktiv co-adjuvans udvalgt fra gruppen bestående af lipid A, lipid A-derivater, monophosphoryl lipid A;
(b) mindst et buffermiddel, hvor det mindst ene buffermiddel omfatter et flygtigt salt ved 10mM og pH 6 udvalgt fra gruppen bestående af ammoniumacetat, ammoniumformat, ammoniumcarbonat, ammoniumbicarbonat;
(c) mindst et glasdannende middel bestående af trehalose i en vægt-til-volumen-koncentration på fra ca. 5 % til ca. 15 % og
(d) mindst et antigen bestående af ricintoksin, hvor sammensætningen er lyofiliseret til dannelse af en tørret vaccinesammensætning, og yderligere hvor den tørrede vaccinesammensætning kan fremkalde en immunreaktion hos et individ.

DRAWINGS

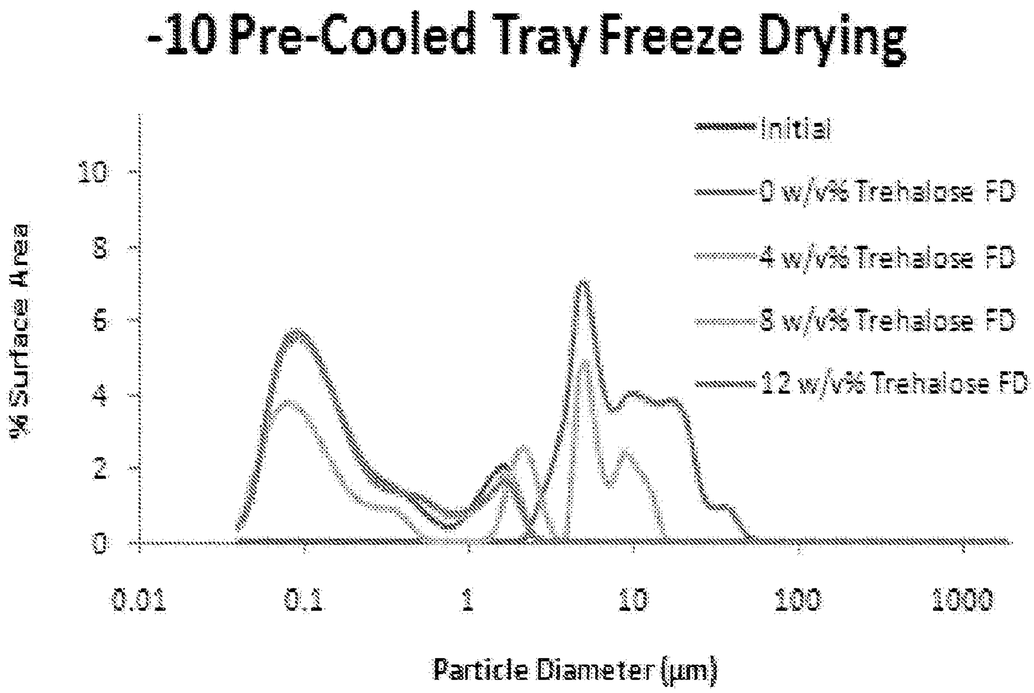


FIG. 1

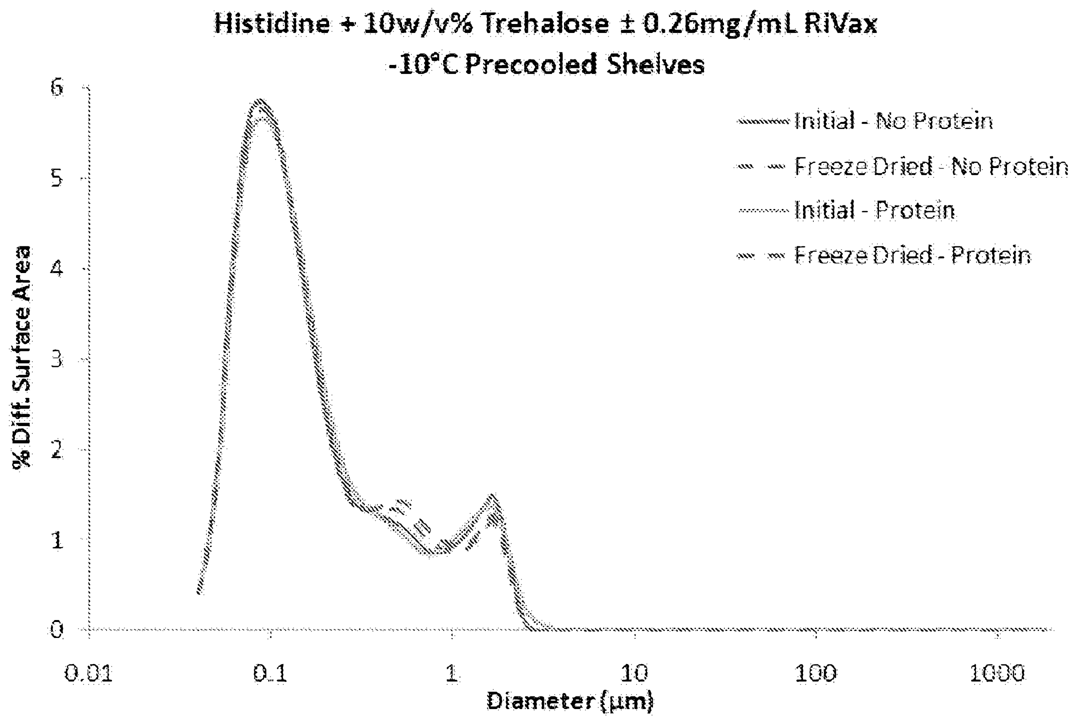

Histidine + 10w/v% Trehalose ± 0.26mg/mL RiVax
-10°C Precooled Shelves
% Diff. Surface Area
Diameter (µm)
Initial - No Protein
Freeze Dried - No Protein
Initial - Protein
Freeze Dried - Protein
0
1
2
3
4
5
6
0.01
0.1
1
10
100
1000

FIG. 2

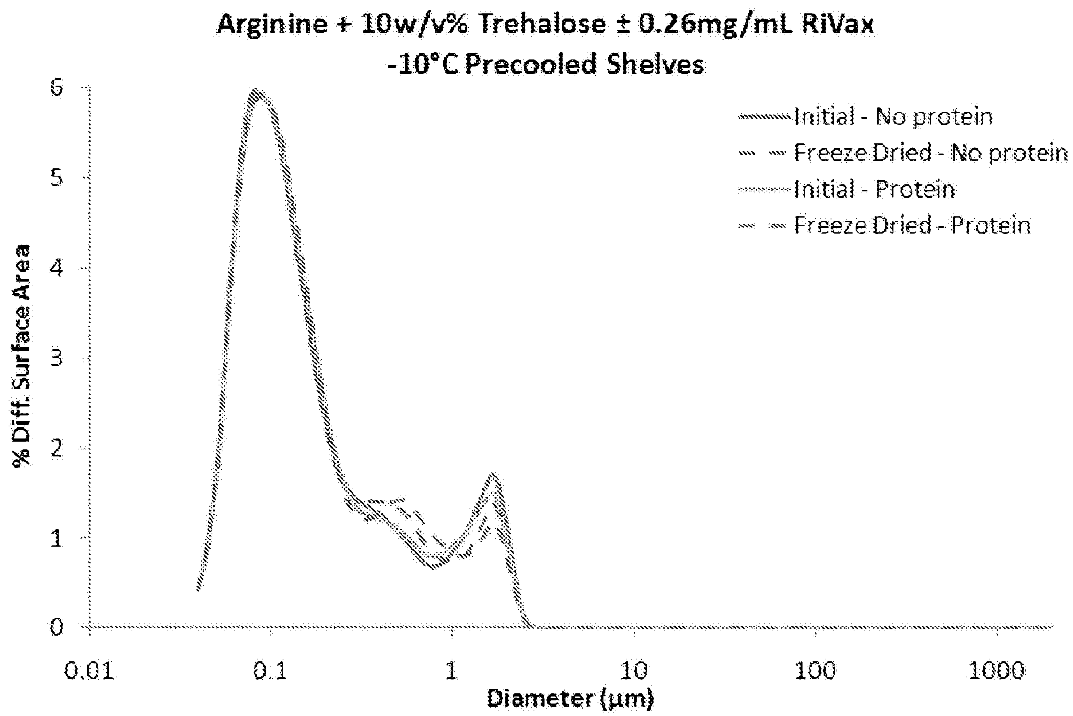

Arginine + 10w/v% Trehalose ± 0.26mg/mL RiVax
-10°C Precooled Shelves
Initial - No protein
Freeze Dried - No protein
Initial - Protein
Freeze Dried - Protein
% Diff. Surface Area
6
5
4
3
2
1
0
0.01
0.1
1
10
100
1000
Diameter (µm)

FIG. 3

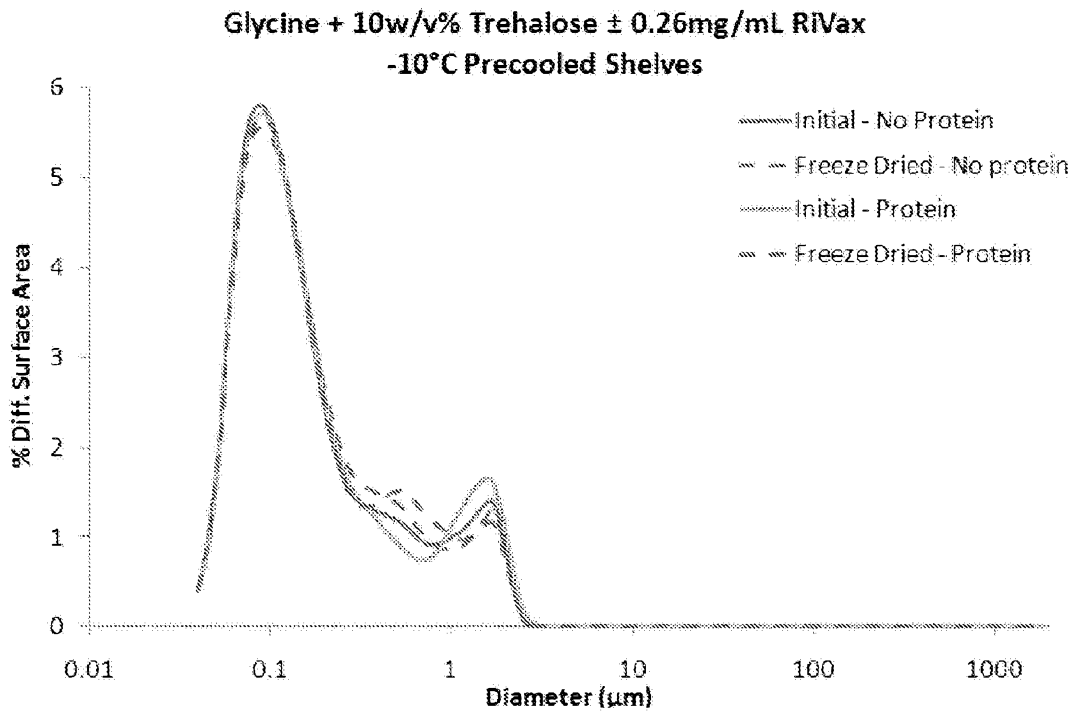

Glycine + 10w/v% Trehalose ± 0.26mg/mL RiVax
-10°C Precooled Shelves
% Diff. Surface Area
Diameter (µm)
Initial - No Protein
Freeze Dried - No protein
Initial - Protein
Freeze Dried - Protein
0
1
2
3
4
5
6
0.01
0.1
1
10
100
1000

FIG. 4

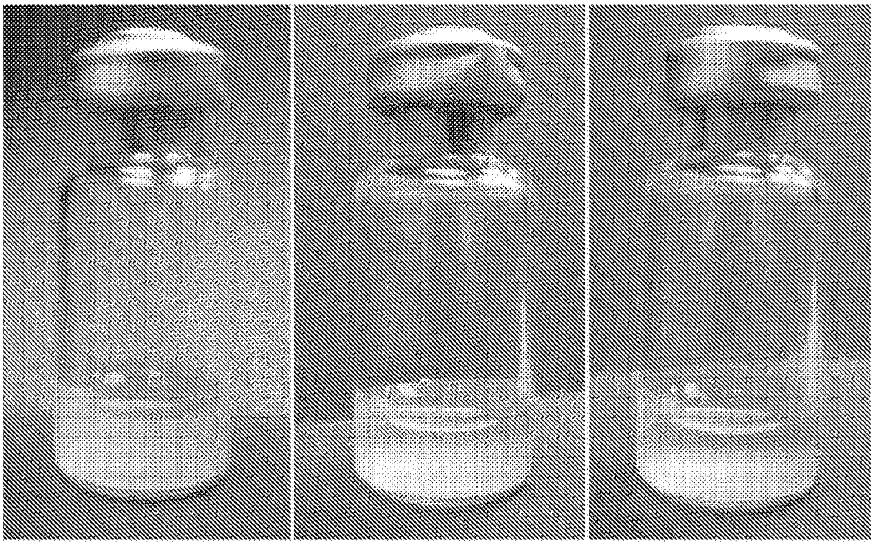
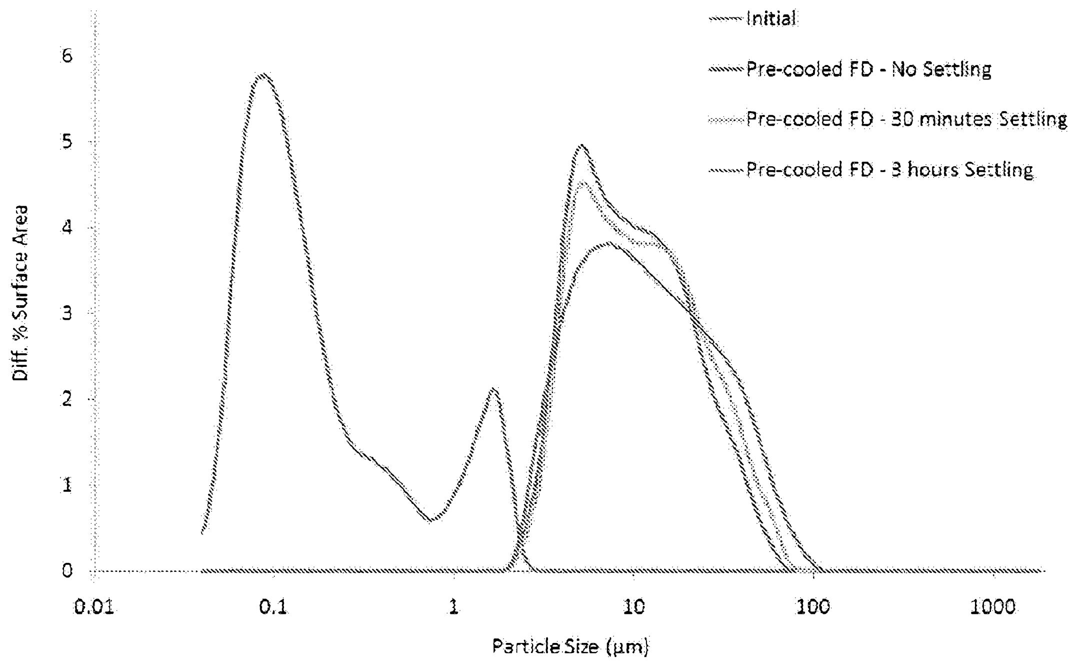


FIG. 5(a) FIG. 5(b) FIG. 5(c)



Initial
Pre-cooled FD - No Settling
Pre-cooled FD - 30 minutes Settling
Pre-cooled FD - 3 hours Settling
Diff. % Surface Area
0
1
2
3
4
5
6
0.01
0.1
1
10
100
1000
Particle Size (µm)

FIG. 6

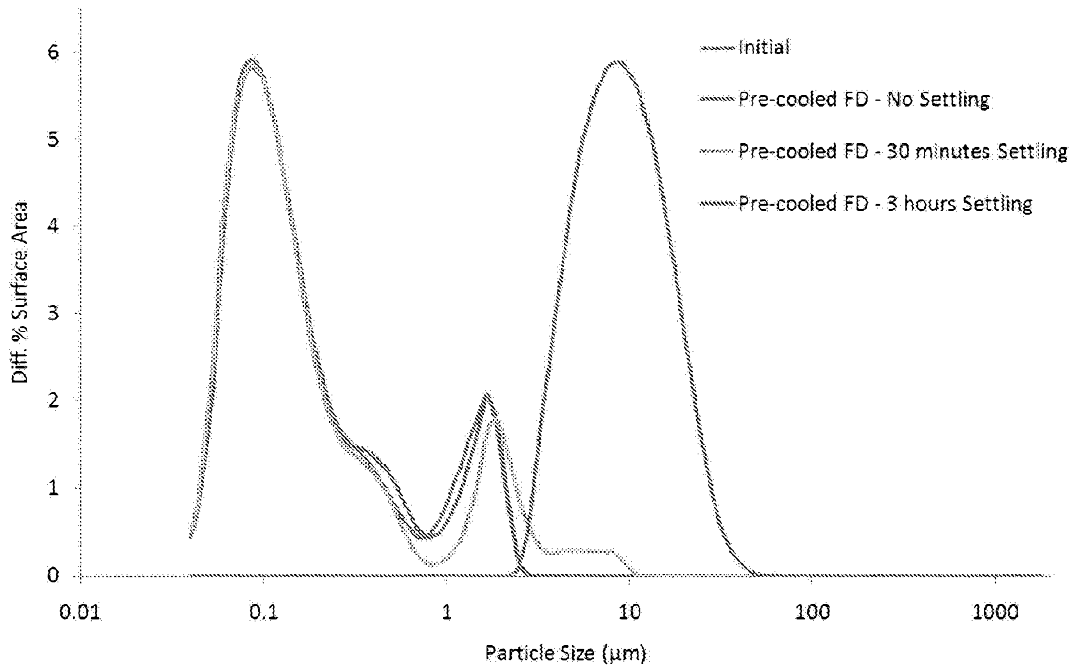

Initial
Pre-cooled FD - No Settling
Pre-cooled FD - 30 minutes Settling
Pre-cooled FD - 3 hours Settling
Diff. % Surface Area
0
1
2
3
4
5
6
0.01
0.1
1
10
100
1000
Particle Size (µm)

FIG. 7

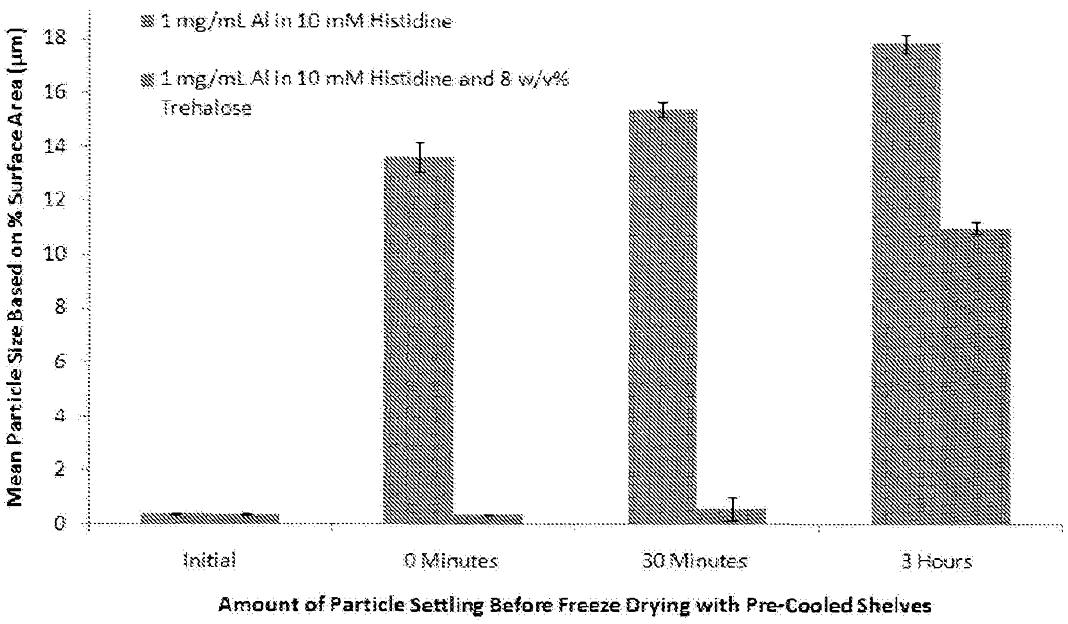

1 mg/mL Al in 10 mM Histidine
1 mg/mL Al in 10 mM Histidine and 8 w/v% Trehalose
Mean Particle Size Based on % Surface Area (μm)
0
2
4
6
8
10
12
14
16
18
Initial
0 Minutes
30 Minutes
3 Hours
Amount of Particle Settling Before Freeze Drying with Pre-Cooled Shelves

FIG. 8

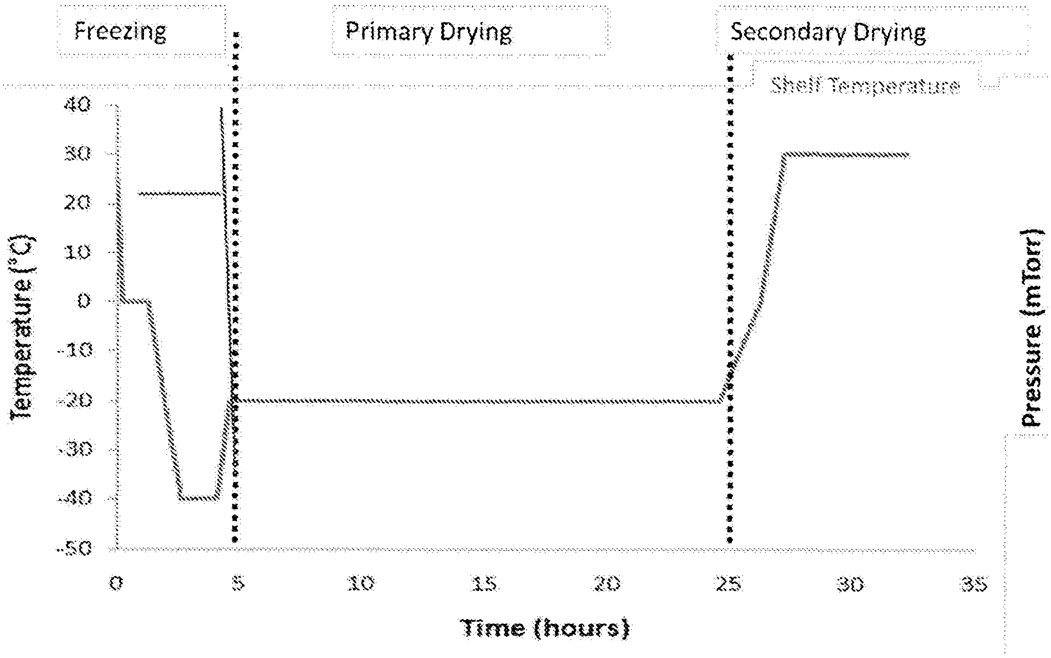

Freezing
Primary Drying
Secondary Drying
Shelf Temperature
Temperature (°C)
40
30
20
10
0
-10
-20
-30
-40
-50
Pressure (mTorr)
0
5
10
15
20
25
30
35
Time (hours)

FIG. 9

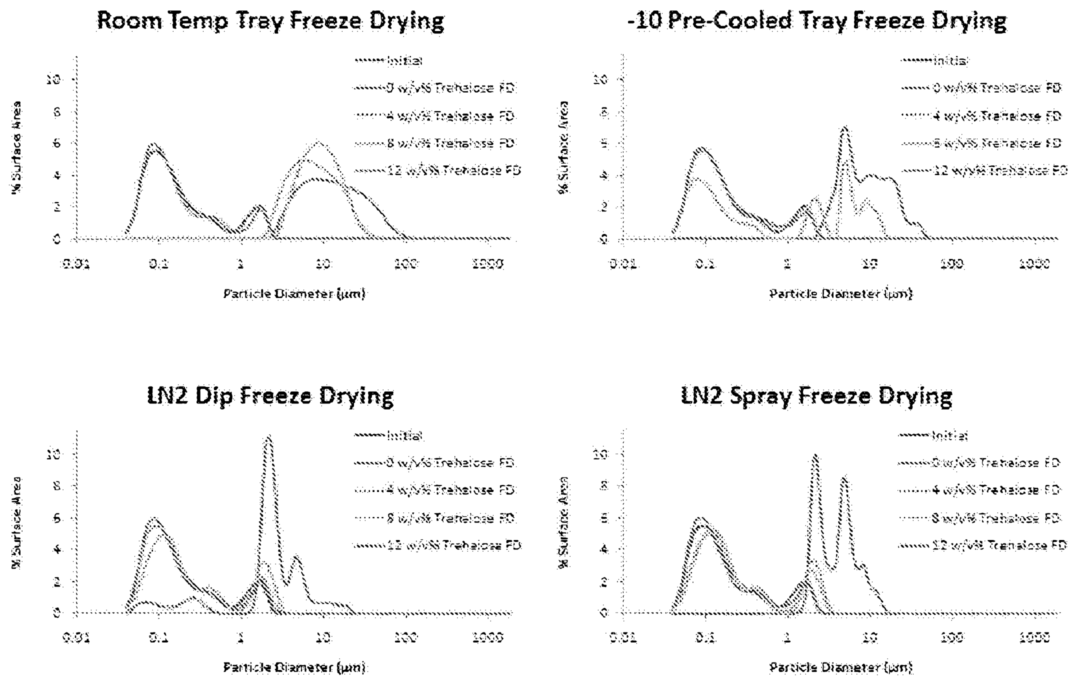

Room Temp Tray Freeze Drying
-10 Pre-Cooled Tray Freeze Drying
LN2 Dip Freeze Drying
LN2 Spray Freeze Drying
Initial
0 w/v% Trehalose FD
4 w/v% Trehalose FD
8 w/v% Trehalose FD
12 w/v% Trehalose FD
% Surface Area
Particle Diameter (µm)
0.01
0.1
1
10
100
1000

FIG. 10

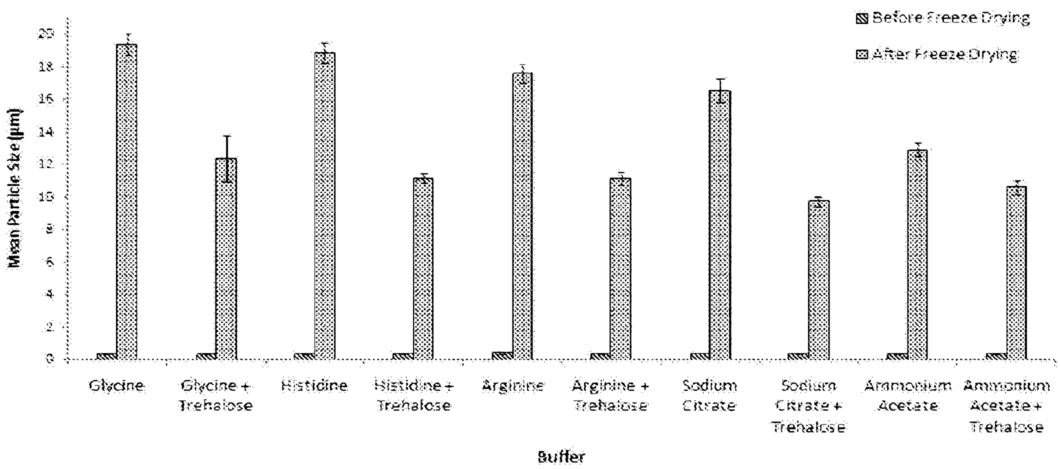

Before Freeze Drying
After Freeze Drying
Mean Particle Size (µm)
20
18
16
14
12
10
8
6
4
2
0
Glycine
Glycine + Trehalose
Histidine
Histidine + Trehalose
Arginine
Arginine + Trehalose
Sodium Citrate
Sodium Citrate + Trehalose
Ammonium Acetate
Ammonium Acetate + Trehalose
Buffer

FIG. 11

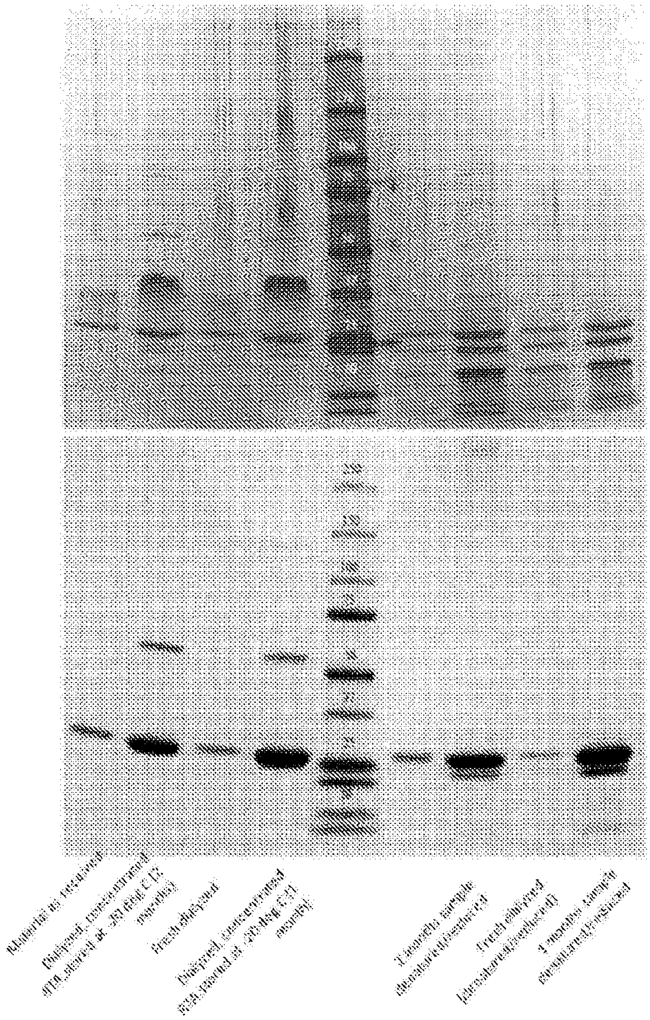


FIG. 12

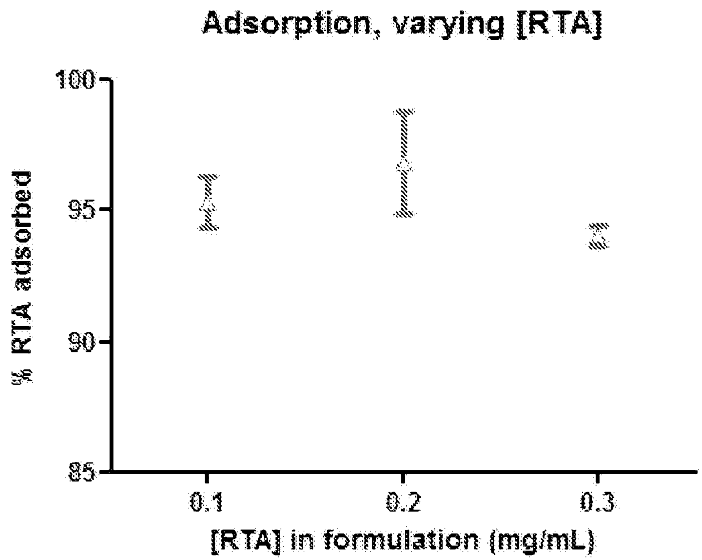
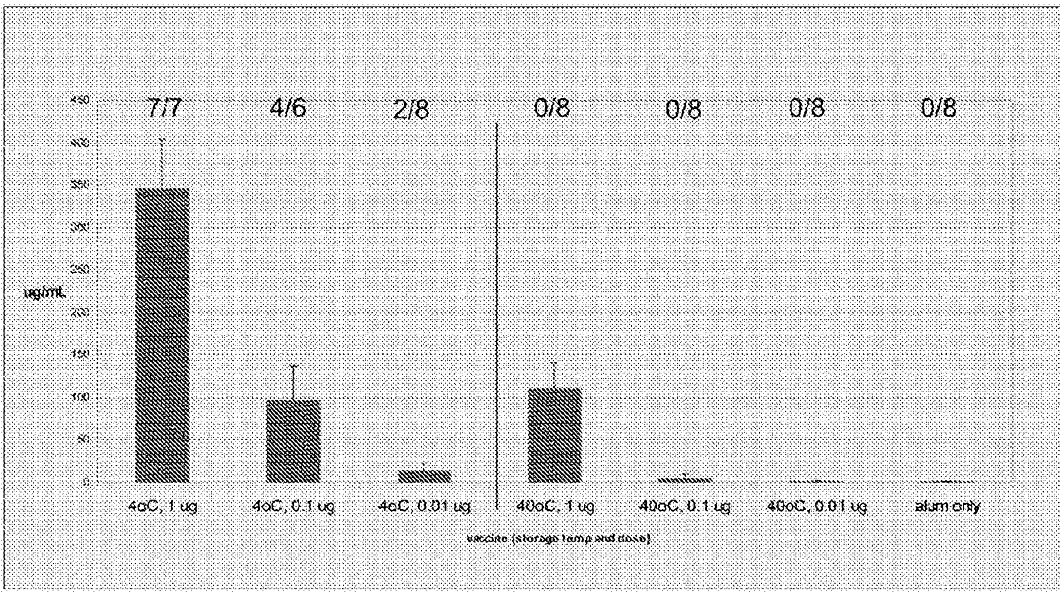

Adsorption, varying [RTA]
% RTA adsorbed
100
95
90
85
0.1
0.2
0.3
[RTA] in formulation (mg/mL)

FIG. 13



7/7
4/6
2/8
0/8
0/8
0/8
0/8
ug/mL
4oC, 1 ug
4oC, 0.1 ug
4oC, 0.01 ug
40oC, 1 ug
40oC, 0.1 ug
40oC, 0.01 ug
alum only
vaccine (storage temp and dose)

FIG. 14

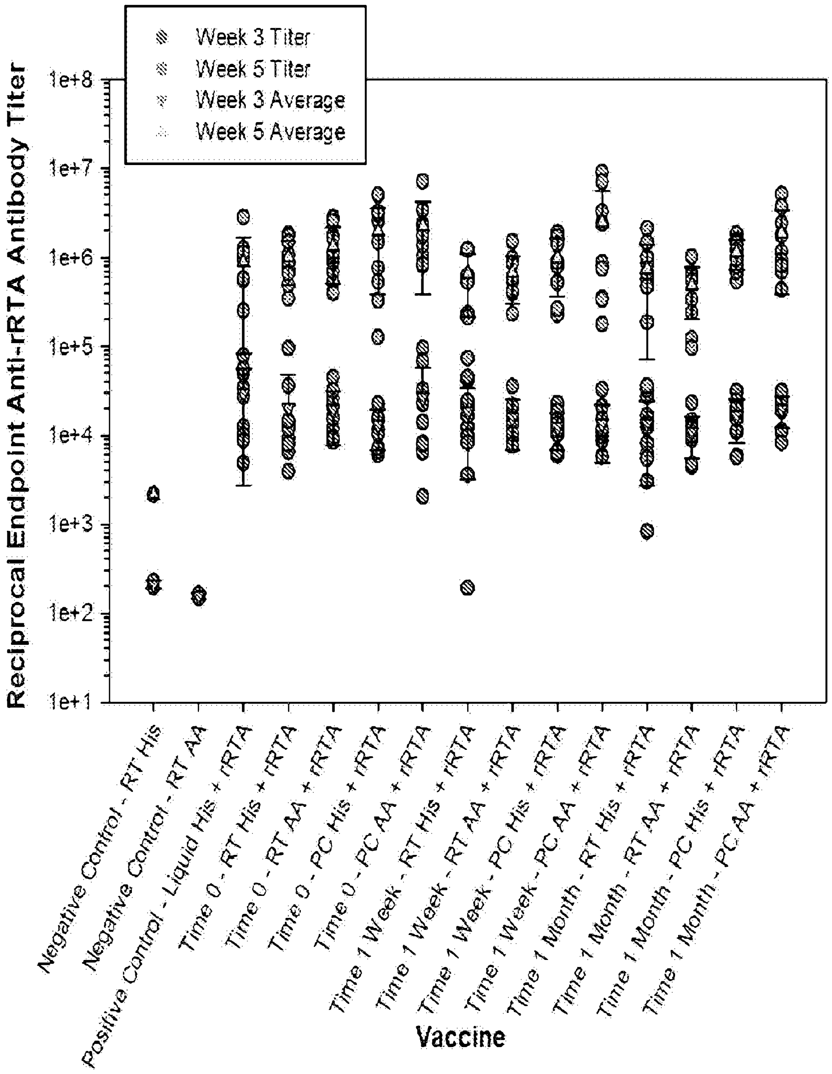

Week 3 Titer
Week 5 Titer
Week 3 Average
Week 5 Average
Reciprocal Endpoint Anti-rRTA Antibody Titer
1e+8
1e+7
1e+6
1e+5
1e+4
1e+3
1e+2
1e+1
Negative Control - RT His
Negative Control - RT AA
Positive Control - Liquid His + rRTA
Time 0 - RT His + rRTA
Time 0 - RT AA + rRTA
Time 0 - PC His + rRTA
Time 0 - PC AA + rRTA
Time 1 Week - RT His + rRTA
Time 1 Week - RT AA + rRTA
Time 1 Week - PC His + rRTA
Time 1 Week - PC AA + rRTA
Time 1 Month - RT His + rRTA
Time 1 Month - RT AA + rRTA
Time 1 Month - PC His + rRTA
Time 1 Month - PC AA + rRTA
Vaccine

FIG. 15

	RT His + rRTA, Time 0			PC His + rRTA, Time 0	
Mouse #	Endpoint Titers	Neutralizing IC_{50} Titers	Mouse #	Endpoint Titers	Neutralizing IC_{50} Titers
31	80000	0	51	160000	25
32	160000	50	52	80000	500
33	160000	300	53	320000	600
34	40000	50	54	40000	25
35	40000	350	55	40000	0
36	40000	50	56	320000	400
37	320000	600	57	320000	50
38	160000	50	58	40000	50
39	80000	50	59	160000	100
40	160000	400	60	10000	0
	# mice with neut. capacity	9 out if 10		# mice with neut. capacity	8 out of 10

	RT His + rRTA, Time month 1			PC His + rRTA, Time month 1	
Mouse #	Endpoint Titers	Neutralizing IC_{50} Titers	Mouse #	Endpoint Titers	Neutralizing IC_{50} Titers
111	16000	0	131	8000	25
112	32000	0	132	4000	10
113	64000	150	133	160000	150
114	64000	25	134	80000	10
115	128000	150	135	80000	25
116	32000	25	136	40000	100
117	500	0	137	160000	100
118	2000	0	138	80000	200
119	32000	10	139	80000	150
120	256000	600	140	40000	100
	# mice with neut. capacity	6 out of 10		# mice with neut. capacity	10 out of 10

FIG. 16

	Liquid His + rRTA	
Mouse #	Endpoint Titers	Neutralizing IC_{50} Titers
21	640000	600
22	160000	0
23	80000	0
24	160000	250
25	160000	150
26	5000	0
27	160000	50
28	40000	50
29	40000	0
30	80000	0
	# mice with neut. capacity	5 out of 10

FIG. 17