Ultra stable glassy state vaccines containing adjuvants

by

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Ultra stable glassy state vaccines containing adjuvants

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Vaccines often require a narrow temperature range for storage during the cold chain. Damage to vaccines can occur if the vaccines are frozen, or exposed to elevated temperatures which could ultimately lead to a loss in vaccine efficacy. Lyophilized vaccines allow for a wider range of storage temperatures without having vaccines experience a decrease in efficacy. By utilizing rapid freezing kinetics and high concentrations of the glass-forming excipient trehalose, the particle size distribution of aluminum hydroxide adjuvant particles was maintained during lyophilization and reconstitution. Lyophilized recombinant ricin toxin A, dominant negative inhibitor, and human papillomavirus vaccines were equally as immunogenic as their liquid counterparts. The lyophilized vaccines were able to remain stable without protein structural changes or a decrease in immunogenicity after storage at an elevated temperature of 40-50 °C, where liquid vaccines exhibited alterations in protein antigen structure and decreased immunogenicity. The addition of the toll-like receptor agonist, glycopyranoside lipid A was able to increase antibody titers and the rate of seroconversion for the anthrax vaccines but failed to do so for the human papillomavirus vaccines, showing that the immune response may be antigen specific. Although, no commercially available vaccines are lyophilized in the presence of an aluminum salt adjuvant, the work presented in this thesis provide evidence that lyophilization can be used successfully with aluminum hydroxide and glycopyranoside lipid A adjuvants.

Dedication

To my family who has encouraged me every step of the way and to my dog Wally who has kept me smiling during grad school.

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Introduction

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Currently, there are thirty diseases preventable by vaccination [193], and numerous new vaccines currently are under development. Because vaccines prevent disease at a relatively low cost, they have become one of the most cost effective healthcare interventions [193], and offer the hope of combating a number of challenging diseases, including malaria, tuberculosis, human immunodeficiency virus and cancer. For the full promise of vaccines to be realized, formulations must be developed that allow optimal immune responses, while at the same time providing for retention of activity during storage, transportation, and delivery. This chapter will discuss topics in vaccine formulation such as types of vaccines, current and future adjuvants, particulate formulations, route of delivery, endotoxin levels, preservatives, stability, and challenges associated with analytical techniques needed for vaccines.

1.1 Vaccine versus protein formulations

There are many strategies for developing formulations appropriate for therapeutic proteins [185, 189, 30, 68, 174, 91, 32, 4]. Vaccine formulations have much in common with these formu-

lations, but differ in a critical aspect: the desirability of an immune response. A strong immune response to a vaccine is a requirement, whereas an immune response to a therapeutic protein formulation could be detrimental to the patient and disease treatment [124].

To help stimulate a suitable immune response against an administered antigen, adjuvants are frequently added to vaccine formulations. These adjuvants are typically suspensions of nano- or microparticles. Although the addition of such particles allows for a lower amount of antigen to create an appropriate immune response, formulation design is complicated because the physical and chemical stability of adjuvants as well as antigens must be considered.

Vaccines create strong immune responses with relatively low concentrations of protein (10-100 microgram/mL) [63] due to the high native immunogenicity of the antigen being used or the presence of an adjuvant in the formulation. Therapeutic protein formulations require much higher protein concentrations to be an effective treatment for a disease, and antibody formulations often require as much as 100 mg/mL of protein [161].

Although the mechanism of action for protein therapeutics and vaccines is very different, both types of formulations need to be stabilized. Excipients used to stabilize protein therapeutics are often used to also stabilize vaccines. Methods to monitor stability and increase formulation stability will be discussed in later sections.

1.2 Types of vaccines

Depending on the characteristics of the pathogen of interest and target population to be vaccinated, different types of vaccines can be formulated. There are three main types of vaccines: live attenuated, killed/inactivated, and subunit vaccines. Live attenuated vaccines consist of a weakened version of the pathogen. Since live attenuated vaccines are normally immunogenic enough on their own, they rarely require an adjuvant [143]. Live attenuated vaccines can be problematic if they revert back to the stronger form of the pathogen, which could potentially cause harm in non-vaccinated or immunocompromised people [163]. To avoid a pathogen from being able to revert to a stronger form, killed, also known as inactivated vaccines are created using whole pathogens

that have been either heat or chemical treated. The safest type of vaccine is the subunit vaccine where only a portion of the pathogen is used [143]. Although subunit vaccines have less risk in the pathogen causing the disease, they are also less immunogenic because they are highly purified. The low immunogenicity often requires the vaccine to contain an adjuvant or be given in multiple doses [143].

Since subunit vaccines only contain a portion of the actual pathogen, they can come in many forms depending on which portion of the pathogen they include. Examples of specific types of subunit vaccines are toxoid vaccines, conjugate vaccines, and DNA vaccines [143]. Toxoid vaccines are used when an invading pathogen secretes a toxin to the body. Toxoid vaccines contain an inactivated version of the toxin, so that in the event of exposure to the actual toxin the body would be protected by neutralizing the toxin. A conjugate vaccine takes advantage of the immune system being able to recognize bacteria coated in polysaccharides by linking the antigen of interest to polysaccharides. A DNA vaccine carries genetic material, DNA, which the body can then use to produce the desire antigen and create an immune response.

The main focus of this chapter will be on subunit vaccines. The main components of subunit vaccines include the antigen, adjuvant, stabilizer, buffer, and possibly preservative.

1.3 General formulation considerations

Intuitively, one might expect based on physiological conditions that buffer pH values near 7 might be optimal for a vaccine formulation. However, a broader range of pH (e.g., 5-8) may be explored for vaccine formulations. Practical limitations on formulation conditions include the relatively rapid rate of deamidation reactions observed at alkaline pH, and acid-catalyzed degradation reactions that can be accelerated at acidic pH values. Stability for many proteins is optimal in solutions formulated at pH 5-6. Pain on injection may be dependent on formulation pH, tonicity, osmolarity, solution temperature, drug concentration, and injection volume [24], but can sometimes be mitigated by using formulations with reduced buffer capacity. The buffer solution should also be adjusted so that the overall vaccine formulation is isotonic. Isotonicity of the vaccine will reduce

tissue damage and pain of injection. Preservatives can be added to vaccines in cases where potential contamination is a concern, such as in multidose vaccine formulations.

1.4 Adjuvants

Adjuvants are materials that are used along with the antigen in a formulation with the primary goal of eliciting a stronger and more efficacious immune response compared to the antigen alone. In addition, an ideal adjuvant should possess the following properties:

- By eliciting a strong immune response, an adjuvant should be capable of lowering the required antigen dose [184, 128], hence reducing or eliminating any antigen-induced toxicity effects, and reducing the per-dose cost for expensive antigens.
- The adjuvant should induce both cellular and humoral immune responses to the antigen [128].
- Adjuvanted formulations should be capable of producing a rapid onset and prolonged immune response [128].
- Adjuvants should aid in creating an immune response in populations not able to originally create an immune response such as elderly, young children, and immune-compromised people [184, 128].
- Any interactions between the adjuvant and the antigen should not result in a loss of structural or chemical integrity of the antigen [128].
- The adjuvant should be safe and easy to formulate [128].

Currently in the United States, the Food and Drug Administration (FDA) has approved two aluminum based adjuvants. The first approved adjuvant is alum which is most commonly present as the mineral salts aluminum phosphate or aluminum hydroxide. The second approved adjuvant is AS04. AS04 is an adjuvant system containing monophosphoryl lipid A (MPL) adsorbed

to aluminum. In addition to alum and AS04, the European Union (EU) has approved three other adjuvants for use in vaccines. The oil-in-water emulsions MF59 and AS03 have been approved along with virosomes [144].

1.4.1 Aluminum salt adjuvants

Aluminum salt adjuvants have been used safely in vaccines for over 70 years. The two main aluminum salt adjuvants used are aluminum hydroxide and aluminum phosphate. Aluminum hydroxide can be found in Alhydrogel®, Imject® alum or alum-antigen precipitate, however all aluminum hydroxide adjuvants are not equally immunogenic [28]. Aluminum phosphate can be found in AdjuPhos®. The type of aluminum salt chosen for the vaccine formulations is based on the mechanism of antigen adsorption to the adjuvant. The antigen can adsorb to the adjuvant surface through van der Waals forces, hydrogen bonding, electrostatic forces, and ligand exchange. Since van der Waals forces and hydrogen bonding provide much weaker binding of antigen to adjuvant, we will focus on only the other two stronger mechanisms of adsorption. The World Health Organization (WHO) recommends that over 80% of antigen be adsorbed to adjuvant based on a tetanus vaccine [192]. However, studies with aluminum salt-adjuvanted vaccines based on recombinant protective antigen [18], lysozyme [40, 34, 117], dephosphorylated α-casein [117] and ovalbumin [117] have shown that antigen need not be fully adsorbed to adjuvant to be effective [39]. The maximum adsorption of protein to Alhydrogel adjuvant has been found to be related to the molecular weight of the protein, where lower molecular weight proteins can adsorb to a greater extent [85].

To maximize attractive electrostatic interactions and encourage adsorption of antigen to adjuvant, the antigen and adjuvant should have opposite charges [157]. Critical parameters for design of adjuvanted formulations thus include the isoelectric point (pI) of the antigen and the point of zero charge (PZC) of the adjuvant. At these two pHs, the protein and adjuvant, respectively, will exhibit net charges of zero. For aluminum hydroxide to PZC is approximately 11, and for aluminum phosphate the PZC is between 4 and 5.5 [139]. Based on the pH of the vaccine formulation, the

charge of the antigen and adjuvant will change; stronger binding is generally seen at solution pH values where the antigen and the adjuvant are oppositely charged [157]. To optimize the PZC for aluminum salt adjuvants, aluminum hydroxide can be treated with phosphate ions. In the presence of phosphate, aluminum hydroxide surfaces are converted to the more thermodynamically favored aluminum phosphate, thus lowering the PZC [81]. Formulations with a higher concentration of salt can have a reduced amount of protein adsorption due to charge screening [85]. The relatively high ionic strength found under physiological conditions can cause antigens that are adsorbed via electrostatic interactions to desorb from aluminum salt adjuvants once injected into the body [81].

Ligand exchange is another means by which antigens can be attached to adjuvant surfaces. Phosphate groups on antigens may exchange with adjuvant hydroxyl groups [81]. To reduce the amount of ligand exchange between the antigen and aluminum salt adjuvant, the aluminum hydroxide adjuvant can be treated with phosphate ions, thus reducing the number of site for potential ligand exchange [81]. Adsorption strength was varied by pre-treating aluminum hydroxide adjuvant with phosphate ions and it was found that the strength of adsorption was inversely proportional to the immune response for HIV gp140 antigen [76], In-labeled alpha casein [126], and hepatitis B surface antigen [57]. Since ligand exchange is a stronger mechanism of adsorption than electrostatics, antigen will not readily elute from the antigen-adjuvant complex once it is injected into the body and comes into contact with fluid [81].

The US Code of Federal Regulations recommends that vaccine formulations contain less than 0.85 mg Al^{+3} per dose when assayed and less than 1.14 mg Al^{+3} when calculated, whereas the World Health Organization (WHO) and European standards recommend less than 1.25 mg Al^{+3} per dose [182]. The toxic levels of aluminum were evaluated to be around 36.42 mg of Al^{+3}, in an acute toxicity study in rats [179], which is 43 times more than the FDA recommended dose. The regulatory agencies have presumably recommended a low dose of aluminum to avoid possibilities of chronic toxicity.

Many vaccines can protect people against a disease through a humoral response wherein antibodies are produced once a pathogen invades the body. The antibodies can help the immune system

clear the invading pathogen from the body. Although it is known that aluminum salt adjuvants create a humoral immune response, the exact mechanism of action remains unknown. Proposed mechanisms include, the depot effect where antigen is slowly released, activation of the NLRP3 inflammasome to release IL-1β and IL-18 cytokines, increased PI3-Syk signaling, and increased danger signals such as uric acid and DNI released during cell death [133]. Some pathogens, however, require the body to initiate a cellular response in order for the pathogen to be cleared. A cellular immune response is important in vaccines protecting against intracellular pathogens [115]. In particular, malaria and tuberculosis vaccines require a cellular immune response to be effective [195]. Since aluminum salts adjuvants create a humoral immune response which is not ideal for vaccines protecting against all pathogens, other vaccine adjuvants need to be investigated [70].

1.4.2 MF59

MF59 was the second approved vaccine adjuvant after alum [144]. MF59 is an oil-in-water emulsion. In the emulsion, squalene oil nanodroplets approximately 160 nm in diameter are surrounded by the non-ionic detergents polysorbate 80 (Tween 80) and sorbitan triolate (Span 85) [156]. When stored at temperatures between 2-8 °C, MF59 is able to retain a constant particle size for up to three years [156]. MF59 is commonly used as an adjuvant in influenza vaccines [129]. In one study, the antigens diphtheria toxoid, tetanus toxoid, group C Meningococcal conjugate, hepatitis B surface antigen and recombinant MB1 were formulated with both aluminum adjuvant and MF59 adjuvant. For all antigens except diphtheria toxoid, formulations containing MF59 adjuvant were able to create a stronger immune response than corresponding formulations containing aluminum, as shown by geometric mean IgG titers after two doses of the vaccine [163]. In addition to creating a stronger immune response, MF59 can also protect against antigenically drifted antigens in influenza vaccines [173, 69, 73].

1.4.3 AS04

AS04 is an adjuvant system created by GlaxoSmithKline Biologicals that contains 3-O-desacyl-4'-monophosphoryl lipid A (MPL) adsorbed to an aluminum salt. Lipopolysaccharide (LPS) is known to stimulate toll-like receptor (TLR) 4, helping create a cellular immune response. MPL comes from the portion of LPS found in the cell walls of gram-negative bacteria [31]. Since LPS is too toxic to be used directly as an adjuvant, MPL is derived from LPS to have a similar effect on TLR 4 without the unwanted toxicity [14]. The AS04 adjuvant can help create both humoral and cellular immune responses [70].

AS04 is currently included in the FDA-approved human papilloma virus (HPV) vaccine Cervarix [51]. The AS04 adjuvant present in a hepatitis B vaccine was tested in comparison to a hepatitis vaccine without AS04. It was found that after one dose of vaccine containing AS04 adjuvant, the patient seropositivity rate was 77%, whereas patients receiving vaccine without AS04 had only a 37% seropositivity rate. After injections at 0 month and 6 months the AS04 group had 98% seroprotected and after injections at 0 month, 1 months and 6 months the group without AS04 had 96% seroprotected, showing that the vaccine formulated with AS04 was equally effective as the vaccine without AS04, but required fewer doses [20].

1.4.4 AS03

AS03 is an oil-in-water emulsion adjuvant system created by GlaxoSmithKline Biologicals. This adjuvant contains squalene and α-tocopherol, a form of vitamin E. Hepatitis B surface antigen (HBsAg) formulated with AS03 had a ten times higher geometric mean titer than antigen alone formulated with alum after two intramuscular doses [121]. A significantly higher antibody titer was also seen when an H5N1 influenza vaccine was formulated with AS03 in comparison to vaccine without an adjuvant [121]. In addition to producing higher antibody titers with HBsAg, the AS03 adjuvanted influenza formulations were able to produced both Th1 and Th2 cytokines in greater amounts than alum [121]. To be most effective AS03 should be injected in the same location and

at the same time as the antigen [121].

1.4.5 Virosomes

Virosomes are virus like particles containing portions of virus envelope without genetic material of the virus. When virosomes are used as an adjuvant, they can create both a humoral and cellular immune response [146]. Virosomes are approximately 100-200 nm in diameter [11]. Virus-like particles can be found in hepatitis A and B, human papillomavirus and influenza vaccines licensed in Europe [122].

1.5 Future adjuvants

Adjuvants are an integral part of an effective subunit and inactivated microorganism vaccine formulations, and scientists have directed their efforts to discover new adjuvant molecules that are safer and more effective than alum. However, new adjuvant research involves thorough in-depth understanding of the mechanism of action, stability pattern, toxicity profile across various doses and populations as well as compatibility with the vaccine candidate in the desired formulation.

1.5.1 GLA

Glucopyranosyl Lipid A (GLA) is a version of lipopolysaccharide (LPS) which has been modified to be non-toxic by removing one phosphate group and residues such as carbohydrates from the hydroxyl group [46]. Monophosphoryl lipid A (MPL) is very similar to GLA in structure and activity except that GLA is more homogenous by having a consistent number and length of acyl chains and is 10-100 times more potent than MPL [46]. Since GLA is similar in structure to LPS, GLA can activate the immune system through interactions with toll-like receptor 4 (TLR 4), creating a Th1 biased immune response. The cellular immune response of GLA is dependent of the type of formulation whereas the humoral immune response has been reported to be independent of the formulation [65]. GLA can be formulated as an aqueous nanosuspension, oil-in-water emulsion, liposome, or alum-adsorbed formulation [65, 119]. Antibody titers of the 2006-2007 FluZone

influenza vaccine were enhanced after one and two injections when GLA formulated as a stable emulsion was added compared to FluZone formulations with and without an emulsion. Additionally, the vaccine containing GLA stable emulsion was able to protect against antigenically drifted H3N2 influenza virus strains not included in the 2006-2007 FluZone vaccine [45].

1.5.2 QS-21

QS-21 is a water-soluble adjuvant. Chemically, it is an acylated 3.28-o-bisdesmodic triterpenoid saponin derived from the bark of the Quillaja saponaria tree [97]. This adjuvant has been tested in several clinical trials for vaccines against infectious diseases such as HIV-1 [60], influenza [114], malaria [175] as well as in cancerous patients with melanoma [80], breast or prostate cancer [96]. QS-21 has been extensively used with MPL in a malaria vaccine with satisfactory results. However, being a natural product, QS-21 exhibits variability in composition depending on the source, and also can be expensive to extract and purify [92]. Also, dose-dependent immune responses for QS-21 pose a challenge in cancer patients, who develop local erythema and flu-like symptoms at doses greater than 150 micrograms [2]. Additionally, QS-21 degrades during long-term storage in aqueous solutions [44]. Synthetic saponins have been investigated to overcome these problems [2].

1.5.3 Immunostimulating complexes

Another adjuvant that contains a saponin is immunostimulating complexes (ISCOM). ISCOM contains cholesterol, phospholipids, saponin, and protein. ISCOMATRIX is similar to ISCOM except it does not contain protein [138]. When the ISCOMATRIX components combine they form approximately 40 nm cage like structures [138]. The ISCOMATRIX has been shown to be stable when refrigerated for 2 years, stored at 40 °C for a few months, after freeze thaw cycles, and during lyophilization [138]. Both humoral and cellular immune responses can be generated with this adjuvant [176]. An increased amount of local reactions to the ISCOMATRIX in a clinical trial for Human Papillomavirus (HPV) vaccine was seen in comparison to the group containing no adjuvant [116]. In vaccine trials for HPV, hepatitis C virus, and influenza, ISCOMATRIX was

found to be safe [116].

1.5.4 Montanide ISA

Montanide ISA 720 is a squalene-based adjuvant designed for human use that consists of mannide monooleate emulsifier and forms stable water-in-oil droplets with the idea of promoting sustained release of antigen at the injection site [8]. In clinical studies involving a malaria vaccine (P. falciparum CSP C-terminal fragment 282-283) formulated with ISA 720 and alum, high antibody titers were obtained along with good lymphocyte proliferation and production of IFN-γ that is critical for the elimination of malaria parasite [147, 105]. Another compound in this category is Montanide ISA 51, which is based on mineral oil that can be metabolized has also been shown to be safe for human use [7].

1.5.5 Microorganism compounds

Components derived from micro-organisms such as bacteria hold promise as "immunopotentiating" adjuvants. For example, specific mutants (produced by site-directed mutagenesis) of heat-labile enterotoxin derived from Vibrio cholera or *E. coli* have been investigated as candidates for mucosal adjuvants that provoked increased serum IgG levels in mice and pigs when administered nasally in a microsphere delivery system [181]. However, toxicity of such molecules has limited their use in humans [181]. Another example in this category is a fusion gene (CTA1 gene from cholera toxin fused with a synthetic analogue of S. aureus protein A encoding gene) that exhibited less toxicity compared to wild-type cholera toxin [106].

1.5.6 Cytokines

Cytokines can also be potential adjuvant candidates. However, a variety of interleukins (IL-1, IL-2, IL-12) evaluated for this purpose exhibited in vivo stability and toxicity issues [181]. Another example is IRX-2 which contains a natural mixture of Th1 cytokines (IL-1, IL-2 and IFN-g) that enhances the antigen-processing capacity of lymph nodes by stimulating the Th1 pathway [123].

1.5.7 Toll-like receptor agonists

Toll-like receptors (TLRs) are pattern-recognition receptors found on cells of the innate immune system. The TLRs bind to a variety of infectious agents and stimulate pathways that finally protect the host cells from the pathogen. Therefore, synthetic or purified TLR agonists have been the interest for adjuvant purposes [172]. One such example of TLR agonist is a repeating sequence of CpG dinucleotides, which has been found to be immunostimulatory and has been tested as an adjuvant in hepatitis B vaccine [47]. Imiquimod and resiquimod are small molecule TLR-7/8 agonist molecules, which are being studied as a topical adjuvant for skin disease [19].

1.5.8 Polymer particles

Micro- and nanoparticle formulations can also be employed for vaccine delivery resulting in sustained-release vaccine formulations. Such formulations involve the use of biodegradable polymers such as poly-lactic acid (PLA), poly-lactic-co-glycolic acid (PLGA), polyethylene glycol (PEG), and polyphosphazene to formulate the micro- or nanoparticles [136]. Since these polymers are biodegradable in aqueous solutions, formulations containing these particles may need to be lyophilized [25]. A wide range of particle sizes can be created with polymer particles [162].

1.6 Vaccine particles

In order for an immune response to be created, vaccine antigen needs to reach lymph nodes where activation of T and B cells can occur. Small particles, less than 100 nm are able to move from the injection site through the extracellular matrix and make their way to draining lymph nodes. Even smaller particles, less than 10 nm are able to increase their mobility by also being able to access blood vessels. Larger particles, greater than 100 nm stay at the site of injection until encountered by antigen presenting cells which can uptake the antigen and transport it to the lymph node [87, 202, 168, 136].

The current literature show conflicting results on how adjuvant particle size affects the im-

mune response of vaccine antigens. There are reports of smaller particles being more effective, larger particles being more effective, and particles of different sizes being equally effective [136]. In general, nanoparticles have been shown to induce a more cellular immune response where microparticles have been shown to create a more humoral response [136]. Potential reasons for why results of many experiments differ on how particle size affect immune response, include differences in particle material, antigen, method of antigen association with particle, particle size uniformity and distribution, and route of injection [136].

The size, shape, and surface molecular organization of antigens have been found to affect the immune response [11]. By using adjuvants of controlled sizes, vaccine particles can be made to be of sizes similar to those of the target pathogen [11, 130]. Virus-like particles and immunostimulating complexes can be on the same order of magnitude of viruses. Emulsions, liposomes and virosomes can be on the same order of magnitude of size as larger viruses, bacteria, fungi and protozoa. Microparticles and mineral salts can be on the same order of magnitude size as bacteria, fungi and protozoans [11]. In addition to adjuvant particles being a similar size to potential pathogens, it is also important for adjuvants to be taken up by antigen presenting cells.

Since the majority of aluminum salt adjuvants are greater than 100 nm, if is important for them to be able to interact with antigen presenting cells in order to be transported to lymph nodes. The diameter of macrophages and dendritic antigen presenting cells has been found to be 10-22 μm [168] which could potentially limit the size of antigen-particle complex to be engulfed. Antigen presenting cells were found to engulf nanoparticles, $\sim$100 nm aluminum hydroxide particles where larger microparticles, $\sim$9.3 μm particles were not taking up by the antigen presenting cells [103]. Dendritic cells have been shown to internalize aluminum hydroxide adjuvant particles 3 μm in diameter more efficiently than 17 μm particles [120]. An additional experiment showed that polystyrene particles 430 nm and 1 μm were more readily internalized than particles 10 and 32 μm, which ultimately lead more immunostimulatory cytokines being produced by cells with internalized particles [160]. The difference seen in antigen presenting cell internalization and cytokine secretion could lead to differences in immunogenicity.

Lyophilization parameters can be varied to create vaccines containing a range of aluminum particle sizes [42]. In a study conducted with a model lyophilized lysozyme vaccine, formulations containing aluminum particles ranging in average size from 2-14 microns all produced similar anti-lysozyme IgG1 titers after two doses [40]. Additionally, an alkaline phosphatase vaccine was lyophilized to create aluminum salt adjuvant particle with mean sizes from 2-17 microns. All aluminum salt adjuvant particle sizes were found to be equally as effective based on the anti-alkaline phosphatase IgG1 titer after two doses [43]. Although the immune response for particles in the 2-17 micron size range appears to be equal, literature does suggest that smaller aluminum hydroxide nanoparticles with a mean diameter of 100-200 nm were more immunogenic than aluminum hydroxide microparticles with a mean diameter of 7-9 microns, when used in ovalbumin and protective antigen vaccines [103].

1.7 Route of delivery

An ideal vaccine should be effective, safe, and administered in a minimally invasive manner. The route of vaccination is a very important consideration as some infectious disease pathogens invade the host cells on mucosal surface; in such cases, the ideal vaccine needs to induce systemic immunity as well as mucosal immunity [52]. Oral administration of vaccine is one of the routes of administration that yields the highest patient compliance, and does not require syringes or trained personnel. However, a vaccine delivered via the oral route must be robust enough to survive the acidic pH in the stomach and proteolytic enzymes, and should be suitably transported across the gastrointestinal tract in order to reach the systemic circulation. Approaches to modulate delivery across the gastrointestinal tract includes altering physicochemical properties of the vaccine for enhanced uptake or formulating the vaccine in micro- or nanoparticles that protect the antigen from acid degradation in stomach. However, particle-based formulations face a major hurdle in crossing the intestinal barrier and therefore generally offer very poor protection at the mucosal site. Several ligand-based delivery systems have been recently explored to identify gastrointestinal surface receptors as vehicles of delivery of antigen via endocytosis to elicit a strong immune response. Such

ligands include lectin based targeting, bacterial adhesins, bacterial toxins, and antibody mediated targeting [151]. Live attenuated vaccines are administered orally as the antigen needs to have an inherent ability to attach to mucosal cells. Presently, the vaccines that have been approved for oral administration include cholera, influenza, polio virus, rotavirus, and Salmonella typhimurium [82].

The nasal route of administration can also produce mucosal and systemic immune responses. It is an attractive alternative to oral vaccines as the antigen is not subjected to acid degradation. Also, this route of administration is easily accessible, highly vascularized and ideal for mass immunization. However, the vaccine still needs to overcome the nasal mucosal barriers to produce systemic effects. Solution, dry powder, or suspension formulations can be delivered via this route. Nasal vaccination possibly demonstrates a more rapid onset compared to oral vaccines [49]. Flumist® is an example of nasal delivery system consisting of temperature-sensitive attenuated influenza virus.

The most common route of vaccine administration is via intramuscular or subcutaneous injection using a syringe and needle. Intramuscular injection optimizes the immunogenicity of the vaccine and greatly reduces any adverse reaction at the site of administration. Although routes of vaccine administration with the use of a needle are effective, alternative routes are being investigated to help increase patient compliance due to pain of injection and patient fear of needles as well as ease of use.

Transcutaneous vaccination has also become a topic of interest for vaccine delivery. The skin is the largest organ in the human body and is the first natural against harmful pathogens. However, the transport of antigens across the stratum corneum represents a significant barrier to this route of vaccine delivery. It is expected that adjuvants such as alum, MPL, and bacterial endotoxins will have limited penetration across the skin due to their large size. However, preclinical transcutaneous studies indicate that cholera toxin (CT) and heat-labile *E. coli* toxin (LT) can be used as adjuvants as they stimulate immune response against other antigens. The most successful delivery via transcutaneous route consisted of physically disrupting the skin barrier with the help of microneedles followed by delivery of the formulation [12].

1.8 Endotoxin levels

Endotoxin comes from lipopolysaccharides (LPS) found in the cell membranes of gram-negative bacteria [109]. LPS commonly contains distinct regions of an O-antigen region, core oligosaccharide, and hydrophobic lipid (Lipid A), with the Lipid A region being responsible for toxicity [109]. Endotoxin can be introduced into formulations when components of the vaccines are produced in gram-negative bacteria, such as recombinant proteins produced in *Escherichia coli* [109]. When the body is exposed to large dose of endotoxin or small doses of endotoxin systematically, an inflammatory reaction occurs which can cause shock, tissue damage, or death [109]. To avoid toxicity caused by endotoxin, endotoxin levels should be kept low in formulations. The threshold pyrogenic dose of endotoxin in humans is 5 EU/kg [110], making it desirable to keep endotoxin levels below this amount. Although specific endotoxin limits have not been set by United States Pharmacopeia (USP), it is recommended to keep endotoxin levels low. Brito and Singh suggested upper endotoxin limits for different types of vaccines based on DTwP and Cholera vaccines as follows: genetic vectors 10 EU/mL, recombinant subunit 20 EU/mL, polysaccharide 20 EU/mL, live attenuated 200 EU/mL, inactivated 500 EU/mL, and toxoid 200,000 EU/mL [26].

Endotoxin present in formulation is most commonly measured by gel clotting in the Limulus Amebocyte Lysate (LAL) test. If levels of endotoxin are too high, endotoxin can be removed throughout steps in the vaccine manufacturing process. Endotoxin is stable at high temperature, and heat sterilization will not inactivate endotoxin unless temperatures exceeding 250 °C for 30 minutes and 180 °C for 3 hours are used [109, 75]. Concentrations of acids and alkalis above 0.1M are capable of inactivating endotoxin. Endotoxin present in protein solutions can be removed by LPS affinity resins, two-phase extractions, hydrophobic interaction chromatography, ion exchange chromatography, gel filtration chromatography, sucrose gradient centrifugation, and membrane adsorbers. If protein is not present in the desired solution for endotoxin removal, ultrafiltration can be used [109].

1.9 Preservatives

Although preservatives are not normally used in single-use vials, preservative are normally added to multidose vials to prevent growth of microorganisms as recommended by the United States Code of Federal Regulations for vaccines not containing live attenuated viruses. Preservative that have been used in US FDA-approved vaccines include thimerosal, phenol, benzethonium chloride, and 2-phenoxyethanol [72]. At an acidic pH thimerosal is able to kill bacteria and at an alkaline or neutral pH thimerosal prevents bacteria and fungus from replicating [150]. Thimerosal is not compatible with aluminum and should therefore not be used with an aluminum salt adjuvant [150]. Vaccines recommended for children under six years old, except for influenza vaccines, have had the thimerosal reduced to trace levels or lower [63]. Thimerosal is currently used in tetanus toxoid vaccine, influenza vaccines, and multidose Menomune-A/C/Y/W-135. Phenol is able to be used against both gram-negative and gram-positive bacteria, mycobacteria, some fungi and viruses [150]. Phenol is currently included in Pneumovax 23. Benzethonium chloride has an optimal antimicrobial activity from pH 4 to 10 and is not compatible with anionic surfactants [150]. Benzethonium is currently included in BioThrax. 2-phenoxyethanol is able to protect against gram-negative organisms but has reduced activity when non-ionic surfactants are present [150]. 2-phenoxyethanol is currently included in inactivated poliovirus vaccine (IPOL).

1.10 Stability

In order for vaccines to be economically feasible and able to be delivered to patients, they generally should have a shelf life of 2 years or longer. To determine the stability of a given formulation, both real time and accelerated stability studies can be conducted. In accelerated stability testing, a stress such as elevated temperature, elevated humidity, light exposure, agitation, freeze-thawing, extremes of pH, or redox conditions [4, 22] is applied to the formulation, and the rates at which the formulation degrades is monitored. Extrapolation of degradation rate data as a function of stress level allows an estimate of shelf life in the absence of stress to be obtained.

Many parameters such as pH, ionic strength, osmolarity and the type and concentration of excipients present may play a role in vaccine stability. pH affects vaccine stability by changing the rate at which hydrolysis and deamidation reactions occur. pH also changes the charge of molecules in solution which can then cause changes in protein structure or changes in adsorption of protein to adjuvant or other surfaces [22]. Lower ionic strength can increase the solubility of biomolecules and the solution ionic strength can change how molecules assemble [22]. Excipients can also be added to formulations for stability [22].

Excipients are commonly added to formulations to increase the formulation stability, maintain pH, modify tonicity, or help increase antigen solubility. Excipients commonly added to increase stability consist of surfactants, sugars, salts, and antioxidants [32]. Surfactants are commonly used to prevent unwanted protein adsorption to surfaces since proteins often denature when adsorbed to surfaces. Sugars in solution are able to protect proteins from denaturing by preferential hydration and excluding sugar molecules from the protein surface. Sugars protect lyophilized formulations by slowing molecular motions in the dried solid state, and by providing hydrogen bonds with protein in the place of water. Salts can be added to formulations to increase the formulation ionic strength and can be added to help maintain a particular pH. Antioxidants are used to protect against oxidation.

To predict the formulation conditions and excipients that maximize the vaccine formulation stability from complex data sets, empirical phase diagrams can be used to better interpret the data [108]. Empirical phase diagrams take mathematical data collected from a variety of spectroscopic techniques and convert it into colors. Similar colors represent similar stabilities. Techniques commonly used in collecting the spectroscopic data for phase diagrams consist of circular dichroism, near UV absorbance, extrinsic fluorescence, dynamic light scattering, turbidity, and intrinsic UV fluorescence [108]. To determine regions of stability, controlled formulation parameters (e.g., temperature, pH, excipient concentration, protein history or other relevant conditions) need to be varied.

Although vaccines can be created with antigen and adjuvant produced separately and then mixed together before administration in the clinic, it is recommended to have antigen and adjuvant

formulated together. If the antigen and adjuvant will be stored separately, both components of the vaccine will need to undergo stability studies separately and then throughout the stability study antigen and adjuvant will need to be combine to test the whole vaccine. Variations in the vaccine such as adsorption of antigen to adjuvant caused by amount of time combine and mixing conditions will be created when the antigen and adjuvant are combined before use by different people. Slightly variations in the mixing procedure used could cause potential changes in the vaccine. These variations in the vaccine could potentially cause a loss in efficacy or safety.

Since vaccines have potential to experience both hot and cold temperatures before being delivered to patients, the vaccine stability should be tested with several cycles of freezing and thawing. Loss of or decreased potency has been observed for vaccines containing an adjuvant (e.g. Alhydrogel) due to freeze-thawing [23]. Several studies in the literature have implicated freezing induced agglomeration of Alhydrogel for loss of potency [53]. A study by Jones et al. subjected Hepatitis B and DTaP vaccine formulations to controlled freeze-thaw cycles; they also evaluated the freezing-induced protection effects provided by additives such as glycol, PEG 300, and glycerin [23].

To increase stability and allow for higher storage temperatures vaccines can be dried. In the dried solid state, degradation reactions occur at a much slower rate and a significantly lower water content is present allowing for less degradation. Methods of drying that have been used consist of lyophilization [30, 40, 43, 5], Xerovac (a dehydration process not involving freezing) [196], spray drying [5, 21, 171], spray freeze drying [5], and carbon dioxide assisted nebulization with a Bubble Dryer® [5, 27] (CAN-BD) (a drying process used to produce an inhalable powder).

1.11 Lyophilization

Lyophilization, also commonly known as freeze drying is the drying technique that will be the focus of this thesis. During the lyophilization process, three main stages occur: freezing, primary drying, and secondary drying. An optional stage of annealing can occur after the freezing stage. The initial liquid formulation first goes through the freezing stage where the shelf temperature is

lowered below the freezing point of the formulation. While the formulation freezes, the formulation components are freeze concentrated. At the end of the freezing stage, the majority of the formulation is frozen solid. The rate freezing occurs will determine the size of ice crystals which will influence the rate of drying. An annealing step can be placed after the freezing stage by increasing the temperature slightly which can allow for Ostwald ripening, leading to larger ice crystals and a faster drying time [95]. During freezing problems may arise due to liquid-ice interfaces, freeze concentration, cold denaturation of protein, mechanical stress, and pH shifts [29].

To begin primary drying, the shelf temperature remains low and chamber pressure is drastically reduced. During primary drying, the majority of water, ∼80% is removed through sublimation. The pressure difference between the ice interface and condenser surface causes water to sublime. Ideally, the formulation temperature will remain below the maximally freeze concentrated glass transition temperature, Tg and collapse temperature to prevent the cake from shrinking or collapsing during the drying phase [30]. During secondary drying the chamber pressure remains reduces while the shelf temperature is increased to supply the additional energy required for removing water more tightly associated with formulation components. At the end of the lyophilization process, the chamber may be purged with an inert gas to help prevent degradation by oxygen and moisture. When lyophilized formulations are ready to use, they can quickly and easily be reconstituted.

To create pharmaceutically elegant and stable lyophilized products, many excipients can be added to the formulation. Common excipients include, glass forming sugars, glass transition temperature modifiers, bulking agents, and surfactants. Sugars such as treahlose or sucrose can help protect protein through the lyophilization process through preferential exclusion in the liquid state and then through the water replacement hypothesis, where sugars can hydrogen bind to protein as water is removed. Additionally, sugars also help create a glassy state in which formulation components are dispersed in a low mobility matrix minimizing component interactions [33]. To further increase the glass transition temperature, polymers such as hydroxyethyl starch (HES) or dextran can be added. Bulking agents such as glycine and mannitol can be added to help low protein concentration form becoming lost. Surfactants help prevent surface adsorption of protein

molecules as well as inhibit protein aggregation during lyophilization and reconstitution. Ideally lyophilized formulations are developed in low salt conditions since salt will significantly decrease the glass transition and eutectic melting temperatures causing the drying stages to require very low shelf temperatures and therefore much longer drying times.

At the end of the lyophilization process, formulations can be found as a solid glassy-state formulation. The glass transition temperature of the lyophilized formulation should be above the intended storage temperature so that the formulation remains in the glassy-state during storage. Degradation reactions are significantly minimized by the high viscosity and low mobility of formulation components in a glassy-state. Degradation is further reduced by having lyophilized formulations contain ∼1% or less water. Lyophilized products are able to have increased stability at higher temperatures than their liquid counterparts because of these qualities.

Despite the positive attributes of lyophilized formulations, only one third of FDA approved vaccines are currently lyophilized and none of these vaccines contain an adjuvant [63]. Since aluminum salt adjuvants are known to aggregate during freezing, lyophilization of vaccines containing adjuvants has been avoided. During freezing, ice crystals are thought to force aluminum salt particles together leading to irreversible aggregation [201]. Clausi et al. was able to show that by using rapid freezing and a high concentration of the glass forming excipient trehalose, aggregation of aluminum salt adjuvants could be avoided during the lyophilization process [42]. Currently, literature has shown potential to lyophilize vaccines containing aluminum salt adjuvants in alkaline phosphatase, lysozyme, and botulium vaccines [43, 40, 41]. In the lyophilized state, these vaccines were able to be stored at elevated temperatures for extended periods of time without a loss in immunogenicity where equivalent liquid formulation were not able to retain immunogenicity.

1.12 Challenges of analytical techniques

When developing antigens to include in vaccine formulations, high resolution techniques such as x-ray crystallography, nuclear magnetic resonance (NMR), and cryo-electron microscopy (cryo-EM) should be used [108]. When vaccine formulations are monitored over time, lower resolution,

faster techniques are more appropriate [108] and will be focused on for the rest of this section. Changes in the vaccine formulations could be an indication of instability leading to a loss of safety and efficacy. Since vaccines frequently contain adjuvants which can scatter light as well as low protein concentrations, analytical techniques can often become difficult.

Primary structure can be looked at by breaking apart the antigen of interest through proteolysis and then analyzing the fragments with mass spectroscopy for areas of degradation. The amino acids, glutamine and asparagine are more prone to deamidation and should be monitored through a loss of carboxylic acid group. The glutamine and asparagine residues should be especially monitored for deamidation when surrounded by a glycine residue allowing for greater flexibility for the deamidation reaction [111]. Oxidation is more common is in the aromatic residues tyrosine, tryptophan and along with cysteine and methionine.

Secondary structure has been examined by infrared spectroscopy. A study conducted with the six model proteins, cytochrome c, ovalbumin, α-chymotrypsinogen A, recombinant human IL-1ra, IgG1, and sTNF-R1 compared the standard solution infrared spectrum at protein concentrations of 15 mg/mL to lower protein concentrations of 1.0 mg/mL and 0.5 mg/mL with protein adsorbed to Alhydrogel adjuvant and found that the spectra were very similar [54]. The technique developed of looking at the secondary structure through infrared spectra of adjuvant-protein pellet would be applicable to vaccines formulated with aluminum adjuvants containing low concentrations of antigen. The secondary structure of proteins adsorbed to aluminum hydroxide, glass, and cellulose was able to be examined by a similar method [16, 66].

Tertiary structure has been examined by tryptophan fluorescence quenching for protein adsorbed to glass, cellulose, silica, and alum [16, 66]. Since proteins contain the amino acid tryptophan which gives off a fluorescent emission depending on how buried the tryptophan residues are in the protein, the amount of unfolding can be monitored by measuring how easily the fluorescence from these residues can be quenched. The Stern-Volmer constant can be used to help determine the amount of quenching taking place. The Stern-Volmer equation uses the ratio of fluorescence intensity without quencher present, Fo, to fluorescence intensity with quencher present, F, equaling

one plus the Stern-Volmer constant, K_{SV}, multiplied by the quencher concentration, $[Q]$. The Stern-Volmer equation is as follows: $Fo/F = 1 + K_{SV}[Q]$ [16].

Aggregation of vaccine antigen and particles present in vaccine formulations can be examined by many different techniques based on the size of particles present in the formulation and the desired information (particle count, particle size distribution, particle images). Imaging particle size techniques using instruments such as Micro-Flow Imaging (MFI) or FlowCAM can count, size, and image particles if particles are greater than 2 microns. Nanosight instruments are capable of sizing particles in the nanometer range. If only the particle size distribution is required, laser diffraction can be used for formulations when particles are much smaller in the range of 0.04 to 2,000 microns. For small particles on the order of nanometers dynamic light scattering can be used.

To monitor the thermal stability of vaccines, differential scanning calorimetry (DSC) can be used to find to melting temperature (Tm). A higher melting temperature would be more desirable for a formulation. Studies have been conducted to compare melting temperatures of formulations with different excipients with and without adjuvant to determine the formulation with the best thermal stability [139]. In addition, enthalpy of unfolding can also be determined for proteins in which the heat-induced conformational change is reversible (i.e. no aggregation) [183]. Peek et al. employed DSC as a method of looking at thermal transitions of proteins adsorbed to Alhydrogel in the absence and presence of stabilizers. The overall increasing Tm of protein-alhydrogel samples in presence of stabilizers (e.g. sorbitol, caprylate, etc.) indicate that proteins adsorbed to adjuvant are stabilized [139]. In another example, measles vaccine powder was analyzed using DSC where the various energy-related (endotherms and exotherms) transformations were seen such as glass transition (Tg), melting (Tm), and recrystallization [104]. However, a powder form may be quite complex consisting of various additives and excipients, and in such cases it becomes challenging to assign peaks to particular components or events.

Chapter 2

Stabilization of a recombinant ricin toxin A subunit vaccine through lyophilization

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2.1 Abstract

Lyophilization was used to prepare dry, glassy solid vaccine formulations of recombinant ricin toxin A-chain containing suspensions of colloidal aluminum hydroxide adjuvant. Four lyophilized formulations were prepared by using combinations of rapid or slow cooling during lyophilization and one of two buffers, histidine or ammonium acetate. Trehalose was used as the stabilizing excipient. Aggregation of the colloidal aluminum hydroxide suspension was reduced in formulations processed with a rapid cooling rate. Aluminum hydroxide particle size distributions, glass transition temperatures, water contents, and immunogenicities of lyophilized vaccines were independent of incubation time at 40 °C for up to 15 weeks. Mice immunized with reconstituted ricin toxin subunit A (RTA) vaccines produced RTA-specific antibodies and toxin-neutralizing antibodies (TNA) regardless of the length of high temperature vaccine storage or the degree of aluminum adjuvant aggregation

that occurred during lyophilization. In murine studies, lyophilized formulations of vaccines conferred protection against exposure to lethal doses of ricin, even after the lyophilized formulations had been stored at 40 °C for 4 weeks. A corresponding liquid formulation of vaccine stored at 40 °C elicited RTA-specific antibody titers but failed to confer immunity during a ricin challenge.

2.2 Introduction

Protein subunit vaccines, like therapeutic proteins [111, 74, 38], tend to be unstable and readily undergo physical and/or chemical degradation [22, 139, 59]. To slow this degradation, vaccines typically must be kept at low (e.g. subzero) temperatures for their entire shelf lives. The stringent cold-chain requirements of many vaccines thus provide a serious impediment to their use in developing countries or in emergency situations [99, 148]. Excursions from the ideal cold-chain temperature are problematic [113]. For example, low-temperature excursions, which may cause accidental freezing, occur in 75-100% of liquid vaccine formulations during their distribution [113]. Freezing may result in loss of antigenicity [170].

The limitations imposed by cold-chain requirements are especially daunting for vaccines against bioterrorism threats. In contrast to vaccines against common diseases, it is not anticipated that bioterrorism vaccines would be administered routinely to patients. Instead, these vaccines would likely be administered only in the event of an imminent or actual bioterrorism attack. To meet the demands of such an emergency, large quantities of vaccines would need to rapidly be made available. In turn, this implies that stockpiles need to be created and maintained under conditions that preserve vaccine stability and efficacy. Thus, for typical vaccines requiring storage at 2-8 °C or sub-zero temperatures, limits on available refrigerated storage capacity and refrigerated transport systems preclude their effective use.

Proteins are generally observed to be relatively weak antigens, and addition of microparticulate adjuvants to vaccine formulations typically is required for an appropriate immune response [128]. Currently, the only adjuvants that appear in vaccines approved for use in the United States are aluminum hydroxide, aluminum phosphate, and monophosphoryl lipid A adsorbed to aluminum

hydroxide [144].

Lyophilization is used to stabilize therapeutic proteins [30] and potentially may extend the shelf life and thermostability of vaccines as well [180, 35, 71]. In the design of a lyophilized vaccine formulation, a primary objective is to use judiciously-chosen excipients [30] to embed the antigen in a glass whose high viscosity and low water content limit degradation reactions. In the first stage of a lyophilization process, temperature is reduced below the freezing point of a formulation, causing ice to crystallize and the remaining solute phase to become progressively more concentrated (approximately 30-100 fold), and viscous (approximately 10^{15}-fold). Eventually, the glass transition temperature at maximal freeze concentration (Tg') is reached, and the solute phase forms a glass, halting further crystallization of water. During the drying stages of lyophilization, the glass transition temperature of the formulation increases as water is removed. Ideally, at the end of the drying cycle the glass transition temperature is well above room temperature, allowing room-temperature storage while maintaining a low-mobility, glassy state. Commonly used glass-forming excipients include sugars such as sucrose and trehalose [30].

The formulation and lyophilization process must be optimized to confer stability not only to the antigen, but also to the adjuvant(s). Unfortunately, colloidal suspensions of aluminum adjuvant particles are unstable, and freezing-induced concentration of adjuvant suspensions causes aggregation during freeze-thawing [170, 201, 107, 42, 153]. Larger particles are less efficiently internalized by dendritic cells [120] and are expected to produce a weaker immune response [127]. For example, recombinant hepatitis B vaccine formulated with aluminum hydroxide lost immunogenicity when lyophilized, and larger particle sizes produced lower immune responses [53]. During lyophilization, aggregation of colloidal aluminum hydroxide suspensions can be inhibited by reducing the extent of freeze-concentration with formulations that contain high concentrations of glass-forming excipients, and also by limiting the time over which the freeze-concentrated suspensions can aggregate by using rapid cooling procedures to accelerate glass formation [42].

Ricin toxin is a potential bioterrorism agent extracted from castor beans (*Ricinus communis*) [112]. The ricin heterodimer consists of two subunits, RTA and RTB [134, 131]. RTA is an RNA N-

glycosidase that selectively inactivates eukaryotic ribosomes, thereby inhibiting protein synthesis. RTB is a lectin that facilitates ricin attachment and entry into mammalian cells. In humans, ricin exposure via injection, inhalation, and possibly ingestion can be lethal [112, 9].

RiVax is a full-length derivative of RTA with attenuating point mutations at residues Y80 and V76 [167]. A liquid vaccine containing RiVax prepared without adjuvant produced RTA-specific neutralizing antibodies in mice [164, 165], and lyophilized RiVax formulations that were reconstituted with a separate aluminum hydroxide adjuvant suspension protected mice against ricin exposure [166]. However, liquid RiVax vaccine formulations are unstable at elevated temperatures [134, 139, 15]. Previous studies of RiVax conformation in solution over a range of temperatures and pHs [139] and studies with RiVax adsorbed to alum [15] have both shown that the protein undergoes structural changes at a temperature around 40 °C.

We hypothesized that the combination of a lyophilization process with controlled cooling rates and the addition of the glass-forming excipient trehalose to colloidal suspensions of aluminum hydroxide could be used to form ultra-stable lyophilized RiVax vaccine formulations. In addition, we tested the hypothesis that aggregation of aluminum hydroxide suspensions would reduce the potency of RiVax vaccines by manipulating cooling rates to induce different degrees of aluminum hydroxide aggregation. Both hypotheses were tested in a murine model.

2.3 Materials and methods

2.3.1 Materials

High purity α,α-trehalose dihydrate and sulfuric acid were from Mallinckrodt Baker (Phillipsburg, NJ). L-Histidine, ammonium acetate, and bovine serum albumin (BSA) were from Sigma-Aldrich (St. Louis, MO). 2% Alhydrogel® (aluminum hydroxide adjuvant) was from Accurate Chemicals and Scientific Corp (Westbury, NY). 3 mL 13 mm glass lyophilization vials, caps, and seals were from West Pharmaceutical Services (Lititz, PA). Concentrated 10X phosphate buffered saline (PBS) and Tween 20 were from Fischer Scientific (Fair Lawn, NJ). Peroxidase-conjugated

affinipure donkey anti-mouse IgG (H+L) was from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). 3,3',5,5'-tetramentylbenzidine (TMB) was from Thermo Scientific (Rockford, IL).

2.3.2 Preparation of vaccine formulations

RiVax stock was received from the University of Kansas (Lawrence, KS) in 10% sucrose, 10 mM histidine 144 mM sodium chloride pH 6 solution. Stock RiVax was dialyzed overnight with three buffer exchanges into 10 mM histidine or ammonium acetate at pH 6, using a 10,000 MWCO SpectraPor7 Dialysis membrane (Spectrum Laboratories, Rancho Dominguez, CA) and concentrated using a Millipore Amicon Ultra-15 MWCO 10,000 centrifugal filter unit.

RiVax and placebo formulations were prepared with 0.85 or 1.0 mg Al/mL from Alhydrogel®, 0, 4, 8 or 12 w/v% trehalose and 0.2 or 0 mg/mL RiVax in 10 mM histidine or ammonium acetate buffer, pH 6. Vaccine formulations used for the stability study contained 0.85 mg Al/mL since this is the maximum allowable limit for aluminum in vaccines in the US [182]. Placebo formulations used for measuring particle size distribution of aluminum hydroxide with varying trehalose concentration used 1.0 mg Al/mL. Histidine buffer was chosen since it was shown previously to stabilize RiVax [139]. Ammonium acetate buffer was chosen because it is volatile and hence sublimes during the lyophilization process [67], reducing the tonicity of reconstituted formulations. In principle, higher concentrations of glass-forming excipients could thus be added to volatile buffer-containing formulations while still maintaining desired tonicity. Formulations were stirred at 2-8 °C for 1 hour, after which time the amount of RiVax protein adsorbed to Alhydrogel® was determined by centrifuging samples containing 0.5 mL vaccine formulation for 30 seconds at 14,500g in order to sediment Alhydrogel® particles with adsorbed RiVax protein. Protein remaining in the supernatant was measured by absorbance at 280 nm, and the protein adsorbed to Alhydrogel® was calculated by the difference. In each of the formulations tested, the 1 hour mixing time was sufficient for approximately 50% of the RiVax to adsorb to the adjuvant.

2.3.3 Lyophilization

Lyophilization vials were filled with 1 mL of formulation. Vials were cooled at one of two rates. For rapid cooling, vials were placed on lyophilizer shelves pre-cooled to -10 °C (FTS Systems Lyophilizer, Warminster, PA). Shelf temperatures were decreased at a rate of 0.5 °C/minute to -40 °C and then held at -40 °C for 1 hour. For slow cooling, vials were placed on room temperature lyophilizer shelves, cooled to 0 °C, held at 0 °C for 1 hour, cooled to -40 °C at a rate of 0.5 °C/minute and then held at -40 °C for 1 hour. To minimize radiation and edge vial effects, sample vials were surrounded with "dummy" vials. Primary and secondary drying was conducted as previously described [42]. After drying the chamber was backfilled with nitrogen until atmospheric pressure was achieved. Rubber stoppers were inserted under nitrogen atmosphere, and the vials were sealed with aluminum caps and stored at -80 °C until use.

2.3.4 Stability study

Lyophilized vaccine samples were used immediately after being removed from -80 °C storage (denoted as "Time 0" samples) or placed in a 40 °C incubator for accelerated degradation studies. Formulations subjected to accelerated degradation conditions were removed after incubation at 40 °C for 1 week, 4 weeks, 8 weeks, or 15 weeks, and then stored at -80 °C prior to administration to mice or further analysis.

2.3.5 Particle sizing

Laser diffraction particle size analysis (LS 230, Beckman Coulter, Miami, FL) was performed on the initial liquid suspensions of aluminum hydroxide and lyophilized formulations reconstituted in 1 mL of 0.22 μm-filtered DI water. Previous studies showed no difference in particle size distributions between formulations with and without protein (data not shown) so no protein samples were used in the size analysis. The optical model used for calculating particle size distributions used a solution refractive index of 1.33 and a sample refractive index of 1.57 [61, 198]. Approximately 6 mL of sample was required. For each run, laser diffraction intensities were recorded three times for

90-seconds each and averaged. Each formulation was run in triplicate.

Microflow image analysis (FlowCAM, Fluid Imaging Technologies, Yarmouth, ME) was used for additional particle size characterization to visualize particles 2-2,000 μm. 0.1 mL of vaccine formulation was analyzed using a 100-μm flow cell with 10x objective and collimator. Dark and light settings of 17 and 20 were used, respectively.

2.3.6 Differential scanning calorimetry

Glass transition temperatures of lyophilized samples were determined using differential scanning calorimetry (Diamond DSC, Perkin Elmer, Waltham, MA). Triplicate samples were prepared inside an aluminum pan under dry nitrogen. Pans were cycled twice between 25 °C and 150 °C at a scan rate of 100 °C/minute. The second heating scan was used to determine the onset glass transition temperature.

2.3.7 Moisture content

Residual moisture in lyophilized vaccines was determined by Karl Fischer analysis (DL 37 coulometer, Mettler, Columbus, OH). Dimethylformamide with known moisture content was used to reconstitute the lyophilized vaccine. The total water present in the sample was determined in triplicate using pyridine-free vessel solution (PhotoVolt, Minneapolis, MN).

2.3.8 Murine immunization studies to assess immunogenicity of vaccine formulations

Murine studies were conducted under University of Colorado at Boulder *Institutional Animal Care and Use Committee* (IACUC) protocol #1103.07. Female Swiss Webster mice 5-6 weeks old were from Taconic (Hudson, NY) and allowed to acclimate for at least 1 week. Mice were housed five per cage and were allowed food and water ad libitum. Mice (10 per group) were injected subcutaneously on Days 0 and 21 with 50 μL of various vaccine formulations, each containing 0 or 10 μg RiVax. Blood was collected under isofluorane anesthesia on Days 0, 21, and 35 via the retro

orbital cavity. Serum was separated by centrifugation at 11,000 rpm for 14 minutes at 4 °C and stored at -80 °C until use.

2.3.9 Total antibody enzyme linked immunosorbent assay (ELISA)

Nunc MaxiSorb 96-well plates (Thermo Fischer Scientific, Rochester, NY) were coated with 50 μL/well of 1 μg RiVax/mL diluted in PBS and incubated at 2-8 °C overnight. Plates were washed four times with PBS containing 0.05% Tween 20. Plates were blocked with 300 μL/well of PBS with 1% BSA, incubated at room temperature for 2 hours, and washed again. Serum was initially diluted in PBS with 1% BSA, 0.05% Tween 20, either 800-fold for serum collected on days 0 and 21, or 10,000-fold for serum collected on Day 35. A series of in-plate 2.33-fold dilutions was made for each sample. Plates were incubated for 2 hours at room temperature and washed. 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 with shaking, followed by washing. 40 μL TMB was added to each well and incubated for 30 minutes, followed by quenching with 40 μL of 2N sulfuric acid. Plates were read at 450 nm on a Molecular Devices Kinetic Microplate Reader (Sunnyvale, CA).

To determine titers, average OD 450 values as a function of dilution were fit to a 4-parameter logistic equation using SigmaPlot software. The constraints 0 <min <0.15 and max <3.3 were used. The cutoff value used was 0.25, which was at least 2.5 times the highest concentration of mouse serum dilution of each day 0 group average.

2.3.10 Vero cell cytotoxicity assay

Vero cells were cultured in Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum and were maintained in a humidified incubator (37 °C, 5% CO_2). For cytotoxicity assays, the cells were trypsinized, adjusted to approximately 0.5-1.0 $\times$ 10^5 cells/mL, seeded (100 μl/well) onto white 96-well plates (Corning Life Sciences, Tewksbury, MA), and allowed to adhere overnight. Vero cells were then treated with either ricin at 10 ng/mL, ricin-serum antibody mixtures or medium

alone for 2 hours at 37 °C. The cells were washed and then incubated for 40 hours. Cell viability was assessed using CellTiter-Glo reagent (Promega, Madison, WI) according to the manufacturer's instructions, except that the reagent was diluted 1:5 in PBS prior to use. Luminescence was measured with a SpectraMax L luminometer (Molecular Devices, Sunnyvale, CA). All treatments were performed in triplicate, and 100% viability was defined as the average value obtained from wells where cells were treated with medium only. The neutralizing titer is defined as the dilution of mouse serum that inhibited ricin cytotoxicity in 50% of ricin treated cells (IC_{50}).

2.3.11 Ricin challenge study

Ricin challenge studies were conducted at the Wadsworth Center (Albany, NY) under Wadsworth Center's IACUC guidelines and protocol 10-384. Mice were vaccinated as described above and on day 49 mice were injected intraperitoneally with 100 ng/g of ricin diluted in PBS. Thereafter, the animals were allowed food and water ad libitum. Blood (<5 μl) was collected from the tail veins of the animals at 24 hour intervals to measure blood glucose levels with an Accu-Chek Aviva handheld blood glucose meter (Roche, Indianapolis, IN). Mice were euthanized when they became overtly moribund and/or when blood glucose levels fell below 25 mg/dL. For statistical purposes, readings at or below the meter's limit of detection of ∼20 mg/dL were set to that value.

2.4 Results and discussion

2.4.1 Aggregation of aluminum hydroxide during lyophilization

Initial experiments were used to examine the effects of various concentrations of the glass-forming excipient trehalose on the aggregation of aluminum hydroxide suspensions during lyophilization. Prior to lyophilization, surface area-weighted particle size distributions (SA-PSDs) for aluminum hydroxide suspensions in formulations without added antigen showed two surface-area weighted populations, one with a size of about about 100 nm, and a secondary population with a size of about 1-2 μm (Figure 2.1), similar to observations from earlier studies [42]. According to

previous literature, Alhydrogel® consists of primary needle-like particles with diameters of about 2 nm [90]. These particles from stable aggregates with diameters of 1-5 μm in suspension in the as-recieved Alhydrogel® suspensions [90]. After lyophilization and reconstitution, aggregation of the aluminum hydroxide suspensions was evident in SA-PSDs for samples containing 0, 4, or 8% trehalose that had been cooled slowly. These SA-PSDs were shifted to larger sizes, with the main peak at roughly 10 μm. In contrast, minimal SA-PSD shifts were seen in slowly cooled samples containing 12% trehalose. Most likely, formulations with lower concentrations of the glass-forming excipient trehalose experience more concentration during freezing, which contributed to the observed increases in aggregation of aluminum hydroxide particles in these samples.

In samples that were cooled rapidly, shifts of SA-PSDs to larger sizes occurred only in samples containing the lowest levels of trehalose (0 or 4%). Thus, aggregation of aluminum hydroxide suspensions could be inhibited either by increasing the concentration of the glass-forming excipient trehalose, or by increasing the rate of glass formation by cooling rapidly [42]. In all cases, little difference was observed between samples buffered with histidine or volatile ammonium acetate (Figure 2.1). In liquid formulations prior to lyophilization, and in reconsituted formulations that had been lyophilized using rapid cooling, 90% of the total adjuvant surface area came from particles of size less than 1.5 μm equivalent spherical diameter. In contrast, larger particles contribute much more to the total surface area in formulations lyophilized with slow cooling, with 90% of the surface area associated with particles less than 18.5 μm in equivalent spherical diameter.

For subsequent studies, formulations were prepared using 8% trehalose, and adjuvant particles were sized, counted, and microscopically imaged using the FlowCAM instrument. This allowed samples with two different particle size distributions to be prepared based on the cooling rate applied during lyophilization. In FlowCAM analysis of samples prior to lyophilization, very few particles >2 μm could be detected (Figure 2.2). After reconstitution, samples that had been lyophilized with rapid cooling showed large numbers (approximately 2×10^6/ml) of particles with an average equivalent spherical diameter of approximately 4 μm. Slowly-cooled samples showed even larger numbers (approximately 4-9$\times10^6$/ml) of even larger particles with an average equivalent

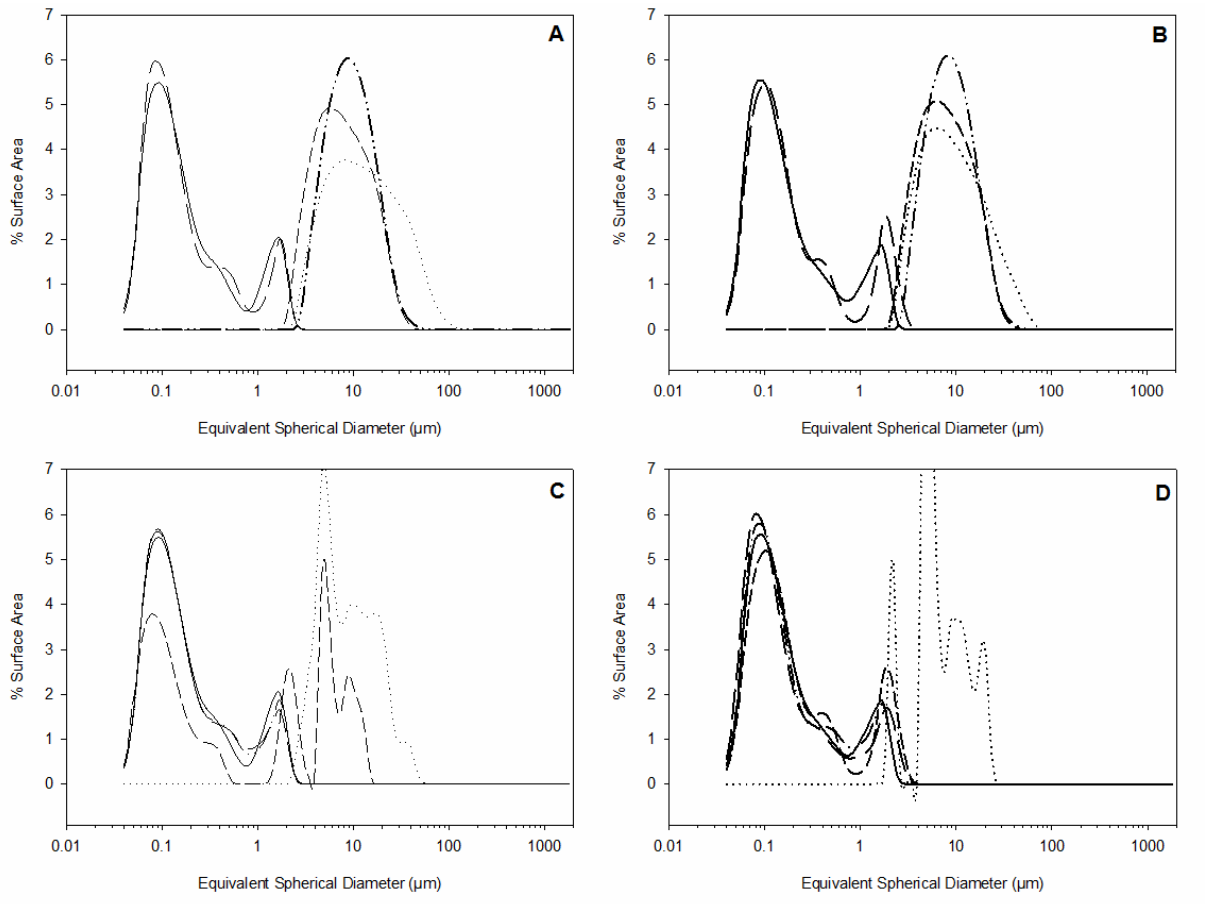

Figure 2.1: Aluminum hydroxide particle size distributions. Lyophilization with rapid cooling and higher trehalose concentrations produce aluminum hydroxide particle size distributions measured after lyophilization and reconstitution that are more similar to the initial liquid particle size distribution (——). Formulations consist of 1 mg Al/mL, 0 (· · · · ·), 4 (– – – –), 8 (– · · – · ·), or 12 (— — —) w/v% trehalose in 10 mM histidine or ammonium acetate buffer at pH 6. (A) lyophilization with slow cooling in histidine buffer; (B) lyophilization with slow cooling in ammonium acetate buffer; (C) lyophilization with rapid cooling in histidine buffer; and (D) lyophilization with rapid cooling in ammonium acetate buffer

Table 2.1: Onset glass transition temperature and water content of placebo vaccines lyophilized with slow cooling in histidine buffer after storage at 40 °C for various periods of time.

Time Stored at 40 °C	Onset Glass Transition Temperature (°C)	Water Content (%)
No Storage Time	107.7 ± 1.7	0.29 ± 0.02
1 Week	112.4 ± 1.5	0.45 ± 0.02
4 Weeks	111.9 ± 1.6	0.67 ± 0.25
8 Weeks	113.0 ± 4.7	0.63 ± 0.05
15 Weeks	110.1 ± 3.2	0.64 ± 0.02

spherical diameter of approximately 6 μm.

2.4.2 Physical stability of lyophilized formulations at elevated temperatures

For storage stability of lyophilized formulations, it is important that the glass transition temperature is well above the storage temperature. Water is a potent plasticizer, and even minute amounts of water may dramatically lower glass transition temperature, and cause cake collapse and vaccine degradation. The physical stability of the lyophilized cakes was assessed by visual appearance, glass transition temperature, and water content. Even after 15 weeks of incubation at 40 °C, there were no visual signs of cake collapse. The onset glass transition temperature was approximately 110 °C and remained constant over the storage time (Table 2.1), suggesting that the lyophilized vaccines were stored in a glassy state and no water entered the cake over time (e.g. from the stopper) [141]. The onset glass transition temperatures were very similar to that of pure trehalose (110-120 °C) [132]. The initial water content of the vaccines was less than 1% wt/wt and remained below this value throughout the storage period (Table 2.1). Laser diffraction and FlowCAM analysis of particle size in lyophilized samples reconstituted after up to 15 weeks of incubation at 40 °C showed no change compared to samples analyzed immediately after lyophilization (data not shown).

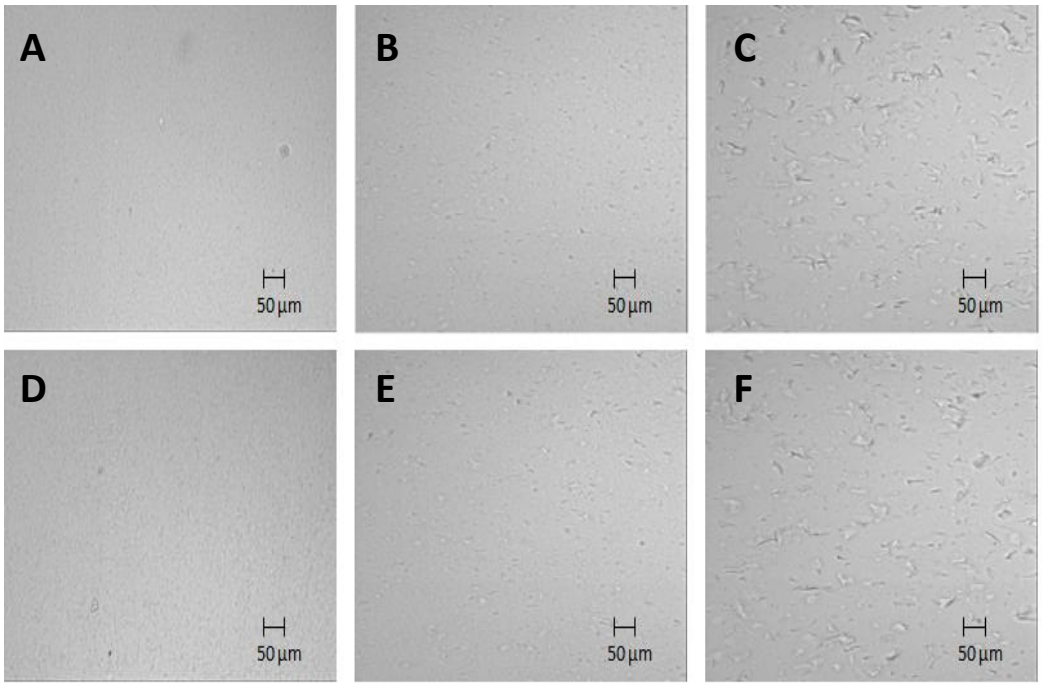

Figure 2.2: Aluminum hydroxide particle images. FlowCAM images of undiluted placebo formulation before lyophilization in histidine buffer (A) and ammonium acetate buffer (D), after reconstitution of placebo vaccine lyophilized with rapid cooling in histidine buffer (B) and ammonium acetate buffer (E), after reconstitution of placebo vaccine lyophilized with slow cooling in histidine buffer (C) and in ammonium acetate buffer (F). All formulations contain 0.85 mg Al/mL with 8w/v% trehalose in 10 mM buffer pH 6.

2.4.3 Immunogenicity of lyophilized RiVax vaccines after high temperature storage

Immunization of mice with liquid vaccine formulations of RiVax produced RTA-specific antibody titers of approximately 3×10^4 three weeks after the first injection, and 9×10^5 two weeks after a booster dose. Groups of mice injected with liquid RiVax or lyophilized RiVax vaccines had response rates of 80-100% and 100% after one and two injections, respectively (Figure 2.3A). No significant differences in endpoint RTA-specific antibody titers were detected between mice immunized and boosted with any of the vaccines (liquid or lyophilized) that had not been subjected to high temperature storage, based on a one way ANOVA on ranks test ($p=0.112$). The reciprocal endpoint antibody titer responses to any of the four lyophilized vaccines that had been stored for 15 weeks at 40 °C were not significantly different based on Mann-Whitney Rank Sum Test ($p>0.05$) from the response to the un-stored lyophilized vaccine of the same group. No differences in response were detected between groups immunized with lyophilized vaccines containing histidine or ammonium acetate buffers based on Mann-Whitney Rank Sum Test ($p=0.182$). When lyophilized vaccines were compared by buffer group over incubation time, significant differences were not detected between "small" and "large" particles based on the Mann-Whitney Rank Sum Test or t-test for normally distributed groups, except for the histidine-containing lyophilized vaccines after 15 weeks of incubation ($p=0.022$) and ammonium acetate-containing lyophilized vaccines after 4 weeks of 40 °C incubation ($p=0.002$). In both of these cases, the lyophilized vaccines that exhibited less aggregation of aluminum hydroxide particles (those made with rapid cooling) were slightly more immunogenic. An explanation for the minimal dependency of immune response on particle size is that, even when aggregation occurred, the majority of the aluminum hydroxide particles were still smaller than the upper size limit (c.a. 10 μm) for phagocytosis by macrophage cells [58] and dendritic cells [120]. A similar lack of dependency of immune response on adjuvant particle size was reported previously for lyophilized lysozyme and alkaline phosphatase vaccines containing aluminum hydroxide or aluminum phosphate particles with average sizes ranging from 1 to 17 μm [40, 43].

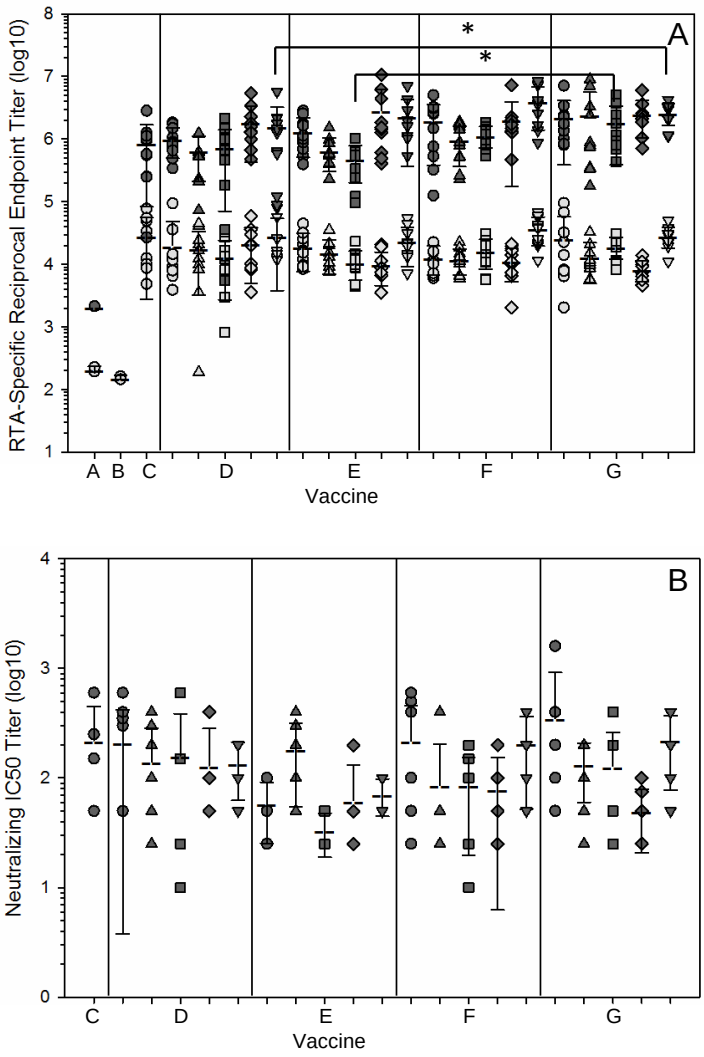

Figure 2.3: RTA-specific and neutralizing antibody titers. (A) RTA-specific antibody titers after one injection (light gray) and after two injections (dark gray) for each vaccine after no high temperature storage (○), 1 week (△), 4 weeks (□), 8 weeks (◊) and 15 weeks (▽) of incubation at 40 °C. Average titers are shown as the average of only the mice that responded with the standard deviation of those mice. Significant differences ($p<0.05$) between groups when comparing the lyophilization method with the same storage condition and buffer is shown by $*$. (B) Toxin neutralizing titer after 2 injections for vaccines with no high temperature storage (○) and vaccines stored at 40 °C for 1 week (△), 4 weeks (□), 8 weeks (◊) and 15 weeks (▽). Vaccine groups: Negative control vaccine without antigen lyophilized with slow cooling in histidine buffer (A), Negative control vaccine without antigen lyophilized with slow cooling in ammonium acetate buffer (B), Positive control freshly prepared liquid RiVax vaccine in histidine buffer (C), RiVax vaccine lyophilized with slow cooling in histidine buffer (D), RiVax vaccine lyophilized with slow cooling ammonium acetate buffer (E), RiVax vaccine lyophilized with rapid cooling in histidine buffer (F), and RiVax vaccine lyophilized with rapid cooling in ammonium acetate buffer (G).

Neutralizing antibody titers were measured in serum two weeks after administration of a booster dose. All liquid and lyophilized vaccine groups, regardless of formulation buffer or particle size, produced neutralizing antibody titers that were not significantly different between groups based on a one way ANOVA on ranks test ($p=0.310$). Neutralizing titer responses to lyophilized vaccines did not decrease over incubation time at 40 °C based on Mann-Whitney Rank Sum Test ($p>0.05$) (Figure 2.3B).

To determine whether the lyophilized vaccines were able to elicit protective immunity, mice were subjected to a prime-boost regimen as described above and then 28 days later challenged with an intraperitoneal injection of ricin. Hypoglycemia was used as a quantitative measure of ricin intoxication [142]. Before the ricin challenge, blood glucose levels were similar in all groups of mice based on one way ANOVA ($p=0.502$) (Table 2.2). Following the ricin challenge, naive mice experienced a rapid drop in blood glucose levels and expired within 24 hours (Figure 2.4). In contrast, mice immunized with a freshly prepared liquid version of the vaccine experienced a slight reduction in blood glucose levels but survived the ricin challenge. There was no statistically significant difference ($p>0.05$) based on one way ANOVA in the blood glucose levels over time for mice immunized with liquid vaccine that was not subjected to high temperature storage, lyophilized vaccine formed by slow cooling in ammonium acetate buffer, or lyophilized vaccine formed by rapid cooling in histidine buffer. There were statistically significant differences in the blood glucose levels between time 0 and 24 hours for mice that recieved lyophilized vaccine formed by slow cooling in histidine buffer ($p=0.029$), lyophilized vaccine formed by rapid cooling in ammonium acetate buffer ($p<0.05$), and liquid vaccine that had been stored at 40 °C ($p<0.001$). Mice immunized with the lyophilized vaccines that had been stored for 4 weeks at 40 °C were protected (80-100%) against the ricin challenge, although the animals experienced a transient reduction in blood glucose levels. On the other hand, only 30% of mice immunized with the stored liquid vaccine survived the ricin challenge. This was consistent with results for titers where mice immunized with the liquid vaccine stored at 40 °C decreased by 47% and 11% for antibody and neutralizing titers respectively from mice administered with the original liquid vaccine. When bound to aluminum hydroxide, RiVax

undergoes changes in tertiary structure at 40 °C as seen by a red shift in fluorescence peak position [15]. Likewise, protein aggregation, secondary, and tertiary structural changes are observed in aqueous solutions of RiVax at 40 °C [139]. These conformational changes in the protein structure most likely result in loss of epitopes critical to inducing rRTA-specific and neutralizing antibodies as well as a protective immune response. Since both the liquid and lyophilized heat-stressed vaccines were identical formulations with respect to excipients, antigen, and adjuvant content, we conclude from these studies that the lyophilized vaccine is more stable than the liquid formulation when stored at high temperatures.

Contrary to our initial expectations, the ability of lyophilized RiVax to protect against ricin challenge was not affected by the degree of aggregation of colloidal aluminum hydroxide. Even after storage for 4 weeks at 40 °C, mice immunized with lyophilized vaccines containing aluminum hydroxide with larger particle sizes (slow-cooled lyophilization process) and smaller particle sizes (rapidly-cooled lyophilization process) showed equivalent blood glucose profiles and ricin challenge survival rates. All four lyophilized formulations were equally effective in terms of generation of rRTA-specific antibodies, neutralizing antibodies, and a protective response against challenge by ricin toxin.

2.5 Conclusions

Lyophilization-induced aggregation of colloidal aluminum hydroxide can be controlled by changing the concentration of the glass-forming excipient trehalose, or cooling rate during the process. RTA-specific antibodies and neutralizing antibodies were elicited in immunized mice regardless of whether the aluminum hydroxide aggregated. Antibody responses were not affected by high temperature storage. However, in ricin challenge studies, mice immunized with lyophilized vaccine that had been stored at high temperature were significantly better protected than mice immunized with liquid vaccine that had been incubated at high temperature.

The instability of colloidal suspension of aluminum hydroxide during freezing has discouraged the development of lyophilized, adjuvanted vaccine formulations, despite the advantages of superior

Table 2.2: Blood glucose (BG) levels of mice before and after exposure to lethal doses of ricin. Average values and the range of values for each vaccine group are shown.

Vaccine		Time= 0 hours	Time= 24 hours	Time= 48 hours	Time= 72 hours
Lyophilized with Slow Cooling in Histidine Buffer (4 weeks at 40 °C)	Average (mg/DL)	130.7	108.1	129.7	122.9
	Range of Values	94-151	88-137	95-158	105-139
Lyophilized with Rapid Cooling in Histidine Buffer (4 weeks at 40 °C)	Average (mg/DL)	125.1	100.7	115.7	116.7
	Range of Values	93-152	80-121	33-136	71-147
Lyophilized with Slow Cooling in Ammonium Acetate Buffer (4 weeks at 40 °C)	Average (mg/DL)	118.4	100.0	114.7	114.6
	Range of Values	104-135	76-122	96-139	98-138
Lyophilized with Rapid Cooling in Ammonium Acetate Buffer (4 weeks at 40 °C)	Average (mg/DL)	135.8	111.6	122.6	124.9
	Range of Values	115-160	33-126	105-148	115-138
Liquid in Hisitdine Buffer (No high temperature storage)	Average (mg/DL)	132.4	113.2	129.2	133.1
	Range of Values	103-185	89-139	112-149	111-162
Liquid in Histidine Buffer (3.5 weeks at 40 °C)	Average (mg/DL)	124.4	75.0	71.7	86.0
	Range of Values	98-149	16-97	52-117	50-124
Sham Immunized with PBS	Average (mg/DL)	132.0			
	Range of Values	111-159			

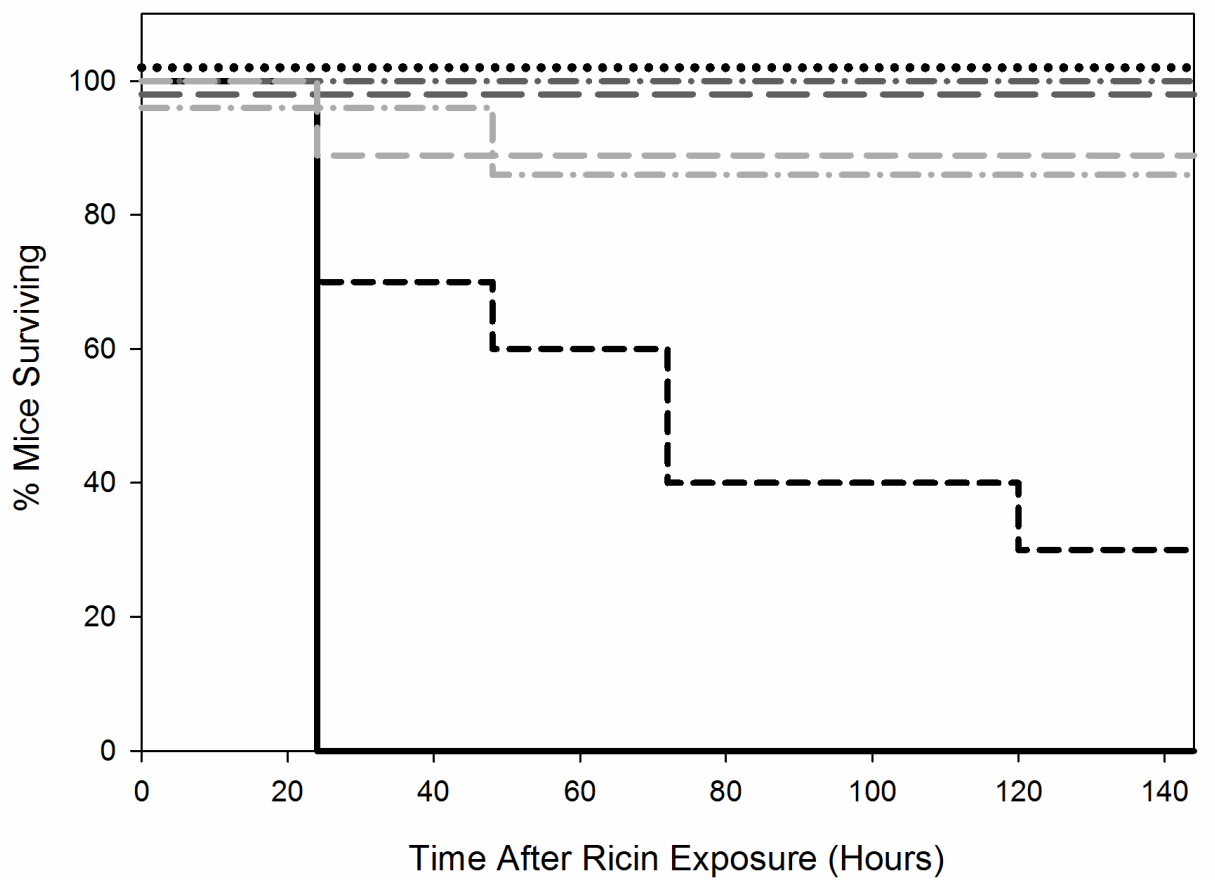

Figure 2.4: Ricin challenge study. Mice immunized with lyophilized or liquid vaccine not exposed to high temperature storage had the highest survival rate during a ricin challenge. Vaccine formulations: No immunization (black ——), liquid vaccine with no high temperature storage (black · · · · ·), liquid vaccine with 3.5 weeks of high temperature storage at 40 °C (black – – – –), RiVax vaccine lyophilized with slow cooling in histidine buffer with high temperature storage for 4 weeks at 40 °C (dark gray – · – ·), RiVax vaccine lyophilized with slow cooling in ammonium acetate buffer with high temperature storage for 4 weeks at 40 °C (dark gray — — —), RiVax vaccine lyophilized with rapid cooling in histidine buffer with high temperature storage for 4 weeks at 40 °C (light gray – · – ·) and RiVax vaccine lyophilized with rapid cooling in ammonium acetate buffer with high temperature storage for 4 weeks at 40 °C (light gray — — —).

thermal stability and reduced cold-chain requirements that such formulations might offer. Currently 67 vaccines are approved by the US Food and Drug Administration. Of these vaccines, 36% contain an aluminum salt adjuvant and 30% are lyophilized, but there are not currently lyophilized vaccines that contain an aluminum adjuvant [63]. The current work demonstrates that, through judicious choice of processing and formulation conditions, the instability of colloidal aluminum hydroxide suspensions can be mitigated, offering the potential for creation of thermally stable lyophilized formulations of vaccines containing aluminum hydroxide adjuvants.

2.6 Acknowledgments

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Chapter 3

Glassy-state stabilization of a dominant negative inhibitor anthrax vaccine containing aluminum hydroxide and glycopyranoside lipid A adjuvants

This chapter will be submitted as K.J. Hassett, D.J. Vance, N.K. Jain, N. Sahni, L.A. Rabia, M.C. Cousins, S. Joshi, D.B. Volkin, C.R. Middaugh, N.J. Mantis, J.F. Carpenter, and T.W. Randolph. "Glassy-state Stabilization of a Dominant Negative Inhibitor Anthrax Vaccine Containing Aluminum Hydroxide and Glycopyranoside Lipid A Adjuvants" to the Journal of Pharmaceutical Sciences.

3.1 Abstract

During transport and storage, vaccines may be exposed to higher or lower temperatures than their recommended storage temperatures, potentially causing losses in vaccine efficacy. Dominant Negative Inhibitor (DNI), a candidate antigen for a vaccine against anthrax, was formulated with the adjuvants aluminum hydroxide and glycopyranoside lipid A (GLA), to study how formulation as a glassy lyophilized powder might prevent these efficacy losses. Freezing and thawing of the vaccine induced aggregation of the adjuvants and decreased its immunogenicity when tested in mice. Liquid formulations of the DNI vaccine lost immunogenicity when stored at 40 °C for 8 weeks, as measured by a decrease in neutralizing antibodies in vaccinated mice. Concomitant with the loss in efficacy, a loss in protein structure was detected by fluorescence spectroscopy after 1 week of storage at 40

°C, and increased deamidation was observed. Lyophilization increased the stability of the vaccine, permitting storage at 40 °C for up to 16 weeks without detectable changes in DNI protein structure, additional deamidation, or decreases in immunogenicity. Compared with vaccines prepared using aluminum hydroxide as the only adjuvant, vaccines containing both aluminum hydroxide and GLA were able to elicit higher immune responses with a greater percentage of mice responding to the vaccine after a single dose.

3.2 Introduction

The recommended storage temperature range for vaccines is typically very narrow [118], and exposure to temperatures either above or below the recommended storage window may damage vaccines. The vast majority, 75-100%, of vaccines are exposed to freezing temperatures during transport through the cold chain [113], which may cause vaccine formulations to experience at least one freeze-thaw cycle. Freeze thawing of vaccines has been shown to cause aggregation of aluminum salt adjuvant particles [101, 35, 153], perturbations in protein antigen structure [35, 170], and losses in immunogenicity [35, 23].

In addition to experiencing inadvertent freeze-thawing, vaccines may also be exposed to elevated temperatures, causing protein antigens to experience physical [83, 186, 84, 98] or chemical [59] degradation, resulting in a loss in vaccine immunogenicity [79, 186]. To study the thermal sensitivity of vaccines, accelerated stability studies are typically conducted at temperatures significantly higher than the recommended storage temperatures. Accelerated stability studies are also commonly used as a predictor of long term stability and shelf life at optimal storage temperatures [77].

Maintaining proper cold-chains is challenging, especially in developing countries. To alleviate this challenge, vaccines should be formulated to withstand a broad range of temperatures. Lyophilization is one strategy that can be applied to protect proteins and other therapeutic agents against temperature extremes, thereby relieving the constraints of the cold chain [30]. Formulation of live, attenuated measles vaccines in dry powders [98] represents an example of this approach.

Degradation in lyophilized formulations is inhibited because of the low water content and high viscosities ($>10^{15}$ centipoise) found in glassy lyophilized formulations [30].

Many vaccines require administration of multiple doses to confer adequate protection. Especially in developing countries, this requirement is problematic, and often patients do not complete multidose regimens [125, 194]. Presumably, better patient compliance would be obtained if vaccines required fewer doses. Adjuvants are often added to vaccines to increase vaccine potency and have the potential to decrease the required number of vaccine doses [128]. Aluminum salts, and aluminum hydroxide combined with monophosphoryl lipid A (MPL) have been approved for use as adjuvants in FDA-approved vaccines [144]. However, no FDA-approved vaccines that contain adjuvants currently are marketed in a lyophilized formulation [63], in part because of the loss of vaccine efficacy that may occur during the requisite freezing step in the lyophilization process. Recent work has shown that by controlling the kinetics of freezing and glass formation through judicious choice of formulation and process conditions, highly stable, efficacious lyophilized vaccines containing aluminum salt adjuvants may be produced [79, 43, 40, 41].

Aluminum salt adjuvants are known to provoke primarily a humoral response. To produce a more robust cellular immune response to a vaccine, other adjuvants typically must be added [46]. One such co-adjuvant is monophosphoryl lipid A (MPL), a non-toxic derivative of lipopolysaccharide (LPS) that can act as a toll-like receptor-4 agonist [46]. Glycopyranoside lipid A (GLA) is a synthetic version of MPL that is more homogenous and active than MPL [46]. To date, there are no reports of commercial lyophilized vaccine formulations that combine both an aluminum hydroxide adjuvant and a cellular immunity stimulant such as GLA.

To examine the possibility of creating stable lyophilized vaccines containing both aluminum hydroxide and GLA, the anthrax vaccine candidate, Dominant Negative Inhibitor (DNI), was used as a model antigen. During an anthrax infection, three individually non-toxic proteins, protective antigen (PA), lethal factor (LF) and edema factor (EF) act together to harm cells. PA forms complexes with LF and EF, allowing the proteins to be transported inside cells. Once inside cells, LF disrupts critical cellular pathways and EF induces cellular swelling. DNI is a recombinant

version of PA that contains the mutations K397D and D425K. These mutations allow DNI to bind LF and EF, but block the entry of the complex into cells [158, 199]. Previous studies have shown DNI to be an effective vaccine antigen [10].

We first hypothesize that both heat and freeze-thaw stresses will damage adjuvanted liquid vaccine formulations of DNI, leading to a loss in protein structure and a decrease in immunogenicity. Second, we propose that glassy-state formulations of DNI-based vaccines will be more robust against these thermal stresses. Finally, we hypothesize that incorporation of the toll-like receptor-4 agonist GLA together with microparticulate aluminum hydroxide in DNI vaccine formulations will confer additional potency, and that this additional functionality can also be protected against thermal stresses through lyophilization.

3.3 Materials and methods

3.3.1 Materials

High purity α,α-trehalose dihydrate and sulfuric acid were purchased from Mallinckrodt Baker (Phillipsburg, NJ). Ammonium acetate, triethanolamine, and bovine serum albumin (BSA) were purchased from Sigma-Aldrich (St. Louis, MO). Two percent Alhydrogel® (aluminum hydroxide adjuvant) was obtained from Accurate Chemicals and Scientific Corp (Westbury, NY). Lyophilized synthetic monophosphoryl lipid A (glycopyranoside Lipid A (GLA) adjuvant) was purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). Three mL 13 mm glass lyophilization vials, caps and seals were from West Pharmaceutical Services (Lititz, PA). Concentrated 10X phosphate buffered saline (PBS), and Tween 20 were from Fischer Scientific (Fair Lawn, NJ). Water for injection was purchased from Baxter Healthcare Corporation (Deerfield, IL). Peroxidase-conjugated affinipure donkey anti-mouse IgG (H+L) was from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). 3,3',5,5'-tetramentylbenzidine (Ultra TMB) was from Thermo Scientific (Rockford, IL).

3.3.2 Vaccine formulation

Dominant negative inhibitor (DNI) protein manufactured by Baxter Pharmaceutical Solutions LLC (Bloomington, IN) was received as a lyophilized formulation containing 25 mg DNI, 113 mg mannitol, 33 mg sucrose, and 2.4 mg dibasic phosphate. Lyophilized DNI was reconstituted in 3 mL of filtered DI water and dialyzed overnight with three buffer exchanges in a 10 mM ammonium acetate buffer pH 7 using 3,500 MWCO Slide-A-Lyzer dialysis cassettes from Thermo Scientific (Rockford, IL).

All vaccines were formulated to contain 10 mM ammonium acetate pH 7 with 0.2 mg/mL DNI and 0.5 mg/mL Al from Alhydrogel. For isotonicity, 9.5 w/v% trehalose was added. In addition to aluminum hydroxide, 0.05 mg/mL GLA was added as a second adjuvant to half of the vaccine formulations. GLA was prepared at 1 mg/mL by suspending lyophilized GLA in a 0.5% triethanolamine pH 7 solution using probe sonication [13]. To create the vaccine formulations containing GLA, suspended GLA was added to Alhydrogel suspensions, vortexed for 5 seconds and then rotated end over end for 30 minutes at 4 °C. 0.2 mg/mL DNI protein antigen was added to buffered adjuvant solutions and rotated end over end for 30 minutes to allow protein to adsorb completely to adjuvant particles.

3.3.3 Protein adsorption

Protein adsorption was measured by mass balance after centrifuging the vaccine formulation at 9,000 x g for 4 minutes at 4 °C to remove particles and adsorbed protein, and measuring the unbound protein concentration in the supernatant through use of the Bradford assay. A standard curve was created using known concentrations of DNI. The amount of protein adsorbed to adjuvant was calculated by subtracting the amount of unbound protein from the known amount of protein in the vaccine.

3.3.4 Lyophilization

Vaccine formulations were lyophilized with 1 mL of formulation per vial. Lyophilizer shelves were pre-cooled to -10 °C (FTS Systems Lyophilizer, Warminster, PA) and vials were placed on the shelves. Vaccine formulations were surrounded by vials filled with DI water to minimize radiative heat transfer effects for vials near the edge of the lyophilizer shelves. The shelf temperature was decreased at a rate of 0.5 °C/min to -40 °C and then held at -40 °C for 1 hour to allow the samples to freeze completely. Primary drying was initiated by decreasing the chamber pressure to 60 mTorr and increasing the shelf temperature to -20 °C at a rate of 2 °C/min. Samples were held at -20 °C for 20 hours. Secondary drying was conducted by increasing the shelf temperature to 0 °C at a rate of 0.2 °C/min followed by an increase to 30 °C at a rate of 0.5 °C/min and holding the shelf temperature at 30 °C for 5 hours. After drying, the shelf temperature was returned to 25 °C and the chamber was back-filled with nitrogen until atmospheric pressure was reached. Chlorobutyl rubber stoppers were inserted into vials under a nitrogen atmosphere. Before storage at -80 °C, vials were sealed with aluminum caps.

3.3.5 Freeze-thaw study

Freeze-thaw stability was examined for liquid vaccine formulations. Formulations were cycled between -20 °C and 4 °C, leaving formulations at each temperature for one day to permit complete freezing or thawing. Vaccines experienced 0, 1, or 5 freeze-thaw cycles.

3.3.6 Elevated temperature incubation study

To test the stability of vaccines at elevated temperatures, liquid and lyophilized vaccines were stored at 4, 40 or 70 °C for 0, 1, 2, 4, 8, or 16 weeks. Time 0 lyophilized vaccines refer to vaccines reconstituted and used immediately after removal from storage at -80 °C.

3.3.7 Particle size analysis

Particle size distributions from 0.04-2,000 μm were measured using laser diffraction particle size analysis (LS 230, Beckman Coulter, Miami, FL). Initial liquid, and reconstituted lyophilized placebo vaccine formulations with and without GLA were measured. For each run, laser diffraction intensities were recorded three times for 90-sec each and averaged. Triplicate samples of each formulation were analyzed.

Particles in the size range of 2-2,000 μm were measured and counted by microflow image analysis (FlowCAM, Fluid Imaging Technologies, Yarmouth, ME). Particle levels in the initial liquid formulations, and in reconstituted formulations of lyophilized vaccines that had been incubated at 40 °C were measured in triplicate. 0.2 mL of samples diluted 10 times were run with a 100-μm flow cell using a 10x objective and collimator. Dark and light settings of 15 and 16 were used, respectively. For freeze-thaw studies, triplicate 1 mL of vaccine formulation diluted 100 times were analyzed with a 300-μm flow cell with 4x objective. Dark and light settings of 20 were used.

3.3.8 Differential interference contrast microscopy

A Zeiss Axiovert 200M widefield microscope was used to take differential interference contrast images of vaccine formulations after 0, 1, or 5 freeze-thaw cycles. A 20x objective was used.

3.3.9 Differential scanning calorimetry (DSC)

Onset glass transition temperatures of placebo lyophilized formulations were obtained using differential scanning calorimetry (Diamond DSC, Perkin Elmer, Waltham, MA). Triplicate samples were prepared inside an aluminum pan under dry nitrogen. Pans were cycled twice between 25 °C and 150 °C at a scan rate of 100 °C/min. The second heating scan was used to determine the onset glass transition temperature.

3.3.10 Fluorescence analysis

Seven hundred μL of vaccine formulations from the freeze-thaw and incubation studies were added to 2 mm pathlength cuvettes. The vaccine formulations were left in the cuvettes overnight at 4 °C to allow settling so that intrinsic fluorescence measurements could be performed in a fluorimeter (Photon Technology International, Birmingham, NJ). For all vaccines, emission spectra were collected from 305-410 nm in 1 nm increments while the temperature was ramped from 10-90 °C in 2.5 °C increments. An equilibration time of 1 min was used at each temperature. Slit widths were set at 3 nm for excitation and emission.

Eighty nine μL of each vaccine formulation and one μL of 350X SYPRO Orange dye (Molecular Probes, Inc., Eugene, OR) were added to PCR tubes. PCR tubes were transferred to a Stratgene RT-PCR instrument (Agilent Technologies, Inc., Santa Clara, CA) and SYPRO Orange fluorescence was measured at 610 nm upon excitation at 492 nm while the temperature was ramped from 25 to 90 °C in 1 °C intervals. An equilibrium time of 90 seconds was used at each temperature. The fluorescence intensity was normalized using a maxima-minima algorithm.

All experiments were performed in duplicate and the signals of the samples were corrected for their respective blanks. The transition temperatures (Tm) were calculated using the second-order derivative of the peak position or SYPRO orange fluorescence intensity versus temperature data. Only the major transition (Tm) was analyzed for vaccine formulations which showed more than one transition. Due to some irreversibility, these values are not thermodynamic Tm, but should be referred to as apparent Tm and used in a comparative manner only.

3.3.11 Deamidation studies

Vaccine formulations subjected to freezing and thawing, and high temperature incubation were tested for deamidation. Each formulation contained 1 mg/mL DNI protein in 10 mM ammonium acetate pH 7 with 9.5 w/v% trehalose. Formulations contained either 0 or 0.5 mg/mL Al. Liquid vaccine formulations from the freeze-thaw study were frozen and thawed in the presence of

aluminum hydroxide adjuvant. Vaccine formulations from the incubation study were in liquid or lyophilized forms during incubation at 40 °C with or without aluminum hydroxide adjuvant.

To desorb protein from aluminum hydroxide adjuvant particles the adjuvant-DNI complexes first were pelleted by centrifugation at 10,000xg for 3 minutes (Sorvall Centrifuge, Thermo Scientific). The supernatant was removed and assayed for protein content by UV-visible absorption spectroscopy. The pellet was resuspended in 1 mL of desorption media (10 mM ammonium acetate, 1 M phosphate and 5 M guanidine hydrochloride). The resulting suspension was incubated at room temperature for 3 hours, followed by centrifugation at 10,000xg for 3 minutes. The supernatant was collected and assayed for DNI content. These steps were repeated 2 more times. The percent desorption was calculated by dividing the total content of protein in the collected supernatant by the initial amount of protein initially bound to the aluminum hydroxide particles. The supernatants were combined and exchanged into 10 mM ammonium acetate, pH 7.0 using Amicon centrifugation filters (10 kDa MWCO) prior to analysis by capillary isoelectric focusing (cIEF).

To characterize charge variants of the DNI protein, which presumably result from deamidation, cIEF experiments were performed on an iCE280 instrument from Convergent Biosciences (Toronto, Canada). All experiments were performed with duplicate samples at 4 °C using a temperature controlled auto-sampler. The final protein concentration used was 0.1 mg/ml. Samples of DNI protein were mixed with Pharmalyte 3.0-10.0 (final concentration of 4%, obtained from GE Healthcare), acidic and basic pI markers of 4.65 and 8.18 (Protein-Simple, Canada), and methyl cellulose (final concentration of 0.35%, Protein-Simple, Canada). 6M urea was added to provide better separation of the charge variants. The optimized separation conditions included pre-focusing at 1500V for 1 minute followed by 8 minutes of focusing at 3000V. Quantification of charge variants was performed using Chrom Perfect software. The number of deamidated residues per molecule was calculated by multiplying the fraction of the total area for each peak by the number of deamidated residues represented by the peak and summing the values.

3.3.12 Vaccine immunogenicity

Murine studies were conducted under the University of Colorado at Boulder *Institutional Animal Care and Use Committee* (IACUC) protocol #1209.02. Female BALB/c mice 5-6 weeks old from Taconic (Hudson, NY) were allowed to acclimate at least one week before use. Ten mice were in each group. Blood samples were collected from the mice under isofluorane anesthesia on days 0, 14 and 28 through the retro orbital cavity. The collected serum was separated by centrifugation at 10,000 rpm for 14 minutes at 4 °C and stored at -80 °C until analysis. On days 0 and 14, mice were injected subcutaneously behind the neck with various formulations. To study the effects of freeze-thawing on the immunogenicity of DNI vaccines, formulations in the presence or absence of GLA were subjected to 1 or 5 freeze thaw cycles prior to administration to mice. To study the effects of incubation of DNI vaccine formulations at elevated temperatures, mice were injected with liquid vaccine formulations as positive controls, placebo lyophilized formulations as negative controls, liquid vaccine formulations that had been stored for 8 weeks at 40 °C, and lyophilized vaccine formulations that had been incubated at 40 °C for 0, 1, 4, 8 and 16 weeks prior to reconstitution.

3.3.13 Total antibody enzyme linked immunosorbent assay (ELISA)

Nunc MaxiSorb 96 well plates (Thermo Fischer Scientific, Rochester, NY) were coated with 50 μL/well of 1 μg/mL DNI diluted in PBS and incubated at 2-8 °C overnight. Plates were washed 3 times with PBS containing 0.05% Tween 20. Plates were blocked with 300 μL/well of PBS with 1% BSA, incubated at room temperature for 2 hours, and washed again. Serum was initially diluted in PBS with 1% BSA, 0.05% Tween 20. 50-fold dilutions were used for serum collected on days 0 and 14, and 750-fold or 250-fold dilutions were used for serum collected on Day 28 for mice injected with or without GLA, respectively. A series of in-plate 2-fold dilutions were made for each sample. Plates were incubated for 1.5 hours at room temperature and washed. 40 μL of HRP-conjugated donkey anti-mouse antibody diluted 10,000 times was added to each well and incubated for 1.5 hours at room temperature with shaking, followed by washing. 40 μL TMB was added to each well

and incubated for 15 minutes, followed by quenching with 40 μL of 2N sulfuric acid. Plates were measured at 450 nm on a Molecular Devices Kinetic Microplate Reader (Sunnyvale, CA). Each serum sample was analyzed in triplicate.

To determine titers, average OD 450 values as a function of dilution were fit to a 4-parameter logistic equation using SigmaPlot software (Systat Software Inc., San Jose, CA). The constraints 0 <min <0.15 and max <3.3 were used. The cutoff value was calculated individually for each mouse as five times the value given on day 0 at a dilution of 100. The evaluate statistically significant differences between groups, a t-test was used for normally distributed groups and a Mann-Whitney Rank Sum Test on non-normally distributed groups.

3.3.14 Neutralizing antibodies

J774 cells grown in DMEM plus 10% FBS were seeded (5x10^3 per well) in white 96 well cell culture plates and incubated at 37 °C overnight. Serum samples were mixed at a 1:100 dilution into media containing lethal toxin (300 ng/mL, 1:1 PA:LF), then diluted two-fold in a separate dilution plate into toxin-containing media, down to a 1:12,800 dilution. The media was removed from the cell wells, and toxin-serum mixtures were transferred into them and incubated for 24 hours at 37 °C. Some cells received media or toxin-containing media only, and served as live and dead controls, respectively. Cell viability was assessed using Cell Titer Glo (Promega, Madison, WI) and a Spectramax L Luminometer (Molecular Devices, Sunnyvale, CA). Neutralizing titers were defined as the inverse titer that protected at least 50% of the cells from lethal toxin.

3.4 Results

3.4.1 Freeze thaw studies - Vaccine characterization

Initially, all liquid vaccine formulations appeared identical based on differential interference contrast microscopy regardless of adjuvant present (Figure 3.1). After one freeze-thaw cycle loose clumping of adjuvant particles was observed. After five freeze-thaw cycles, large particles (>10

μm) were seen in all formulations, irrespective of the presence or absence of GLA.

The concentration of particles of size greater than 5 microns in each formulation was measured using a FlowCAM microflow imaging instrument (Figure 3.2). All vaccine formulations started with particles of similar mean particle diameters ($\sim$7-10 μm) and concentrations ($\sim$1 million particles/mL). After one freeze-thaw cycle, a small increase in mean particle size (to $\sim$12 μm) was observed. After five freeze-thaw cycles, the mean particle size in each of the formulations was approximately 20 μm. Concomitant with the formation of larger particles, there were decreases in the number of smaller particles found in the formulations.

Regardless of the number of freeze-thaw cycles, DNI was completely adsorbed to adjuvant in all formulations, both initially and after 1 or 5 freeze-thaw cycles. After formulations containing DNI adsorbed to aluminum hydroxide particles were pelleted by centrifugation and resuspended in PBS for 1 hour at 37 °C, $\sim$20% of the DNI desorbed.

3.4.2 Effect of freeze-thawing on antigen structure

Intrinsic tryptophan and extrinsic SYPRO Orange fluorescence studies were conducted to examine protein structure after 0, 1, and 5 freeze-thaw cycles. All formulations, regardless of the number of freeze-thaw cycles, or the presence of adjuvants, exhibited thermal transitions at approximately 45 °C. No increases in deamidation were detected after 1 or 5 freeze thaw cycles in vaccine formulations.

3.4.3 Freeze thaw studies - Immunogenicity

Liquid formulations were subjected to a 0, 1 or 5 freeze-thaw cycles and injected into mice. All mice responded with anti-DNI antibodies after two injections of the vaccine regardless of the number of freeze-thaw cycles, but more non-responders were seen after one injection of the Alum+DNI formulation that had been subjected to five freeze-thaw cycles. After one injection, a significant decrease in titer was seen for both the Alum+DNI and Alum+GLA+DNI vaccines exposed to 5 freeze-thaw cycles when compared to vaccines not exposed to freezing and thawing (p=0.007 and

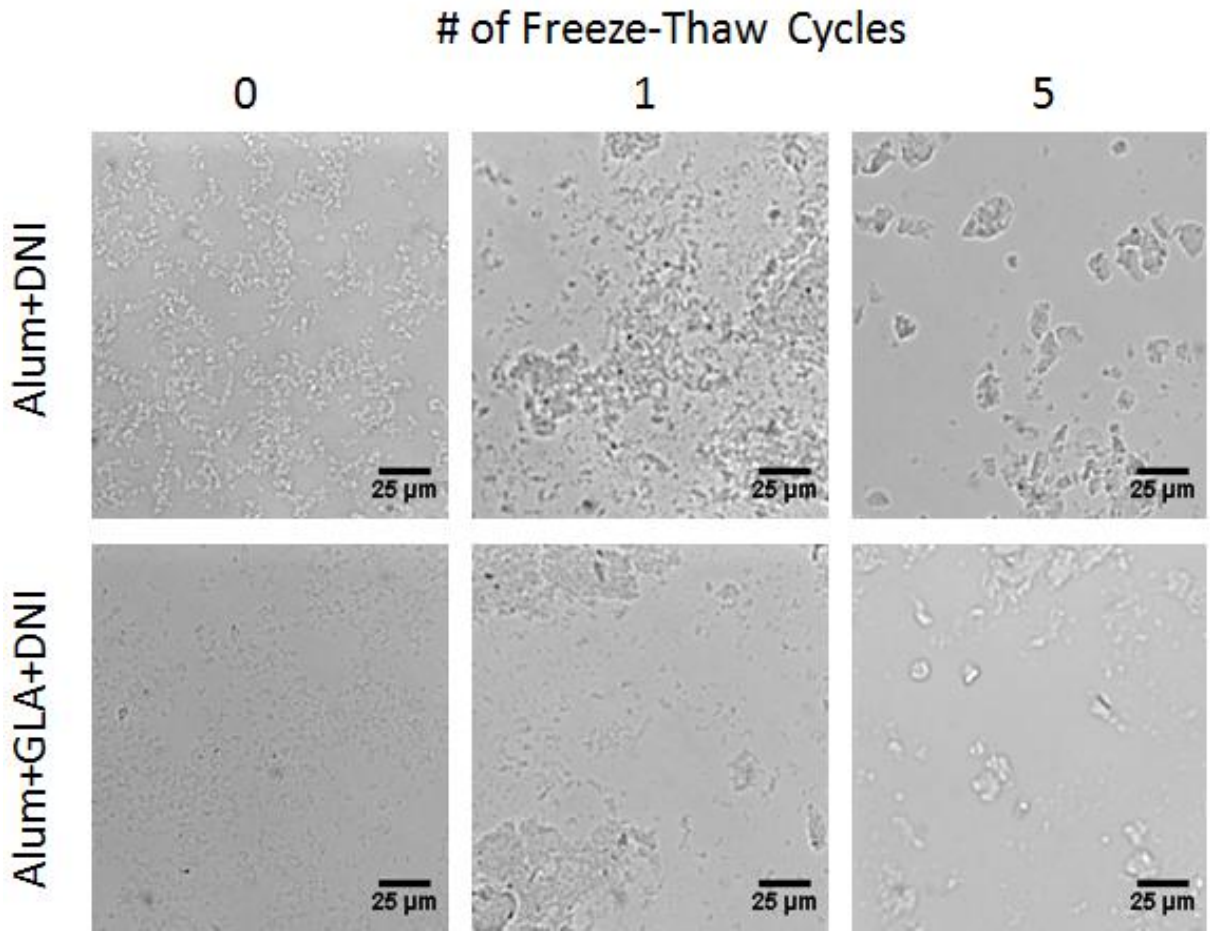

Figure 3.1: Aluminum hydroxide adjuvant particles aggregate during freezing and thawing as seen by differential interference contrast microscopy images after 0, 1, and 5 freeze-thaw cycles. More particle aggregation is observed with increasing the number of freeze-thaw cycles.

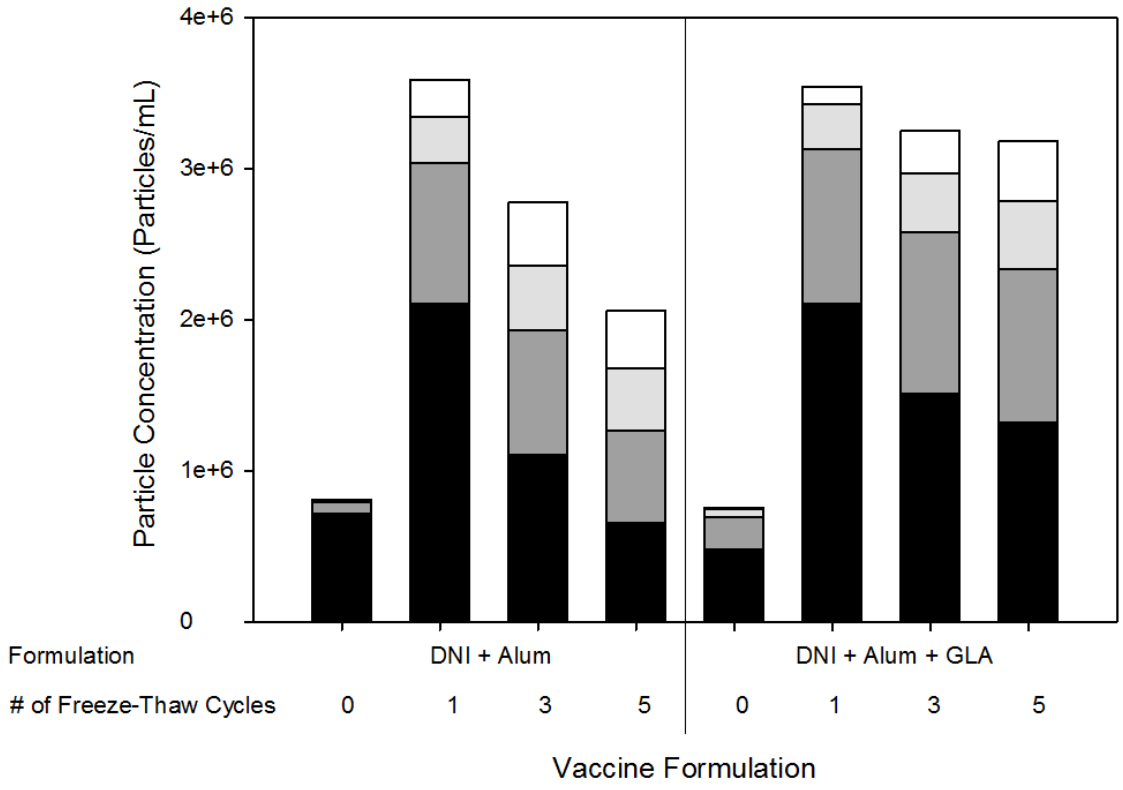

Figure 3.2: Aluminum hydroxide particle size and concentration after 0, 1, 3, and 5 freeze-thaw cycles. After more freeze-thaw cycles occur, a decrease in 2-5 μm particles is detected and an increase in larger particles is seen. Particles 5-10 μm (black), 10-20 μm (dark gray), 20-30 μm (light gray), 30+ μm (white).

p=0.011 respectively) (Figure 3.3A). Neutralizing titers did not show a significant decrease in titer from the initial liquid vaccine for any of the formulations (Figure 3.3B).

3.4.4 Elevated temperature studies - Vaccine characterization

To ensure that lyophilized vaccines were stored in a glassy state, glass transition temperatures were measured in lyophilized placebo formulations (without protein). The glass transition temperature for placebo vaccine formulations without and with GLA were 115.5 ± 1.6 °C and 117.3 ± 3.8 °C, respectively. The glass transition temperatures of the formulations were very similar to that of pure trehalose, 110-120 °C [132], showing that the water content of the formulations was minimal. If water were to be present in the formulation, the glass transition temperature would be drastically reduced as water acts as a potent plasticizer [132]. Since the glass transition temperatures were significantly higher than the storage temperatures (4, 40 or 70 °C), the lyophilized vaccine formulations remained in a glassy state during storage.

Previous work showed that formulations containing aluminum salt adjuvants can be lyophilized and reconstituted without significant changes to the initial liquid particle size distribution, provided that sufficient amounts of trehalose are used in combination with rapid cooling methods before lyophilization [42, 79]. Particle size distributions were determined for initial liquid and reconstituted lyophilized placebo formulations with and without GLA (Figure 3.4). Vaccine formulations without GLA initially had similar particle size distributions and after lyophilization and reconstitution, whereas vaccine formulations containing GLA exhibited slight increases in particle size after lyophilization and reconstitution.

For each lyophilized and reconstituted formulation, a significant increase compared to the initial liquid formulations in the number of particles greater than 2 microns in size was observed by FlowCAM analysis (Figure 3.5). A greater number of particles were detected in formulations containing GLA. Even more particles were found when DNI was added to the formulation. After the initial increase in particles following lyophilization, no further increase in particle counts could be detected after incubation at 40 °C for up to 16 weeks.

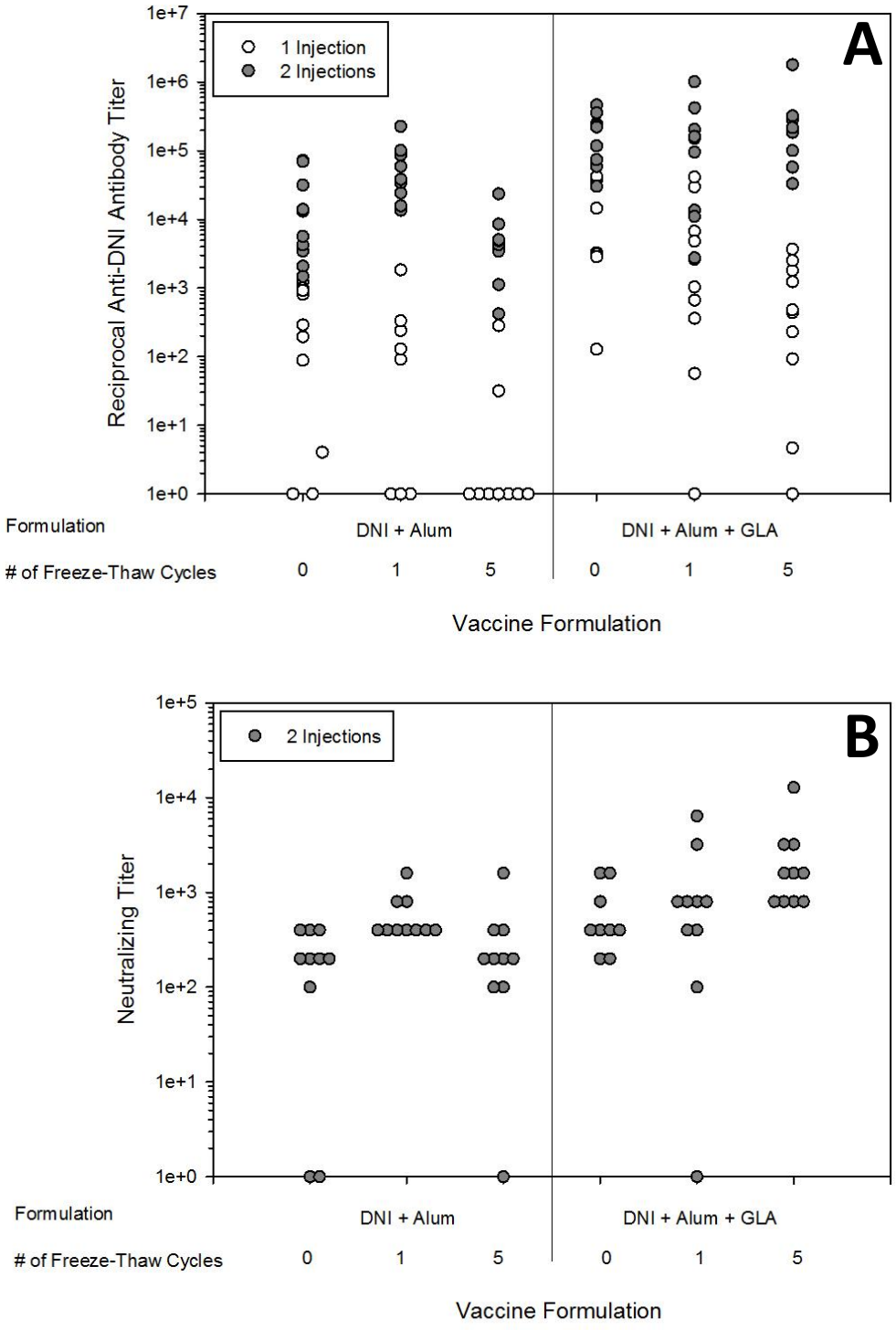

Figure 3.3: Total anti-DNI antibody titers (A) and neutralizing antibody titers (B) after one vaccine injection (white circles) and after two vaccine injections (gray circles) for liquid vaccine after 0, 1, and 5 freeze-thaw cycles. Reduced immunogenicity is detected with 5 freeze-thaw cycles after one injection.

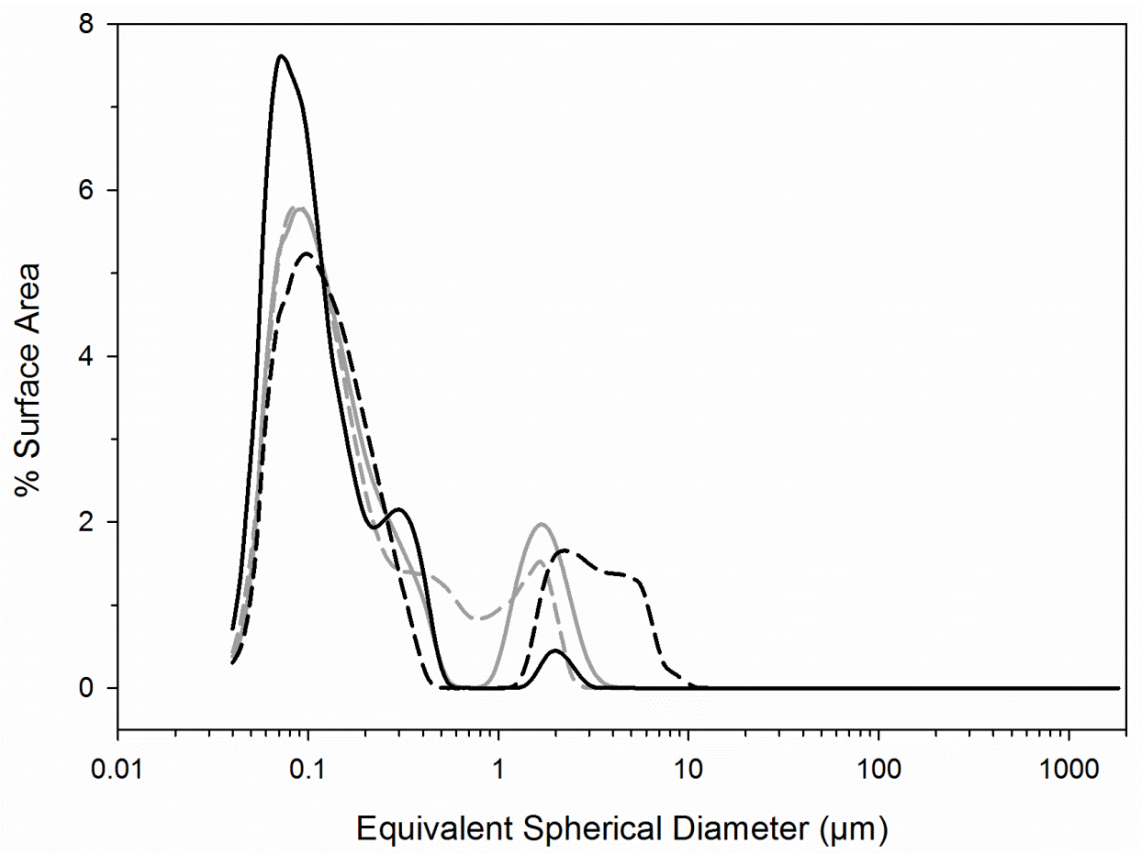

Figure 3.4: Particle size distributions of placebo vaccine (gray) and placebo vaccine with GLA (black) before (solid line) and after lyophilization and reconstitution (dashed line). Initial liquid particle size distributions are very similar for both formulations. After lyophilization and reconstitution, only a slight increase in particle size distribution is seen in the formulation containing GLA.

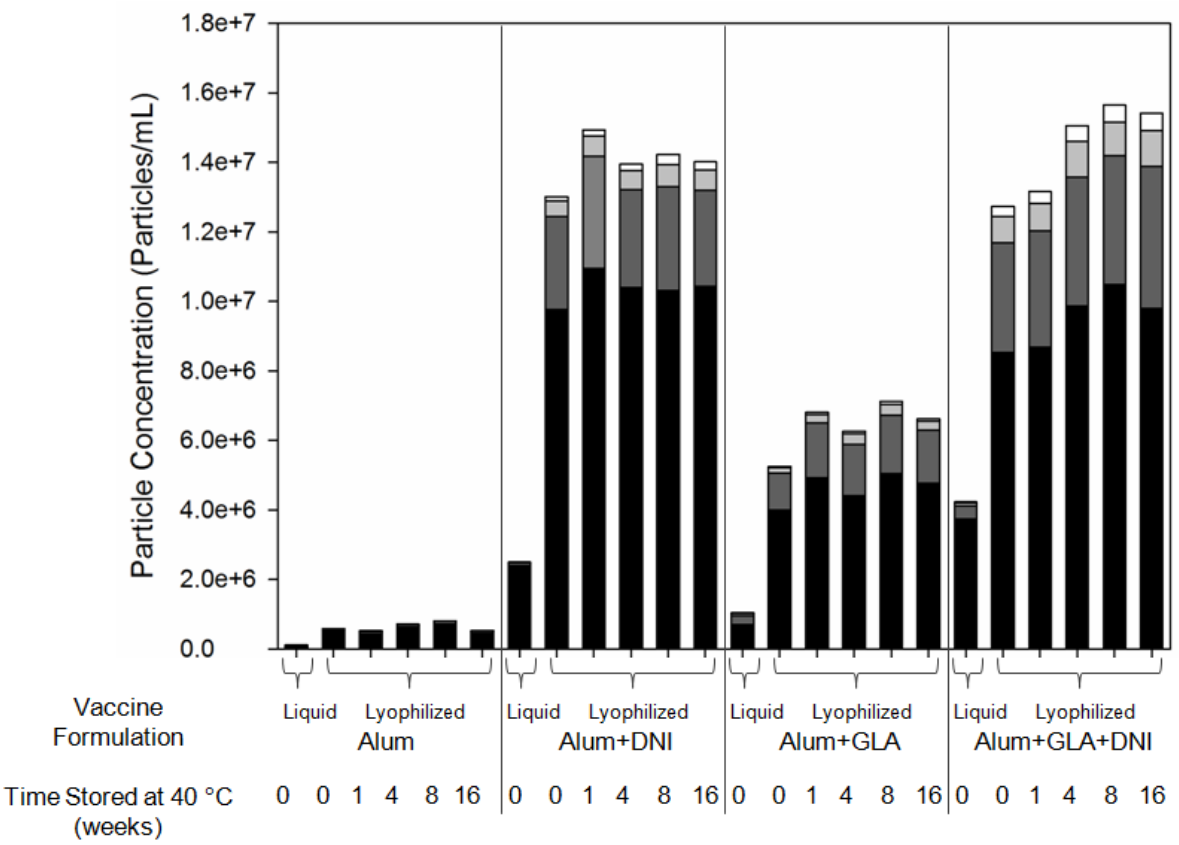

Figure 3.5: Particle size and concentration for particle greater than 2 microns for liquid and reconstituted lyophilized DNI vaccines formulations. Particles 2-5 μm (black), 5-10 μm (dark gray), 10-15 μm (light gray) and greater than 15 μm (white). An increase in particle number was seen when the formulations are lyophilized and reconstituted but no change was seen when the vaccine is incubated at 40 °C for up to 16 weeks.

DNI adsorption to adjuvant particles was measured in liquid formulations prior to lyophilization, in reconstituted lyophilized formulations, and in lyophilized and reconstituted vaccine formulations that had been incubated at 40 °C for up to 16 weeks. Essentially complete (90-100%) adsorption of the DNI protein to aluminum hydroxide adjuvant was observed for all conditions tested. When aluminum hydroxide particles with adsorbed DNI were collected by centrifugation, resuspended in PBS and incubated at 37 °C for one hour, only 70% of DNI remained adsorbed, suggesting that DNI may at least partially desorb in vivo after injection.

3.4.5 Effect of elevated temperatures on antigen structure

Prior to storage at elevated temperatures, cooperative thermal transitions were observed in all samples when analyzed by either intrinsic tryptophan or extrinsic SYPRO Orange fluorescence spectroscopy. In all of the formulations, the transition occurred at a temperature of ∼45 °C. Intrinsic fluorescence spectra of DNI adsorbed to adjuvant in liquid suspensions showed ∼2 nm red-shift in peak positions compared to those for DNI in solution, indicating conformational perturbation in DNI upon adsorption to the adjuvant surface.

After storage at 4 °C for 1, 2, 4, 8, and 16 weeks, thermal transitions occurring at ca. 45 °C were still evident for all samples (Figure 3.6, Table 1). However, if liquid samples were stored at 40 or 70 °C prior to fluorescence analysis, no thermal transitions were observed, suggesting that unfolding of the protein had already occurred prior to analysis. In liquid samples containing DNI (without adjuvant), clear transitions were observed after incubation at 40 °C for up to 4 weeks, a weak transition was detected after 8 weeks of incubation, and no transitions could be detected after 16 weeks. No transitions were detected after liquid samples had been stored at 70 °C for any length of time. In contrast, after storage at any temperature for a period of up to 16 weeks, lyophilized vaccine formulations displayed cooperative thermal transitions, indicating improved structural integrity of the DNI in lyophilized form compared to the liquid formulations. Examples of thermal scans of intrinsic and extrinsic fluorescence used to calculate Tms are shown in Figure 3.6.

Table 3.1: Vaccines incubated at 4, 40 or 70 °C for 0-16 weeks exhibiting fluorescent melting temperatures 40-50 °C measured by intrinsic and extrinsic SYPRO Orange methods (●) or only the intrinsic methods (⊙). Sample without detectable transitions are denoted with (○) symbols. Lyophilized vaccines maintained melting temperatures during incubation, where liquid vaccines did not exhibit detectable melting temperatures when stored at temperatures above 4 °C.

Sample	Storage Length (Weeks)	Storage Temperature 4 °C	40 °C	70 °C
Liquid unbound DNI	No Storage Time	●		
	1	●	●	○
	2	●	●	○
	4	●	●	○
	8	●	⊙	○
	16	●	○	○
Liquid Alum + DNI	No Storage Time	●		
	1	●	○	○
	2	●	○	○
	4	●	○	○
	8	●	○	○
	16	●	○	○
Liquid Alum + GLA + DNI	No Storage Time	●		
	1	●	○	○
	2	●	○	○
	4	●	○	○
	8	●	○	○
	16	●	○	○
Lyophilized Alum + DNI	No Storage Time	●		
	1	●	●	●
	2	●	●	●
	4	●	●	●
	8	●	●	●
	16	●	●	●
Lyophilized Alum + GLA + DNI	No Storage Time	●		
	1	●	●	●
	2	●	●	●
	4	●	●	●
	8	●	●	●
	16	●	●	●

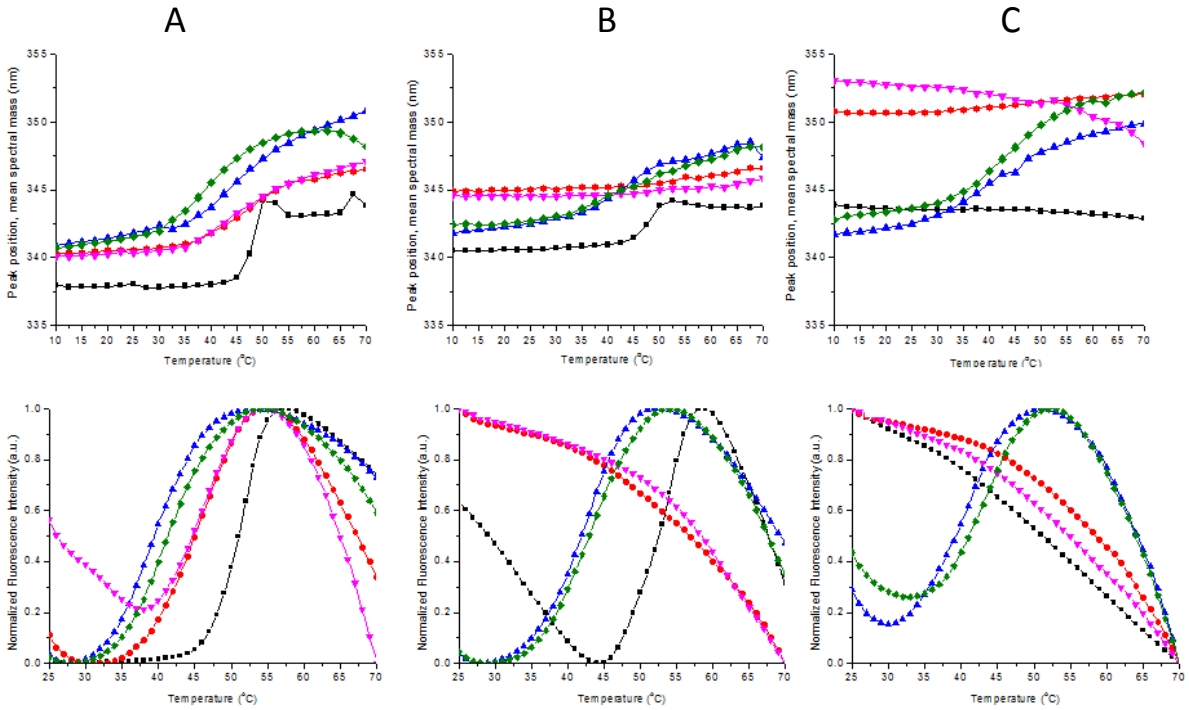

Figure 3.6: Examples of intrinsic (top row) and extrinsic SYPRO Orange (bottom row) fluorescence melting curves for DNI vaccine formulations stored at 4 °C (A), 40 °C (B), and 70 °C (C) for 4 weeks. Samples are liquid DNI (black), liquid vaccine formulation (red), lyophilized vaccine formulation (blue), liquid vaccine formulation with GLA (purple) and lyophilized vaccine formulation with GLA (green).

Deamidation was significantly slowed in the lyophilized state compared to the liquid state. No liquid or lyophilized formulations showed an increase in deamidation during storage at 4 °C for up to 4 weeks. However, when liquid formulations were stored at 40 °C, deamidation was detected after 1 week of storage, but no increase in deamidation could be detected in lyophilized formulations. Liquid formulations containing aluminum hydroxide adjuvant deamidated significantly faster than formulations without adjuvant (Figure 3.7).

3.4.6 Elevated temperature studies - Immunogenicity

The immunogenicity of the vaccine formulations was determined from titers of both total anti-DNI antibodies as well as neutralizing antibodies. Liquid vaccines stored at 40 °C were able to induce production of anti-DNI antibodies, but very few mice injected with liquid vaccine stored at 40 °C for 8 weeks responded with neutralizing titers (Figure 3.8). Although the protein antigen in the liquid vaccine stored for 8 weeks at 40 °C was able to produce antibodies with affinity for native DNI, these antibodies were not effective at neutralizing toxic anthrax proteins, which was consistent with the structural data that suggested a loss of antigen structure when stored at 40 °C.

Lyophilization did not acutely affect the immunogenicity of the vaccines. Liquid vaccines and vaccines reconstituted immediately after lyophilization produced equivalent immune responses, both in the presence and absence of GLA ($p=0.307$ and $p=0.775$ respectively). However, unlike the liquid formulations, lyophilized formulations were able to retain immunogenicity equivalent to un-incubated liquid samples during storage at 40 °C up to 16 weeks, both with ($p=0.793$) and without GLA ($p=0.347$).

After two injections, the liquid vaccine formulation containing GLA produced a significantly stronger immune response than the formulation without GLA ($p\leq0.02$), as measured by total anti-DNI and neutralizing antibodies. In addition to exhibiting higher titers, mice injected with the vaccine formulation containing GLA were more likely to respond to the vaccine after one injection, whereas mice injected with the formulation without GLA often required two injections for a significant response.

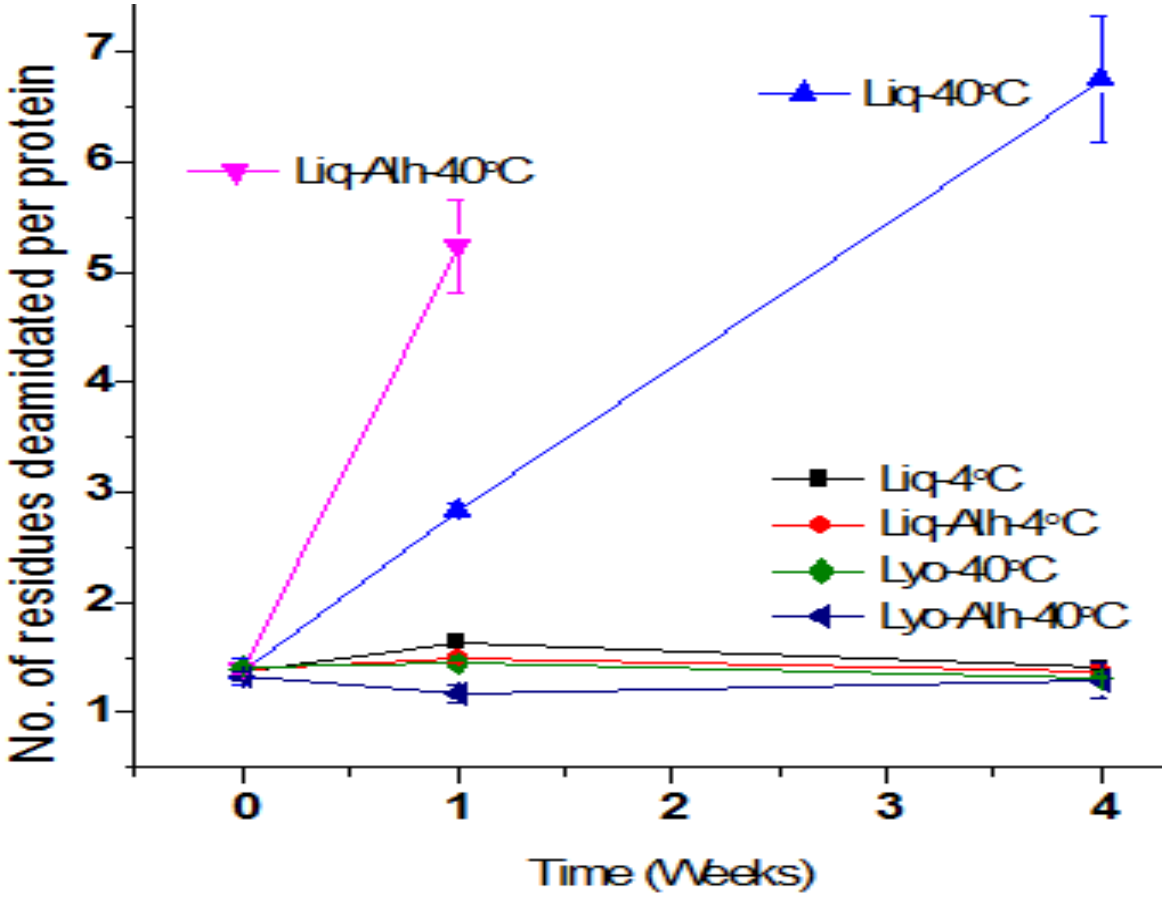

Figure 3.7: Deamidation of DNI increases with storage at higher temperatures and in the presence of aluminum hydroxide adjuvant when stored in the liquid state. Lyophilized vaccines show no increase in deamidation with incubation, even at higher temperatures.

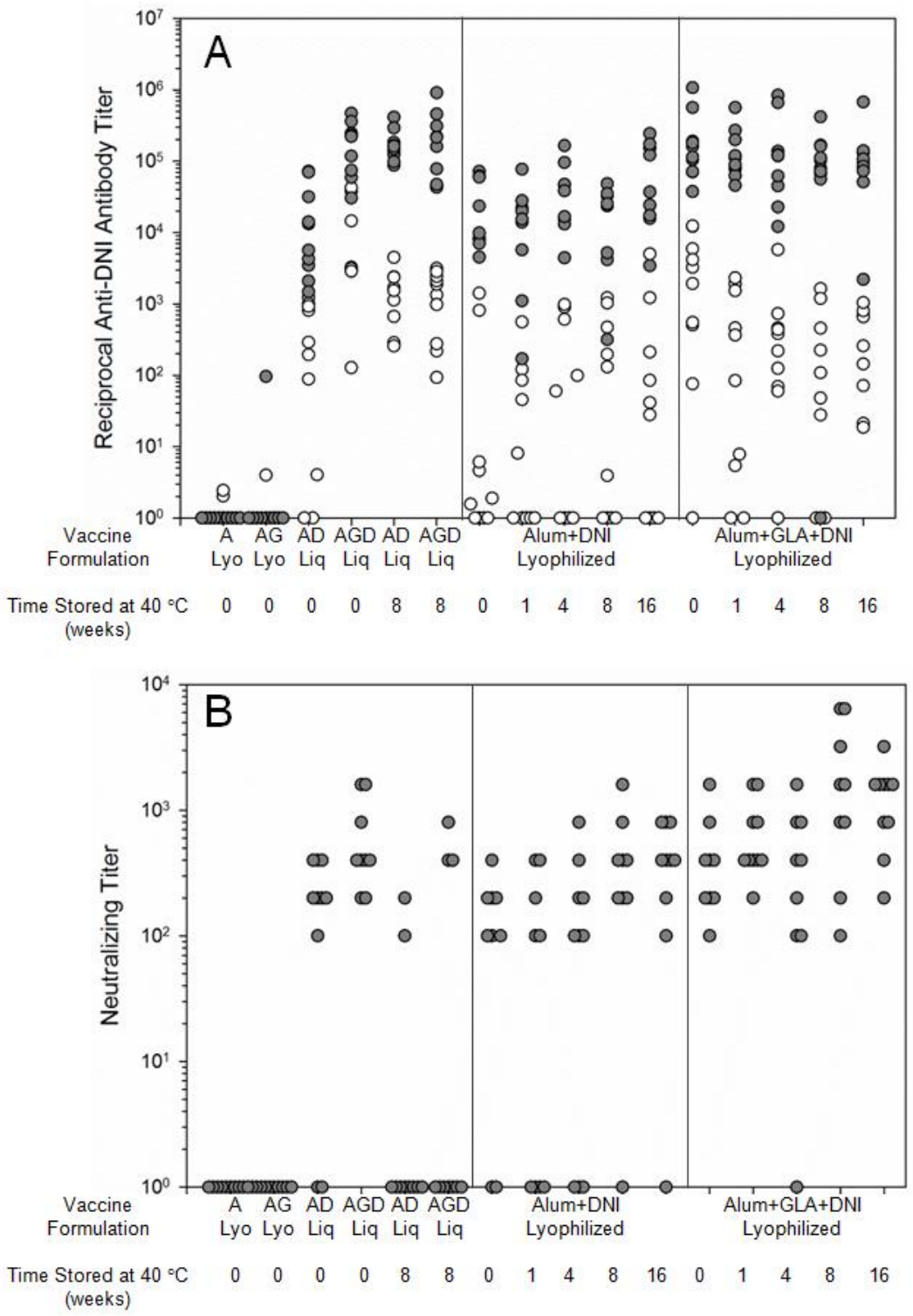

Figure 3.8: Total anti-DNI antibody titers (A) and neutralizing antibody titers (B) after one vaccine injection (white circles) and after two vaccine injections (gray circles) for liquid and reconstituted lyophilized Alum (A), Alum+GLA(AG), Alum+DNI (AD) and Alum+GLA+DNI (AGD) vaccines stored at 40 °C for 0-16 weeks. Lyophilized vaccines remain immunogenic even after storage at 40 °C for 16 weeks, where liquid vaccines show a decrease in immunogenicity after 8 weeks of incubation.

3.5 Discussion

Since all lyophilized vaccines experience freezing once during the lyophilization process and a large fraction of vaccines experience freezing temperatures at least once during passage through the cold chain, freeze-thaw studies were conducted. Vaccine formulations were first exposed to one freeze-thaw cycle to mimic damage caused due to freezing during the lyophilization process. The structure of DNI within these formulations appeared to be unaffected by freeze-thawing, or lyophilization and reconstitution based on Tm values for DNI determined from fluorescence scanning and the observed lack of increased deamidation. Freezing and thawing the vaccine formulations caused an increase in number of particles as well as an increase in larger sized particles. Since the rate of freezing used in the freeze-thaw study was different from the lyophilization cycle, the increase in particle formation was different for each study. After vaccines were frozen and thawed once, their immunogenicities were similar to that of the initial liquid vaccine. Additionally, lyophilized and reconstituted versions of the same vaccine formulation generated immune responses similar to those of the initial liquid form. These results demonstrate that the freezing stage of lyophilization should not cause damage to the vaccine.

Vaccine formulations were frozen and thawed five times to mimic more extensive damage that could happen as a result of thermal excursions during shipping and storage. After five freeze-thaw cycles, vaccine formulations exhibited no alterations in protein melting temperature or deamidation. Larger particles were formed at the expense of smaller particles with more freeze-thaw cycles. Although no difference in immunogenicity was detected after two injections from initial liquid vaccine, reduced anti-DNI antibody titers were detected after a single dose for both Alum+DNI and Alum+GLA+DNI formulations. After five freeze-thaw cycles, the fraction of mice responding with anti-DNI antibodies to the Alum+DNI vaccine after a single injection decreased from 80% to 20%, although 100% responded after two injections. Similar results were seen with a tetanus toxoid vaccine in which higher concentrations of trehalose resulted in a greater percentage of residual antigenicity after five freeze-thaw cycles [170]. The exact cause of the reduced immunogenicity is

still debatable.

Liquid vaccine formulations lost potency following exposure to higher temperatures. Protein structure of liquid formulations was perturbed after 1 week of storage at 40 °C. In contrast, DNI structure was preserved in vaccine formulations lyophilized, even after storage at 70 °C for 16 weeks. Additionally, lyophilization prevented deamidation of the DNI protein, even in the presence of aluminum hydroxide particles. Previous studies have shown that proteins deamidate faster in the presence of an aluminum salt adjuvant [59, 55]. The immunogenicity of liquid vaccines was compromised by 8 weeks of storage at 40 °C, whereas the immunogenicity of lyophilized vaccines was retained after storage at 40 °C for 16 weeks.

To administer the vaccine formulations in as few doses as possible, reducing transportation needs and cost while increasing patient compliance, GLA could be added to vaccine formulations already containing aluminum hydroxide. The immunogenicity of vaccine formulations containing GLA was higher after one injection than formulations without GLA. The response after one injection with GLA was almost as high as two injections without GLA, demonstrating the ability of GLA to increase the immune response and reduce the required number of doses. Also, a higher percentage of mice responded after one injection to vaccines containing GLA. Similar results were seen when MPL was added to human Papillomavirus vaccines [178, 88].

3.6 Conclusions

Damage can be caused by both elevated and freezing temperatures in liquid vaccine formulations. Freeze-thaw cycles were found to be detrimental to a DNI vaccines immunogenicity and aluminum hydroxide particles. Lyophilized formulations showed much better stability than the liquid formulations upon incubation for 16 weeks at 4, 40 and 70 °C based on fluorescent and deamidation measurements. To complement the structural studies, lyophilized vaccines stored at 40 °C did not lose immunogenicity for storage up to 16 weeks whereas liquid vaccines lost immunogenicity prior to 8 weeks. The immunogenicity of vaccines containing GLA was much higher than vaccines that contained only aluminum hydroxide adjuvant. After only one injection, vac-

cines that contained GLA produced a higher percentage of mice that responded with anti-DNI and neutralizing antibodies.

In lyophilized vaccine formulations, variations in temperature during transport are less detrimental to vaccine potency. In the lyophilized state minimal water is present, avoiding any potential damage by freeze-thaw events. Lyophilized vaccines also permit longer storage at recommended temperatures and can remain immunogenic for short excursions to elevated temperatures if breaks in the cold chain occur.

3.7 Acknowledgments

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Chapter 4

Eliminating the cold chain: A highly-thermostable, adjuvanted HPV vaccine

This chapter will be submitted as K.J. Hassett, N.M. Meinerz, F. Semmelmann, M.C. Cousins, R.L. Garcea, and T.W. Randolph. "Eliminating the Cold Chain: A Highly-Thermostable, Adjuvanted HPV Vaccine" to Nature Biotechnology.

4.1 Abstract

A major impediment to economical, worldwide vaccine distribution is the requirement for a "cold chain" to preserve antigenicity. We addressed this problem using a model human papillomavirus (HPV) vaccine stabilized by immobilizing HPV16 L1 capsomeres, i.e., pentameric subunits of the virus capsid, within organic glasses formed by lyophilization. Lyophilized glass and liquid vaccine formulations were incubated at 50°C for 12 weeks, and then analyzed for retention of capsomere conformational integrity and the ability to elicit neutralizing antibody responses after immunization of BALB/c mice. Capsomeres in glassy-state vaccines retained tertiary and quaternary structure, and critical conformational epitopes. Moreover, glassy formulations adjuvanted with aluminum hydroxide or aluminum hydroxide and glycopyranoside lipid A were not only as immunogenic as commercially available Gardasil® and Cervarix® vaccines, but also retained complete neutralizing immunogenicity after high-temperature storage. The thermal stability of such adjuvanted vaccine powder preparations may thus eliminate the need for the cold chain.

4.2 Introduction

Cervical cancer is the third most common cancer in women worldwide [50]. The majority of cervical cancer occurs in women in less developed countries [50] where the availabilities of vaccines and preventative screenings such as Pap smears are limited [155]. Infection with high-risk types of human papillomavirus (HPV) is the primary etiologic event associated with cervical cancer [187], and therefore affordable, stable vaccines to prevent HPV infection could significantly reduce the disease prevalence in resource-poor regions of the world.

To prevent loss of efficacy, vaccines typically must be refrigerated during transport and storage. The logistical requirements associated with maintenance of carefully controlled cold chains make vaccine delivery in many regions challenging [94], and adds substantial costs. For the commercially available HPV vaccines Gardasil® and Cervarix®, cold chain requirements (storage at 2-8°C without freezing [63]) and cost (currently $360 for a three-dose series in the United States) are impediments for widespread use in many regions of the world [188].

The recommended temperature ranges for vaccine transport through the cold chain are narrow [118]. If liquid vaccine formulations freeze, or if they are exposed to elevated temperatures, loss of efficacy may result [169, 36, 23, 79, 186]. Liquid vaccines that contain microparticulate adjuvants such as aluminum hydroxide may be particularly prone to damage resulting from freezing, in part because of the tendency of these adjuvants to agglomerate [36, 201, 170, 153, 101].

Lyophilization may be used to immobilize vaccine antigens and adjuvants within glassy organic matrices [42], where the combination of low molecular mobility and low moisture content inhibits antigen degradation. Moreover, by utilizing high concentrations of glass-forming excipients and rapid freezing rates, agglomeration of microparticulate adjuvants can be avoided or minimized during the lyophilization process [42]. Reconstituted lyophilized vaccines adjuvanted with aluminum hydroxide have been reported to be equally immunogenic as their liquid counterparts [79, 40, 43].

To generate adequate protective immune responses to vaccines that are based on purified

protein antigens, microparticulate adjuvants typically must be added to formulations. Currently aluminum salts such as aluminum hydroxide are present in all commercially available adjuvanted vaccines. Aluminum salt adjuvants such as aluminum hydroxyphosphate sulfate, the primary adjuvant in the commercial HPV vaccine Gardasil®, primarily provokes a humoral response. To produce a more robust cellular immune response, other adjuvants typically must be added19. One such adjuvant, monophosphoryl lipid A (MPL), is a non-toxic derivative of lipopolysaccharide that can stimulate a cellular immune response through its Toll-like receptor-4 agonist activity [46]. MPL adsorbed to aluminum hydroxide is used to adjuvant Cervarix® HPV vaccines. Glycopyranoside lipid A (GLA) is a synthetic variant of MPL that is more homogeneous and active than MPL [46]. At present there are no commercial vaccines that combine both an aluminum hydroxide adjuvant and a cellular immunity stimulant such as GLA in a lyophilized formulation.

Commercial HPV vaccines contain virus-like particles (VLPs) as the vaccine antigens [155], but subunit capsomere protein preparations also have shown promise as alternative vaccine antigens [149, 200, 62, 88, 197]. Capsomeres are comprised of L1 protein monomers assembled into pentamers to form the basic subunit of the HPV capsid. Although in some formulations capsomeres may be less immunogenic than their VLP counterparts, formulations with MPL appear equally immunogenic [178]. Capsomeres purified after expression in E. coli may offer reduced production costs with respect to currently marketed VLP-based vaccines, which are produced in S. cerevisiae (Gardasil®) or baculovirus-infected insect cells (Cervarix®).

We hypothesized that embedding HPV16 L1 capsomeres within glassy matrices formed during lyophilization would yield a dry powder vaccine formulation with enhanced thermal stability. To test this hypothesis, we prepared HPV16 L1 capsomeres in formulations that contained trehalose as a glass-forming excipient. Relatively high concentrations of trehalose (9.5% wt/vol) were used to promote rapid glass formation during the freezing step of the lyophilization process. Trehalose also served as a tonicity modifier, and hence we used only minimal additional buffer salts (10 mM histidine). In addition, we tested formulations that contained aluminum hydroxide or both aluminum hydroxide and GLA as adjuvants. The formulations were processed using controlled rapid freezing

rates [79, 42] to avoid agglomeration of aluminum hydroxide microparticles, followed by drying under vacuum to form glassy matrices. The lyophilized formulations were reconstituted either immediately after lyophilization, or after 12 weeks of incubation at 50°C, and tested for retention of native capsomere structure using transmission electron microscopy, size exclusion chromatography, fluorescence spectroscopy, epitope binding assays, and immunoassays. The immunogenicities of the formulations were tested in BALB/c mice, and compared to the immunogenicities of commercially available HPV vaccines subjected to similar storage conditions.

4.3 Materials and methods

4.3.1 Materials

High purity α,α-trehalose dihydrate and H2SO4 were purchased from Mallinckrodt Baker (Phillipsburg, NJ). L-Histidine monohydrochloride monohydrate, triethanolamine, ethylene glycol tetraacetic acid (EDTA), TritonTM X-100, Benzonase® nuclease, OptiprepTM density gradient medium and bovine serum albumin (BSA) were purchased from Sigma-Aldrich (St. Louis, MO). Two percent Alhydrogel® (aluminum hydroxide adjuvant) was obtained from Accurate Chemicals and Scientific Corp (Westbury, NY). Lyophilized synthetic monophosphoryl lipid A (glycopyranoside Lipid A (GLA) adjuvant) was purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). Three mL 13 mm glass lyophilization vials, caps and seals were from West Pharmaceutical Services (Lititz, PA). Concentrated 10X phosphate buffered saline (PBS), Tween 20, ammonium sulfate, glycerol, acrylamide, tris(hydroxymethyl)aminomethane (Tris), and NaCl were from Fischer Scientific (Fair Lawn, NJ). Water for injection was purchased from Baxter Healthcare Corporation (Deerfield, IL). Dry powdered milk was purchased though Safeway Inc (Pleasanton, CA). Peroxidase-conjugated affinipure donkey anti-mouse IgG (H+L) was from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). 3,3',5,5'-tetramethylbenzidine (Ultra TMB and Turbo TMB) was from Thermo Scientific (Rockford, IL). Lipofectamine was from Invitrogen (Carlsbad, CA). Plasmid-safe DNase was from Epicentre (Madison, WI).

4.3.2 HPV16 L1 capsomere protein purification

In brief, HPV16 L1 protein was expressed in an untagged form in HMS174 *E. Coli* using the vector HPV16-p3 (Macejak and Garcea, in preparation). Cells were lysed by two passages through a GEA Niro Soavi Panda homogenizer at 800-1000 bar. The soluble fraction was collected after centrifugation of the cell lysate. This fraction was the chromatographed on a Q Fast Flow column (GE Healthcare, Piscataway NJ). The L1 protein eluted in the flow-through, and was then precipitated using ammonium sulfate at 30% saturation. The resuspended ammonium sulfate precipitate was solubilized in a Tris buffer pH 8.5 and chromatographed on a Q sepharose anion exchange column (GE Healthcare, Piscataway, NJ). L1 eluted as pentamers from the sepharose column using a sodium chloride gradient, with a final purity of greater than 95% as estimated by SDS-PAGE. Before formulation, fractions containing L1 were exchanged into a 100 mM histidine buffer pH 7.1 by size exclusion chromatography.

4.3.3 Vaccine formulation

Vaccines were formulated to contain 0.1 mg/mL HPV16 L1 capsomeres in 54 mM histidine HCl pH 7.1 with 9.5 w/v% trehalose. Additionally, some formulations contained 0.5 mg/mL aluminum from Alhydrogel®, or 0.5 mg/mL aluminum from Alhydrogel® and 0.05 mg/mL GLA. Formulations were rotated end over end at 8 rpm in 2 mL polypropylene microcentrifuge tubes at 4°C for 1 hr to allow adsorption of capsomeres to adjuvant.

4.3.4 Lyophilization

One mL aliquots of vaccine formulations at 4°C were filled into 3 mL lyophilization vials and placed on lyophilizer shelves that had been pre-cooled to -10°C (FTS Systems Lyophilizer, Warminster, PA). Vials containing vaccine formulation were surrounded by dummy vials filled with DI water to minimize radiative heat transfer effects for vials near the edge of the lyophilizer shelves. The shelf temperature was decreased at a rate of 0.5 °C/min to -40°C and then held at -40°C for 1 hr to allow the samples to freeze completely. Primary drying was initiated by decreasing

the chamber pressure to 60 mTorr and then increasing the shelf temperature to -20°C at a rate of 2°C/min. Shelf temperatures were held at -20°C for 20 hr. Secondary drying was conducted at a pressure of 60 mTorr by increasing the shelf temperature to 0°C at a rate of 0.2°C/min, followed by increasing to 30°C at a rate of 0.5°C/min and holding the shelf temperature at 30°C for 5 hr. Finally, the shelf temperature was returned to 25°C and the chamber was back-filled with nitrogen until atmospheric pressure was reached. Chlorobutyl rubber stoppers were then inserted into vials under a nitrogen atmosphere, and the vials were sealed with aluminum caps and transferred to a freezer for storage at -80°C.

4.3.5 Differential scanning colorimetry (DSC)

Onset glass transition temperatures of placebo lyophilized formulations were obtained using differential scanning calorimetry (Diamond DSC, Perkin Elmer, Waltham, MA). Triplicate samples were prepared inside an aluminum pan under dry nitrogen. Pans were cycled twice between 25°C and 150°C at a scan rate of 100°C/min. The second heating scan was used to determine the onset glass transition temperature.

4.3.6 Particle size analysis

The particle size distributions in liquid formulations prior to lyophilization or after lyophilization and reconstitution were determined by microflow analysis using a FlowCAM instrument (Fluid Imaging Technologies, Yarmouth, ME). The FlowCAM measures the size and concentration of particles of sizes greater than 2 μm. A 100 micron path-length flow cell was used at a flow rate of 0.08 mL/min with images taken at a rate of 10 frames per second. A 10X objective and collimator were used. Light and dark settings of 17 and 15, respectively, were used to capture particles. To avoid capturing overlapping particles, formulations were diluted ten times for placebo formulations, and 100 times for formulations containing capsomeres. A total sample volume of 0.35 mL of each diluted sample was analyzed.

4.3.7 Transmission electron microscopy (TEM)

Vaccine formulations were adsorbed to formvar/carbon-coated, glow-discharged 400 mesh copper TEM grids. After sample adsorption, grids were washed with 5 mM EDTA and stained with 1-2% uranyl acetate. Images were collected using a Philips CM10 transmission electron microscope operating at 80 kV equipped with a Gatan Bioscan2 digital camera.

4.3.8 Size exclusion high performance liquid chromatography (SE-HPLC) analysis of capsomere integrity

Reconstituted lyophilized HPV16 L1 capsomeres were analyzed using a Beckman Coulter Gold HPLC system (Fullerton, CA) with a TSK-gel G3000SWXL column (TOSOH Bioscience, Montgomeryville, PA) and a running buffer containing 50 mM Tris, 350 mM NaCl, 10% glycerol, pH 8.1 at a flow rate of 0.6 mL/min. UV absorbance at 280 nm was used for detection. Each sample was run in duplicate.

4.3.9 SDS-PAGE

Capsomere proteins stored in the liquid or lyophilized states were analyzed on a 4-20% Mini-PROTEAN BioRad polyacrylamide gel at 0.5 μg/well under non-reducing and reducing (100 mM dithiothreitol) conditions in a Tris-glycine running buffer. The gels were stained with Coomassie Blue.

4.3.10 Fluorescence spectroscopic analysis of thermally-induced denaturation of HPV16 LI capsomeres

The intrinsic fluorescence of HPV16 L1 capsomeres was monitored as a function of temperature to determine the capsomere melting temperature. Fluorescence was excited at 295 nm, and spectra were collected from 305 to 400 nm on a fluorimeter (SLM Instruments Inc. Urbana, IL). Spectra were recorded every 5°C from 20 to 90°C, after an equilibration time of 10 min at each temperature. The wavelength center of spectral mass was calculated at each temperature, and the

apparent melting temperature of the capsomeres was determined as the onset temperature of the thermal transition that was observed around 60°C.

4.3.11 Front-face mode fluorescence analysis of capsomere tertiary structure

For front-face mode spectroscopic measurements, 3 mL aliquots of vaccine formulations were pipetted into quartz cuvettes and placed in a fluorimeter with a holder that maintained the angle of incidence at 53°C. Samples were excited at 295 nm and emission spectra were collected from 310 nm to 400 nm. 10 μL aliquots of a 5 M acrylamide quencher solution was added to the sample and the peak intensity at 331 nm for capsomeres and 340 nm for 8 M urea unfolded capsomeres were monitored after each acrylamide addition. The ratios of fluorescence intensity without any quencher present to the fluorescence intensity with acrylamide present were plotted against the acrylamide concentration and linear regression was used to determine the Stern-Volmer quenching constant, K_{SV} as the slope of the resulting line.

4.3.12 L1 and V5 epitope binding assay

An ELISA-based assay was used to monitor the presence of L1 and V5 capsomere epitopes. Vaccine formulations with or without aluminum hydroxide adjuvant were diluted in PBS, and 0.125 μg/well of HPV16 L1 capsomere protein was coated on 96-well Nunc flat bottom PolySorp Immuno plates and incubated overnight at 4°C. Plates were washed three times with 0.05% Tween 20 in PBS at 300 μL/well. Plates were blocked with 100 μL/well of blocking buffer (5% dry milk, 0.05% Tween 20 in PBS) for 1 hr at 37°C. After blocking, blocking buffer was removed and primary antibodies, either L1 or V5 at a dilution of 1:1000 in blocking buffer, were added 50 μL/well and incubated at 37°C for 1 hr. After washing three times, secondary antibody diluted 1:5,000 in wash buffer (0.05% Tween 20 in PBS) was added 50 μL/well and incubated at 37°C for 1 hr. The secondary antibodies against L1 and V5 were a goat anti-rabbit and a goat anti-mouse HRP conjugated IgG antibody, respectively. After washing five times, 50 μL/well of Turbo TMB was added and plates were incubated at room temperature for 5 min. The reaction was quenched with 50 μL/well 1 M

H2SO4 and plates were read for absorbance at 450 nm on a Molecular Devices Kinetic Microplate Reader (Sunnyvale, CA).

4.3.13 Vaccine immunogenicity

4.3.13.1 Dose-dependency of immune response to lyophilized and reconstituted vaccines

Murine studies were conducted under the University of Colorado at Boulder *Institutional Animal Care and Use Committee* (IACUC) protocol #1209.02. Female BALB/c mice from Taconic (Hudson, NY) were allowed to acclimate at least one week before use and were 10 to 11 weeks old at the start of the immunization study. Blood samples were collected through the retro orbital cavity under isofluorane anesthesia on days 0, 21, and 36, and mice were injected intramuscularly on days 0 and 21 with reconstituted lyophilized capsomeres, capsomeres with aluminum hydroxide, capsomeres with aluminum hydroxide and GLA, liquid Gardasil®, and Cervarix® vaccines. Reconstituted lyophilized vaccines were injected at doses of 1, 3, 5, or 8 g of HPV16 L1 capsomeres, Gardasil® was injected in doses containing 1, 3, or 5 μg of HPV16 VLPs and Cervarix was injected in doses containing 1, 2, 3, or 4 μg of HPV16 VLPs. Serum was separated by centrifugation at 9,400xg for 14 min at 4°C and stored at -80°C until use.

4.3.13.2 Thermal stability of lyophilized HPV16 L1 vaccines

To test their ability to withstand high-temperature excursions from cold-chain conditions, liquid and lyophilized vaccine formulations were incubated at 50°C for 12 weeks. After incubation the lyophilized vaccines were reconstituted with water for injection, and these formulations as well as the liquid formulations were compared to formulations that had not been subjected to incubation at elevated temperatures. Lyophilized HPV16 L1 vaccines and Gardasil® were injected into mice at either 1 or 5 μg/dose, and Cervarix® was injected at either 1 or 4 μg/dose. Two doses of the lyophilized HPV16 L1 and Cervarix® vaccines were administered, and single doses of Gardasil® that had been incubated for 12 weeks at 50°C were given. Serum samples were collected and

processed as described above.

4.3.14 Total antibody enzyme linked immunosorbent assay (ELISA)

Nunc MaxiSorb 96 well plates (Thermo Fischer Scientific, Rochester, NY) were coated with 50 μL/well of 1 μg HPV16 L1 capsomere/mL diluted in PBS and incubated at 2-8°C overnight. Plates were washed 3 times with PBS containing 0.05% Tween 20. Plates were blocked with 300 μL/well of PBS with 1% BSA, incubated at room temperature for 2 hr, and washed again. Serum was initially diluted in PBS with 1% BSA, 0.05% Tween 20, 100-fold for serum collected on days 0, 500-fold for serum collected on day 14, and 1,000 or 5,000-fold for serum collected on day 28 for mice injected without and with adjuvant respectively. A series of in-plate 2-fold dilutions were made for each sample. Plates were incubated for 1.5 hr at room temperature and washed. 40 μL of HRP-conjugated donkey anti-mouse antibody diluted 10,000 times was added to each well and incubated for 1.5 hr at room temperature with shaking, followed by washing. 40 μL Ultra TMB was added to each well and incubated for 15 min, followed by quenching with 40 μL of 1 M sulfuric acid. Absorbances of samples in the wells of the plates were measured at 450 nm on a Molecular Devices Kinetic Microplate Reader (Sunnyvale, CA).

To determine titers, average OD 450 values as a function of dilution were fit to a 4-parameter logistic equation using SigmaPlot 12 (Systat Software, San Jose, CA) software. The constraints 0 <min <0.15 and max <3.3 were used. A cutoff value of 0.5 was used. Groups with normally distributed antibody titers were compared with a t-test and groups without normal distributions were compared with the nonparametric Mann-Whitney Rank Sum Test.

4.3.15 Pseudovirus production

For a detailed protocol see [1]. In brief, 293TT cells were transfected using lipofectamine with DNA plasmids expressing secreted alkaline phosphatase (SEAP), HPV16 L1 and HPV16 L2 capsid proteins. Cells were lysed 2 to 3 days after transfection using TritonTM X-100, Benzonase®, Plasmid-safe^{TM}, and ammonium sulfate. The pseudovirions were salt extracted, and isolated from

the clarified cell lysate by sedimentation in an Optiprep^{TM} gradient. Fractions were collected from the bottom of the gradient tube and assayed for DNA and protein content by PicoGreen assays and BCA assay, respectively.

4.3.16 Neutralizing antibodies

One hundred μL/well of a suspension containing 3x10^5 293TT cells/mL were plated in 96 well tissue culture plates and incubated at 37°C for 2-5 hr. HPV16 pseudovirus was added to dilutions of mouse serum and incubated on ice for 1 hr. 100 μL of pseudovirus/mouse serum solution was added to plated cells and incubated at 37°C for 3 days. The negative control was an anti-bovine papillomavirus (BPV) antibody, and the positive control was heparin, which inhibits infection. After incubation, supernatant was collected from cells. The Great Escape SEAP Chemiluminescence test kit (Clontech, Mountainview, CA) was used for detection of SEAP. Plates were read on a multifunctional BioTek plate luminometer at a set glow-endpoint of 0.20 seconds/well. For a detailed protocol, see [137].

The neutralization titer was defined as the dilution of mouse serum that neutralized 50% of the pseudovirus as determined by SEAP colorimetric measurement. The fractional neutralization was defined as the difference between the anti-BPV values and diluted mouse serum value, divided by the difference between the anti-BPV value and the heparin value. Percent neutralization was then determined as 100 x the fractional neutralization value. Neutralization values were fit to a 4-parameter logistic equation using SigmaPlot 12. Neutralization values could not be fit for serum samples that did not exhibit a sufficient decrease in neutralization under the dilution conditions tested; these samples were assigned a titer value of 300,000. Groups with normally distributed neutralization titers were compared with a t-test, and groups without normal distributions were compared with the Mann-Whitney Rank Sum Test.

4.4 Results

4.4.1 Choice of an incubation temperature for accelerated stability studies

Ideally, accelerated degradation studies for protein antigens are carried out at sufficiently high temperatures so that measurable damage occurs over the course the accelerated stability study, but at temperatures low enough to avoid phase changes within the formulation or gross denaturation of the protein antigen. Thus, we first determined a temperature that was below both the glass transition temperature (Tg) of the lyophilized formulation and the thermal melting point of capsomeres. The onset glass transition temperatures for lyophilized placebo formulations were found to be 97.2 ± 3.4°C and 102.6 ± 5.2°C in the absence and presence of aluminum hydroxide microparticles, respectively. At the low capsomere and GLA concentrations used in our studies, addition of capsomeres and/or GLA to these formulations would not be expected to affect the glass transition temperature significantly. We determined the onset melting temperature of the HPV16 L1 capsomere at approximately 60°C (melting curve not shown). The melting temperatures of HPV VLPs types 6, 11, 16, and 18 included in Gardasil® are reported to be above this temperature [159]. An incubation temperature of 50°C was therefore chosen to evaluate stability.

4.4.2 HPV16 L1 capsomere vaccine characterization

HPV16 LI vaccine formulations that had been lyophilized and immediately reconstituted or lyophilized and stored for 12 weeks at 50°C prior to reconstitution were analyzed by transmission electron microscopy (TEM) for capsomere structural appearance, size exclusion-high performance liquid chromatography (SE-HPLC) for capsomere size, front-face fluorescence for tertiary structure, V5 and L1 antibody immunoassays for conformational epitope reactivity, and FlowCAM flow microscopy for particle size and concentration. After 12 weeks of incubation within the liquid formulations at 50°C, capsomeres were degraded sufficiently such that they could not be reliably detected by SDS-PAGE (Figure 4.1e), and thus further characterization was not conducted.

TEM was used to visualize HPV16 L1 capsomeres. Before lyophilization, HPV16 capsomeres

were uniform in appearance with a diameter of approximately 9 to 10 nm (Figure 4.1a). After lyophilization and reconstitution, they appeared similar to capsomeres in the initial preparation (Figure 4.1b). Storing the lyophilized vaccine for up to 12 weeks at 50°C did not affect capsomere appearance (Figure 4.1c). Capsomere structure could not be analyzed by TEM in vaccine formulations that contained adjuvants.

The chromatographic retention time of the HPV16 L1 capsomeres was monitored by SE-HPLC, and did not to change after incubation for 12 weeks at 50°C in the lyophilized state (Figure 4.1d). Additionally, the subunit L1 protein remained intact after lyophilization and high temperature incubation, as shown by SDS-PAGE (Figure 4.1e).

Stern-Volmer constants (K_{SV}) determined from front-face mode fluorescence acrylamide quenching studies were used to gain insight on the effect of formulation and processing on the tertiary structure of capsomeres. High K_{SV} values reflect facile solvent access to (normally buried) tryptophan residues within the capsomeres, whereas lower Stern-Volmer constants are typically associated with folded proteins wherein quenchers such as acrylamide have limited access to buried tryptophan residues [56]. Changes in K_{SV} thus may reflect an overall alteration in the accessibility of tryptophan residues and folding of the protein [56]. K_{SV} values for formulations that had been lyophilized and reconstituted immediately were unchanged from those of capsomeres in the initial liquid formulation. Likewise, K_{SV} values did not change when the lyophilized capsomere or capsomere and aluminum hydroxide formulations were incubated for 12 weeks at 50°C prior to reconstitution, as shown in Figure 4.4.2. K_{SV} values were slightly lower in the formulations containing aluminum hydroxide microparticles, which may be due to increased steric hindrance as capsomeres adsorb to aluminum hydroxide.

Binding of the antibodies L1 and V5 to HPV16 L1 capsomeres was measured to determine the effect of processing and storage on retention of HPV16 L1 capsomere structure. L1 is a polyclonal antibody that detects multiple epitopes on L1, whereas V5 binds a neutralizing epitope specific to conformationally intact capsomeres or VLPs [191, 152, 190]. Antibody binding to capsomeres was retained in each formulation following lyophilization and reconstitution, as well as after 12 weeks of

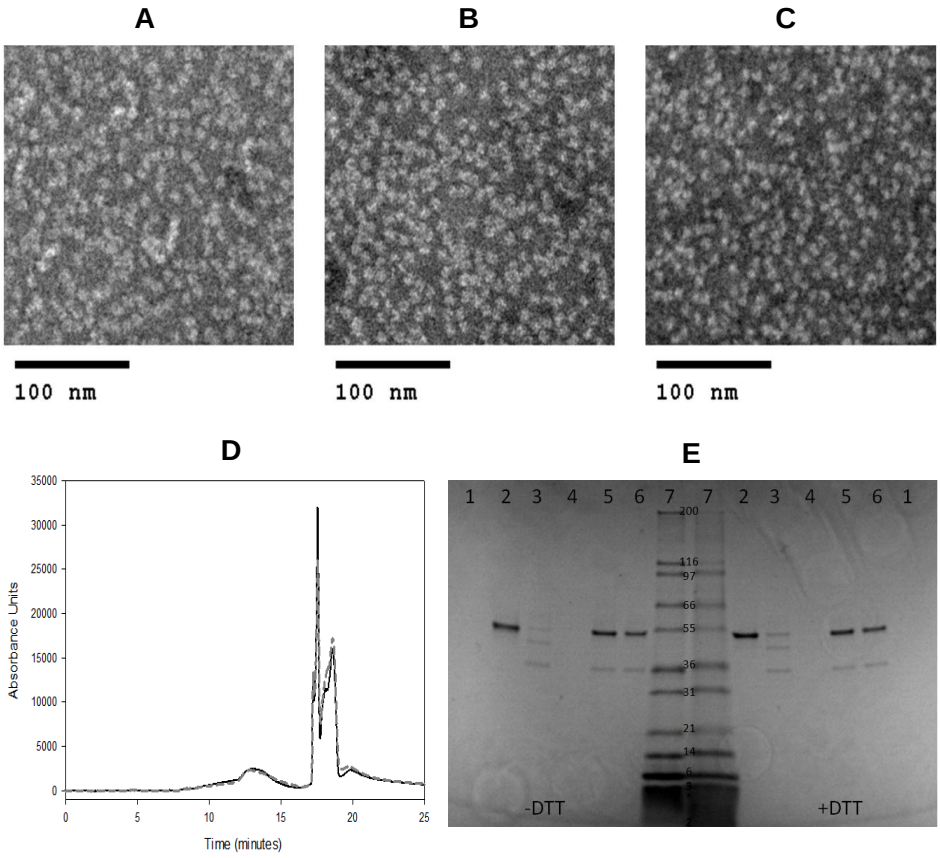

Figure 4.1: Preservation of HPV16 capsomere structure after lyophilization and incubation in the glassy state. TEM images of HPV16 L1 capsomere formulations without adjuvant, before lyophilization (a), immediately after lyophilization and reconstitution (b), and after incubation at 50°C for 12 weeks in the lyophilized state and reconstitution (c) showed no change in capsomere appearance with lyophilization or incubation in the lyophilized state. SE-HPLC retention times (d) for HPV16 L1 capsomeres measured after lyophilization and immediate reconstitution (solid black line) were unchanged following lyophilization and incubation at 50°C for 12 weeks prior to reconstitution (gray dashed line). The capsomere peak appeared at approximately 13 mins; peaks eluting after 17 mins were due to buffer components. Analysis of capsomeres by SDS-PAGE (e) is shown under non-reducing (-dithiothreitol, DTT) and reducing conditions (+DTT). Sample lanes contained buffer (1), liquid HPV16 L1 capsomere vaccines prior to incubation (2), liquid HPV16 L1 capsomere vaccines after 20 weeks incubation at 4°C (3), liquid HPV16 L1 capsomere vaccines after 12 weeks at 50°C (4), reconstituted lyophilized HPV16 L1 capsomeres prior to incubation (5), reconstituted lyophilized HPV16 L1 capsomeres after 12 weeks of incubation at 50°C (6) or molecular weight markers (7). L1 subunits were intact after lyophilization and incubation in the glassy state at 50°C for 12 weeks, but some loss of L1 subunits was observed when liquid formulations were stored at 4°C. Incubation of liquid vaccine formulations at 50°C resulted in complete loss of intact L1 protein.

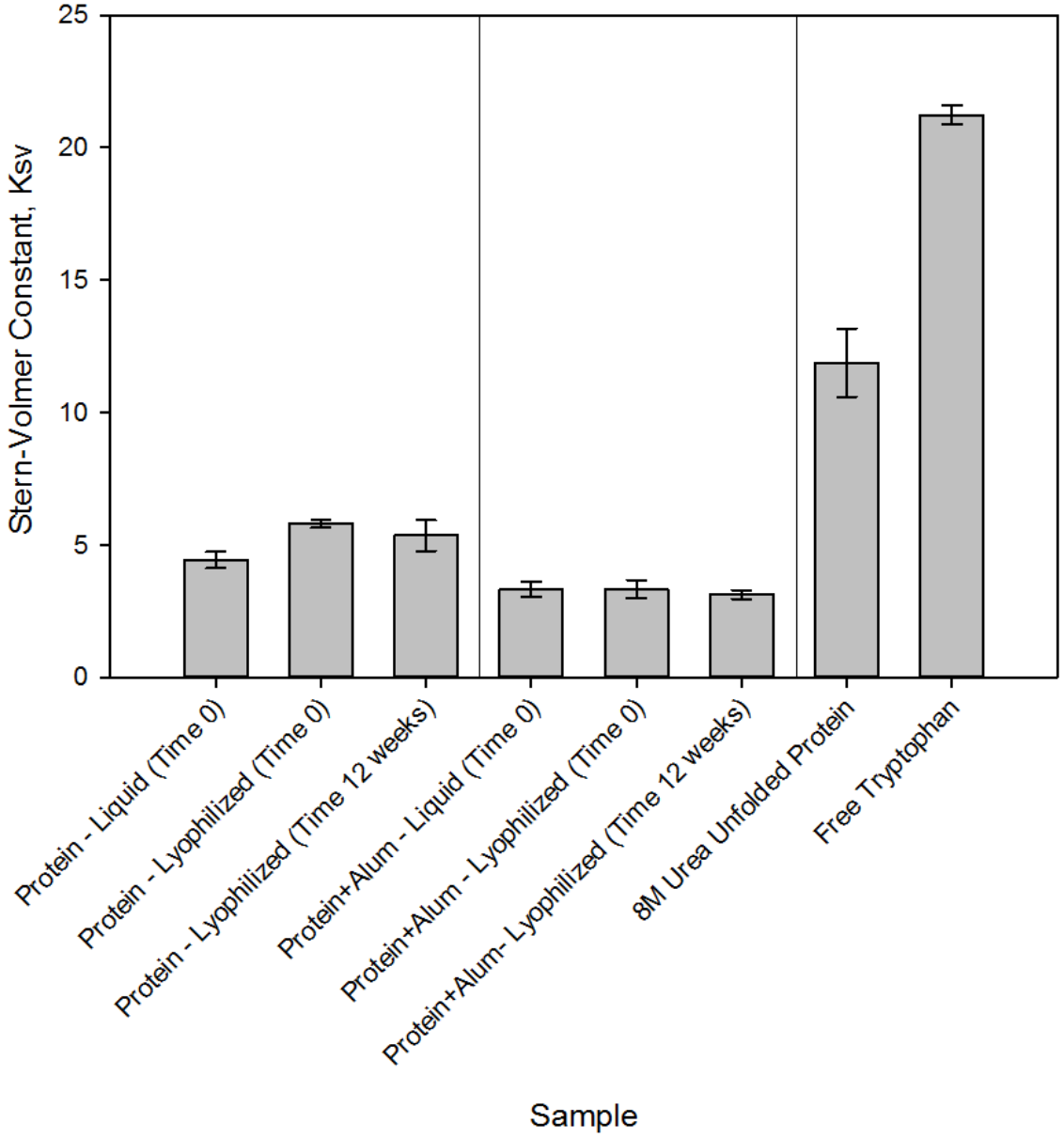

Figure 4.2: Capsomere tertiary structure is maintained after incubation for 12 weeks at 50°C as measured by intrinsic fluorescence. Stern-Volmer fluorescence quenching constants (K_{SV}) measured after reconstitution of lyophilized capsomere formulations (prepared either with or without aluminum hydroxide adjuvant) were the same as those observed in liquid formulations prior to lyophilization, and did not change after 12 weeks incubation at 50°C in the glassy state. K_{SV} values for capsomeres unfolded in 8 M urea or for free tryptophan were much larger, indicative of greater solvent accessibility to normally buried tryptophan residues. K_{SV} values and associated error bars represent mean ± standard deviation, n=3.

incubation at 50°C in the lyophilized, glassy state (Figure 4.4.2). The positive control was a fresh sample of the HPV16 L1 capsomeres and the negative control was a polyomavirus capsid protein, VP1, a structural equivalent to L1 [145, 154].

Microflow imaging analysis showed that placebo formulations containing only trehalose and buffer salts showed low (ca. 10^4/ml) levels of particles of size greater than 2 μm, and no changes in this background level were detected after lyophilization and reconstitution (Figure 4.4.2, panel 1). The concentration of particles >2 μm was slightly larger following lyophilization and reconstitution of adjuvant-free formulations of capsomeres, presumably to a small degree of capsomere aggregation (Figure 4.4.2, panel 2). Addition of suspensions of aluminum hydroxide adjuvant particles to placebo formulations increased the background concentration of particles, but lyophilization had no effect on the size distribution of these particles (Figure 4.4.2, panel 3). In comparison, when suspensions aluminum hydroxide adjuvant particles were added to formulations containing capsomeres (Figure 4.4.2, panel 4), the concentration of particles >2 μm increased by approximately an order of magnitude, likely because capsomeres induced agglomeration of smaller aluminum hydroxide particles via "bridging" interactions. Lyophilization of samples containing both capsomeres and aluminum hydroxide slightly decreased the concentration of particles >2 μm. For all lyophilized samples, particle size distributions were essentially unaffected by 12 weeks of incubation at 50°C (Figure 4.4.2).

4.4.3 Immunogenicity of HPV16 L1 vaccines

Immunogenicities of HPV16 L1 vaccine formulations were quantified and compared against immunogenicities of commercial HPV VLP-based vaccines by measuring total anti-HPV16 L1 capsomere antibody titers by ELISA, and determining neutralizing antibody titers with a pseudovirus neutralization assay. A dose-dependent response was seen for reconstituted, lyophilized HPV16 L1 vaccines following administration of 7, 5, 3, or 1 μg doses of capsomeres, for Gardasil® following administration of 5, 3, or 1 μg doses of HPV16 VLPs and for Cervarix® following administration of 4, 3, 2, or 1 μg doses of HPV16 VLPs, as shown in Figure 4.4.3. All of the doses administered

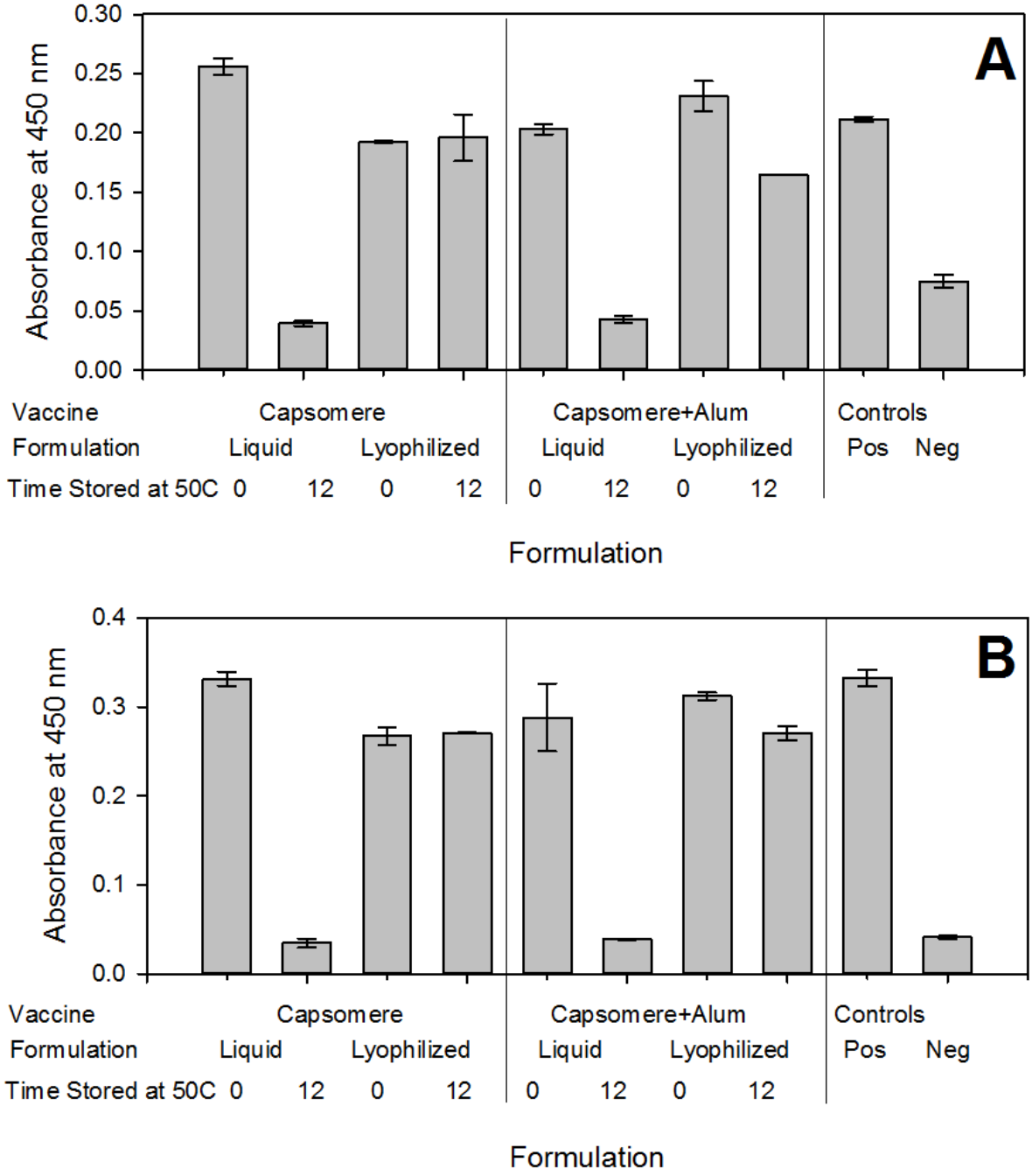

Figure 4.3: Retention of critical epitopes in capsomeres after lyophilization and incubation in the glassy state. Lyophilized capsomere formulations with or without aluminum hydroxide adjuvant retained antibody reactivity with L1 (A) and V5 (B) measured by ELISA-based assays, even when incubated at 50°C for 12 weeks. In contrast, liquid formulations lost reactivity after incubation. Positive controls were freshly prepared HPV16 L1 samples and negative controls were samples containing the polyomavirus structural protein VP1. Results are shown as the mean ± standard deviation, n=2.

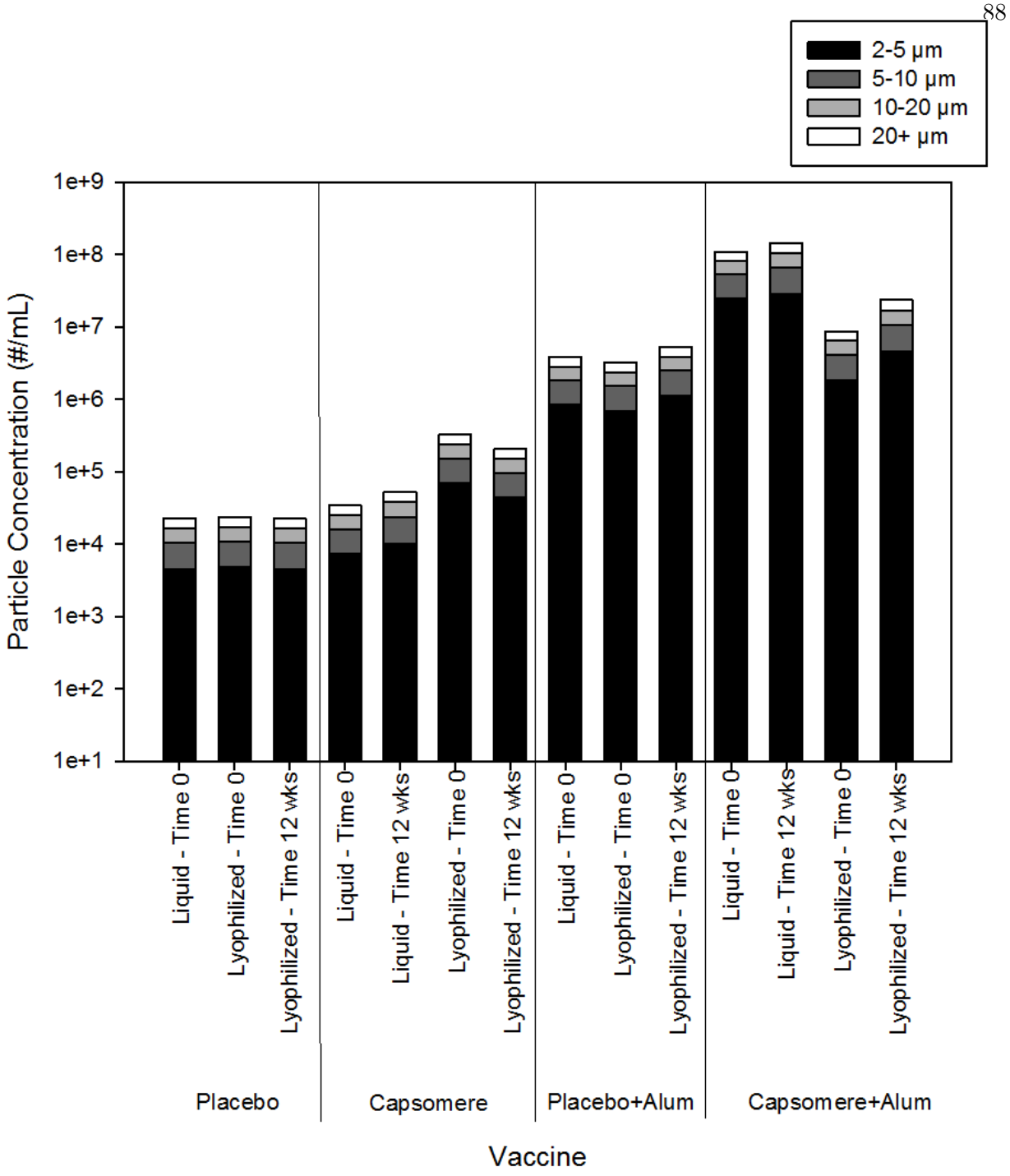

Figure 4.4: Particle size distributions in heat-treated vaccine formulations. The particle size and concentration of particles of size greater than 2 μm in placebo, capsomere, placebo and aluminum hydroxide, and capsomere and aluminum hydroxide formulations did not change after lyophilization or after incubation at 50°C for 12 weeks in the glassy state. Particle concentrations are shown for particles 2 to 5 μm (black), 5 to 10 μm (dark gray), 10 to 20 μm (light gray), and >20 μm (white). Particles size distributions are reported as the mean of three measurements for each sample. Compared with particle size distributions observed in initial liquid samples, lyophilization and reconstitution induced only minor changes in particle size distributions. In addition, particle size distributions in lyophilized samples were largely unaffected by 12 weeks of incubation at 50°C.

were in the linear range for the murine model used.

Lyophilized vaccine formulations containing HPV16 L1 capsomeres without adjuvants elicited anti-HPV16 L1 antibody titers of 10^3 to 10^4, and 10^5 after one and two doses respectively (Figure 4.4.3A). Addition of aluminum hydroxide increased immune responses after both one and two injections (p0.05), except for the 5 μg dose after two injections (p=0.46). The addition of GLA to formulations already containing aluminum hydroxide did not significantly increase the antibody titers (p>0.05) observed after either one or two injections. Neutralizing titers (Figure 4.4.3B) were approximately one order of magnitude lower than the anti-HPV16 L1 titers, but followed a similar pattern. The lyophilized vaccines containing adjuvants performed equally well if not better than commercially available vaccines, based on levels of total anti-HPV16 L1 IgG and neutralizing antibody titers produced in immunized animals.

After incubation for 12 weeks at 50°C, lyophilized HPV16 L1 vaccines adjuvanted with aluminum hydroxide or with aluminum hydroxide and GLA produced anti-HPV16 L1 capsomere antibody titers in BALB/c mice similar to those elicited by their non-incubated counterparts (Figure 4.4.3). The 5 μg dose of vaccines containing capsomeres and aluminum hydroxide produced lower titers than non-incubated controls (p=0.008), but responses following the second dose were equivalent to those produced by non-incubated controls. Compared to non-incubated controls, lyophilized, adjuvant-free capsomere vaccines subjected to high-temperature incubation produced decreased anti-HPV16 L1 capsomere antibody titers when administered in 5 μg doses, but equivalent responses at all other doses. Neutralizing antibody titers elicited in response to lyophilized HPV16-L1 capsomere vaccines were unaffected by incubation at 50°C for 12 weeks, except for those generated in response to the second 5 μg dose of adjuvant-free capsomere vaccines (p=0.032). In contrast, anti-HPV16 L1 antibody titers and neutralizing antibody titers produced in response to 5 μg doses of Gardasil® decreased after high temperature incubation (p=0.04 and p=0.02, respectively), but the differences in anti-HPV16 L1 and neutralizing responses to 1 g doses were not significant (p=0.32 and p=0.39, respectively). Incubation of Cervarix® at 50°C for 12 weeks resulted in dramatic reductions in antibody titers (p <0.02) and nearly complete loss of neutralizing

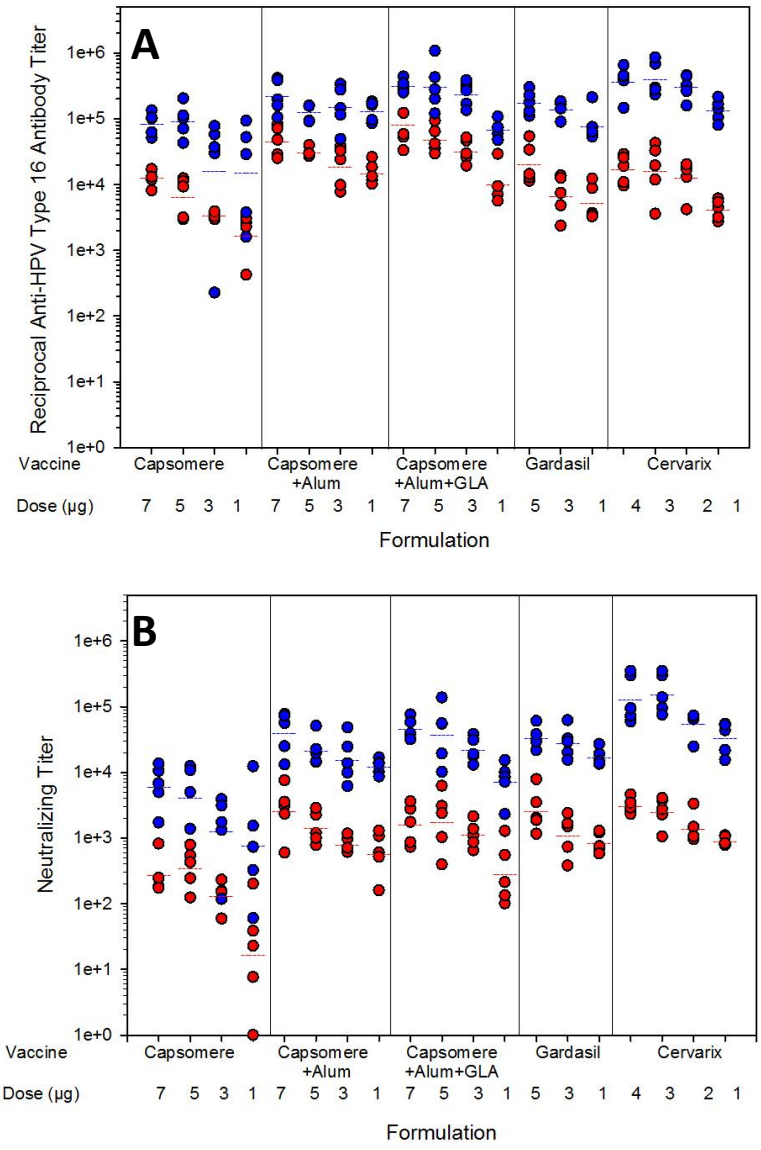

Figure 4.5: Antibody responses to vaccine formulations after immunization of BALB/c mice. Total anti-HPV16 antibody titers (A) were measured by ELISA; neutralizing antibody titers (B) were measured by the pseudovirus neutralization assay. Responses were measured following administration of one (red circles) or two (blue circles) injections. Horizontal bars represent the geometric mean for each group (n=5). From left to right, vaccine formulations contained capsomeres, capsomeres with aluminum hydroxide, capsomeres with aluminum hydroxide and GLA, Gardasil®, or Cervarix®. Adjuvanted capsomere vaccines produce dose-dependent anti-HPV16 antibody titers and neutralizing antibody titers that are similar to those produced by Gardasil®. Following the second dose, HPV16 antibody titers and neutralizing antibody titers produced by Cervarix® were higher than those produced by either Gardasil® or the adjuvanted capsomere vaccines.

titers ($p<0.033$).

4.5 Discussion

Highly effective HPV vaccines are commercially available, but their high cost and cold-chain requirements are barriers for their use in low resource regions of the world where the need is great [188]. HPV L1 capsomere-based vaccines may provide a lower cost alternative. The HPV VLP antigens in Gardasil® and Cervarix® use yeast or baculovirus-infected insect cells for production. In contrast, the ability to purify capsomeres after expression of L1 in *E. coli* may confer cost advantages in the manufacturing process.

HPV vaccines envisioned for use in low resource settings also must be highly efficacious. HPV16 L1 capsomere vaccines have been shown to protect mice from vaginal challenge with HPV16 [197] and, in the current study, HPV16 L1 capsomere vaccines adjuvanted with either aluminum hydroxide or both aluminum hydroxide and GLA elicited anti-HPV16 and neutralizing responses equivalent to those resulting from administration of the commercially-available HPV vaccines Gardasil® and Cervarix®.

Development of stabilizing formulations for the storage and delivery of therapeutic protein [68] and protein-based vaccines [100] remains a challenging endeavor. Lyophilization is widely used to stabilize therapeutic proteins [30]. In contrast, lyophilization is not used for any currently marketed vaccine that contains adjuvants, likely because vaccines containing aluminum salt adjuvants are typically thought to be susceptible to loss of immunogenicity caused by freeze-thawing. However, using the combination of controlled, rapid freezing rates and high concentrations of the glass-forming excipient trehalose, we produced lyophilized glassy vaccine formulations of HPV16 L1 capsomeres without any detectable degradation of capsomere protein. Once dried, the formulations exhibited glass transition temperatures near 100°C. Below this temperature, viscosities in the glassy state are more than 10^{15} times the viscosity of water [6], preventing reactions that might otherwise cause protein degradation and loss of vaccine efficacy. Lyophilized vaccine formulations appeared identical to initial liquid formulations based on TEM, front-face mode fluorescence quenching, and antibody

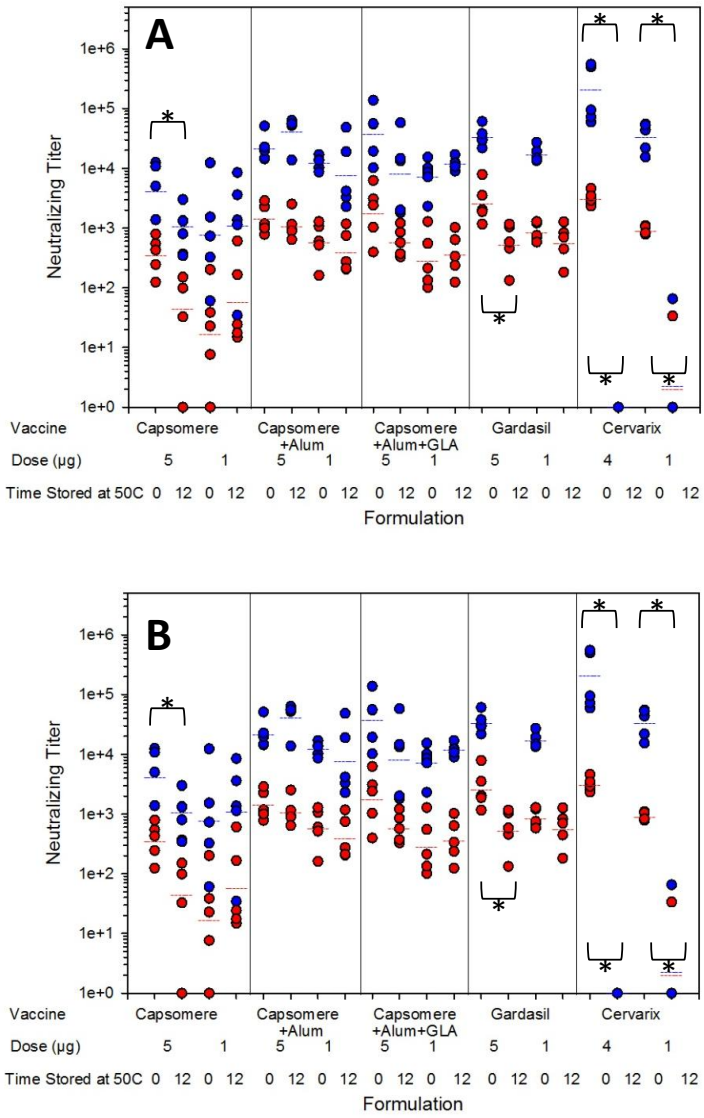

Figure 4.6: Immunogenicity of HPV16 vaccine formulations after incubation for 12 weeks at 50°C. Total anti-HPV16 antibody titers measured by ELISA (A) and neutralizing antibody titers measured by the pseudovirus neutralization assay (B) following one injection (red circles) or two injections (blue circles) of various HPV16 vaccines. Horizontal bars represent geometric mean titer values (n=5). Vaccines with significantly ($p < 0.05$) reduced antibody titers after high-temperature incubation (based on t-test for normally distributed groups or Mann-Whitney Rank Sums test for non-normal groups) are noted with an *. From left to right, vaccine formulations contained capsomeres, capsomeres with aluminum hydroxide, capsomeres with aluminum hydroxide and GLA, Gardasil®, or Cervarix®. Anti-HPV16 antibody titers and neutralizing antibody titers produced in response to both Gardasil® and Cervarix® were decreased when the vaccines were incubated for 12 weeks at 50°C. In contrast, anti-HPV16 antibody titers and neutralizing antibody titers produced in response to lyophilized, adjuvanted capsomere vaccines were unchanged after high-temperature incubation.

epitope reactivity, and yielded anti-HPV16 and neutralizing antibody responses equivalent to those generated by the initial liquid formulations when tested in BALB/c mice.

Gardasil® is reported to be relatively thermally stable, with a half-life at 42°C greater than 3 months [159], which is consistent with the roughly one order-of-magnitude decrease in anti-HPV16 and neutralizing titers we observed after 12 weeks of incubation at 50°C. The immunogenicity of Cervarix® was drastically reduced after incubation, even though previous work showed that minor interruptions in the cold chain do not affect the immunogenicity of Cervarix® [177]. Even after incubation in the glassy state after 12 weeks at 50°C, no alterations in capsomere structure were detected and the multimeric protein complex of L1 subunits remained intact. Additionally, the immunogenicities of adjuvanted HPV16 L1 capsomere vaccines were unaffected by 12 weeks of incubation at 50°C, demonstrating their exceptional stability at high temperatures. The high stability of these vaccines might entirely obviate the need for refrigerated transport and storage conditions, in turn greatly reducing costs for future HPV vaccine programs.

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Chapter 5

Conclusions

5.1 Lyophilization of vaccines

To avoid the narrow constraints of the cold chain, vaccines can be lyophilized. Three vaccines, a recombinant ricin toxin A (rRTA) subunit vaccine, a dominant negative inhibitor (DNI) anthrax vaccine, and a human papillomavirus (HPV) type 16 L1 capsomere vaccine were successfully lyophilized with the stabilizing excipient trehalose in the presence of aluminum hydroxide and glycopyranoside lipid A adjuvants. In the lyophilized state, these vaccine avoid the potential for freeze-thaw damage when exposed to temperatures lower than the recommended storage temperature since they contain minimal water. Additionally, these vaccines avoid damage caused by exposure to elevated temperatures by being trapped in a low mobility glassy matrix. By being able to transport vaccines over a significantly wider temperature range without a loss in efficacy, these vaccines may be delivered to patients much easier through the cold chain, especially in developing countries.

5.2 Immunogenicity of a ricin vaccine with variable aluminum hydroxide adjuvant particle size

Aluminum hydroxide adjuvant particles can be created to be different sizes based on the rate of freezing before lyophilization and the concentration of the glass-forming excipient trehalose used in the formulation. More rapid freezing rates, such as liquid nitrogen freezing methods or pre-cooled lyophilizer shelves, were able to better preserve the aluminum hydroxide adjuvant particle

size distribution after lyophilization and reconstitution. When higher concentrations of the excipient trehalose were used, the particle size distribution could be maintained even when slower rates of freezing, such as room temperature lyophilizer shelves were used. These particle size effects were seen in a histidine and volatile ammonium acetate buffer system.

Although literature has suggested that the size of adjuvant could potentially effect the immunogenicity of vaccines, the immune response observed was independent of the aluminum hydroxide particle size distribution. Similar immune responses were seen by total anti-rRTA, and ricin neutralizing antibody titers for aluminum hydroxide adjuvants particles with a mean of 1 and 10 microns in both buffer systems. These immune responses translated into equal protection during a challenge study.

Since the rRTA vaccines were formulated at a pH of 6, both the aluminum hydroxide adjuvant and rRTA protein had an overall positive charge. The similarity of charge between the antigen and adjuvant, resulted in ∼50% of the antigen adsorbing to adjuvant. Although half the protein was not associated with the adjuvant, an immune response was still mounted and animals were protected during a challenge study.

Through lyophilization with the excipient trehalose, rRTA vaccines were able to be stored in a glassy-state during incubation at 40 °C for 15 weeks. Lyophilized vaccines maintained a constant particle size distribution, glass transition temperature, water content, and immunogenicity after storage time points. A liquid vaccine stored at 40 °C for 3.5 weeks was no longer able to protect mice against ricin challenge, showing that the lyophilized vaccine has improved properties after incubation at an elevated temperature.

5.3 Effect of freeze-thaw cycles on an anthrax vaccine

In addition to potentially increasing in particle size during lyophilization, aluminum hydroxide adjuvant particles were also found to increase in size after freeze-thaw cycles. In the absence of trehalose, aluminum hydroxide adjuvant particles significantly aggregated after one freeze-thaw cycles and continued to aggregate with increasing freeze-thaw cycles. When the excipient trehalose

was added to formulations, aluminum hydroxide particle size was protected for one freeze-thaw cycle, but particle aggregation was seen after multiple freeze-thaw cycles.

After 1 or 5 freeze-thaw cycles, DNI protein was able to retain tertiary protein structure as detected by fluorescence melting temperatures with and without trehalose present in the formulation. Although both formulations retained tertiary structure, the fluorescence spectra was shifted 2 nm when trehalose was not present suggesting structural alterations. Additionally, deamidation was not detected in any vaccines exposed to freeze-thaw cycles.

DNI protein was able to completely absorb to aluminum hydroxide adjuvant and remain adsorbed even after 5 freeze-thaw cycles both with and without trehalose present in the formulation. After freeze-thaw cycles, vaccines were exposed to a phosphate buffered saline solution at 37 °C. Vaccines containing trehalose were able to desorb ∼20% of DNI protein, where vaccines containing sodium chloride show that DNI protein remains completely associated with the aluminum hydroxide adjuvant.

Freeze-thaw caused a decrease in immunogenicity of the DNI vaccines. A significantly greater decrease in immunogenicity was detected in formulations without trehalose and a greater number of freeze-thaw cycles. After one freeze-thaw cycle with trehalose present in the formulation, the immune response was equivalent to vaccine not exposed to freezing temperatures. After five freeze-thaw cycles, a decrease in antibody titers was detected but the vaccine still performed significantly better than the vaccine without trehalose. When sodium chloride was included in the formulation instead of trehalose, a significantly reduction in immunogenicity and seroconversion was detected after one freeze-thaw cycle and immunogenicity continued to decrease with more freeze-thaw cycles.

5.4 Effect of incubation at an elevated temperature on an anthrax vaccine

Liquid DNI vaccines were able to maintain tertiary structure when stored at 4 °C for up to 16 weeks but were not able to maintain structural integrity measured by a loss in fluorescence melting temperature at a storage temperature of 40 °C for one week. DNI protein not adsorbed to aluminum hydroxide adjuvant was slightly more resistant to tertiary structure changes by exhibiting

a fluorescence melting temperature up to one month at 40 °C. Lyophilized vaccines were able to remain structurally intact at 40 or 70 °C for up to 16 weeks, exhibiting a higher stability of the DNI antigen.

The immunogenicity of lyophilized vaccines was greatly improved in the lyophilized state after incubation at 40 °C when compared to liquid vaccines. Liquid vaccines stored at 40 °C for 8 weeks were able to produce high levels of anti-DNI antibodies, but failed to produce protective neutralizing antibodies. Lyophilized vaccines were able to produce both anti-DNI and neutralizing antibodies at levels similar to initial liquid and lyophilized vaccines.

5.5 Stability of a human papillomavirus vaccine at an elevated temperature

Lyophilized vaccines containing human papillomavirus type 16 L1 capsomere protein remained structurally intact after storage at 50 °C for 12 weeks measured by transmission electron microscopy, fluorescence stern-volmer constants, and conformational epitopes. Capsomere proteins were able to be lyophilized without conformational alternations. The L1 capsomere vaccine demonstrates that the lyophilization process works for more complex, larger protein antigens. Additionally, the lyophilized HPV type 16 L1 capsomere vaccines were equally as immunogenic as commercially available vaccines with respect to HPV 16 in a murine model.

5.6 Increasing immunogenicity with a toll-like receptor 4 agonist

Aluminum salt adjuvants are known to help promote humoral immune responses. The addition of the toll-like receptor 4 agonist, glucopyranosyl Lipid A (GLA) can help promote a cellular immune response. The GLA adjuvant was able to be lyophilized for the first time in the presence of an aluminum salt adjuvant in this work. GLA was able to increase antibody titers in the DNI vaccine. Vaccine formulations with GLA were able to have an immune response after one injection equivalent to the immune response after two vaccine injections when only aluminum hydroxide was included in the formulation. Additionally, DNI vaccines that contained GLA in addition to aluminum hydroxide had a significantly higher percentage of mice seroconvert after one vaccine in-

jection than mice receiving a vaccine containing only the aluminum hydroxide adjuvant. Although, GLA helped increase the immune response for the DNI vaccine, the L1 capsomere vaccine did not experience the same increases in immunogenicity showing that adjuvant effects may be specific to the vaccine antigen.

Chapter 6

Future recommendations

The work presented in this thesis shows promise for vaccines to be lyophilized in the presence of aluminum salt adjuvants, but much work can be done in the future to continue to develop vaccines of increased stability and immunogenicity. Aluminum salt adjuvants are just one type of vaccine delivery systems and other systems such as emulsions, polymer particles, or liposomes may be more effective for certain vaccines. Lyophilization strategies will need to be developed to preserve important characteristics of these vaccine formulations. Optimizing the lyophilization process may include the addition of excipients other than trehalose or modifications to the lyophilization cycle.

Although very few adjuvants are contained in approved vaccines, many new adjuvants are currently being investigated for use in vaccines. Each of these adjuvants many present challenges when formulated as part of a lyophilized vaccine. Additionally, methods are needed to characterize adjuvant degradation, activity, and association with antigen or a co-adjuvant such as aluminum salt. Any adjuvant included in a vaccine formulation needs to remain stable with constant properties throughout the storage of the vaccine.

To further investigate the effects of particle size on the vaccine immunogenicity, particles should be created from the same initial starting material with narrow particle size distributions. Since studies presented here contained aluminum hydroxide particles of a wide particle size distribution, it is hard to tell which particles are more immunogenic. The immune response of aluminum salt adjuvants may be further enhanced if an optimal particle size is found.

Degradation of vaccine antigen can occur by oxidation, aggregation, partial unfolding, hy-

drolysis or other pathways that were not included in this thesis and could be studied in future work. With more complete studies including as many degradation mechanisms and antigens as possible, the question of "What factors are the most critical for maintaining a protective immune response?" can be start to be answered. If we know what factors are most important for preserving the immune response during storage, we can tailor stabilization mechanisms to these areas.

To continue work on the HPV vaccine, other HPV types should be investigated such as 18, 6, 11, or other types infecting people in the area desired to administer the vaccine. Although 16 covers a large percentage of HPV that causes cervical cancer, we would ideally want a higher coverage rate in a vaccine. Creating a vaccine with multiple antigens could be difficult if each antigen differs in stability. Promising vaccines should be tested in an additional animal model to support results seen in a murine model.

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[200] Hang Yuan, Patricia A. Estes, Yan Chen, Joseph Newsome, Vanessa A. Olcese, Robert L. Garcea, and Richard Schlegel. Immunization with a pentameric L1 fusion protein protects against papillomavirus infection. Journal of Virology, 75:7848–7853, 2001.

[201] Mary I. Zapata, Joseph R. Feldkamp, Garnet E. Peck, Joe L. White, and Stanley L. Hem. Mechanism of freeze-thaw instability of aluminum hydroxycarbonate and magnesium hydroxide gels. Journal of Pharmaceutical Sciences, 73:3–8, 1984.

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Appendix A

Animal protocol 1103.07

This appendix contains University of Colorado IACUC animal protocol 1103.07 and protocol appendices 1-2. Protocol 1103.07 was used for animal studies with the ricin vaccine.

Reviewed by IACUC Department Representative MJM

Protocol No. 1103.07
(assigned by Animal Resources)

Date of Receipt 3/2/2011

APPROVED

IACUC Approval Date 5/20/2011

IACUC Expiration Date 5/20/2014

University of Colorado at Boulder
Institutional Animal Care and Use Committee
APPLICATION TO USE VERTEBRATE ANIMALS
FOR RESEARCH OR TEACHING*

Date: February 22, 2011

Department: Chemical and Biological Engineering

Principal Investigator: Dr. Theodore W. Randolph

Phone: 303-492-4776
Fax: 303-492-4341

E-Mail: theodore.randolph@colorado.edu

Faculty Responsible for Project (if appropriate):

Project (Course) Title:

Proposed Start Date of Project: April 2011

Projected End Date of Project: August 2011

NOTE: The proposed start date and projected end date do not indicate the actual approval period of the protocol.

Funding source: Soligenix Inc.
Grant number: Project #1546546

Important: The IACUC is required to compare the grant to the protocol. If this protocol is federally funded (e.g. NIH, NSF), please submit an electronic copy of the grant along with the electronic Word version of the protocol via email to richard.husser@colorado.edu

This is a:

_X_ New Application

___ Renewal/Continuation application – Please note: **All** addendums connected with this re-application are expired along with the original protocol.

*The IACUC meets on the 3rd Wednesday of each month. **All protocols must be filed with the IACUC Office by the 1st Wednesday of the month**. **To submit applications by interoffice mail address to "IACUC Administrator, 563 UCB". The office location is ARC, room 37)** Electronic copies of protocols and addendums are available from Animal Resources and online. For further information visit the Animal Resources website at: http://www.colorado.edu/VCResearch/integrity/animalcare/index.html

I. ABSTRACT

Summarize the objectives of this animal research or teaching project using **non-technical** language that a layperson can understand. Include the general methodologies used and the scientific or educational relevance of the project. **Do not use a grant abstract** because it is too specific.

Currently no FDA approved vaccines are both freeze dried and contain an aluminum salt adjuvant [1]. Freeze drying is important for vaccine formulations because it increases the stability of the vaccine, allowing for longer storage and shipping times at elevated temperatures. Aluminum salt adjuvants are important for vaccines because they can enhance the immune response of vaccine components that are cannot create a strong enough immune response on their own [2]. It has been previously shown that freeze dried vaccines containing an aluminum salt adjuvant do not produce a strong enough immune response to be used as a vaccine [3]. We would like to investigate why freeze dried vaccines that contain an aluminum salt adjuvant produce a low immune response so that in the future freeze dried vaccines with aluminum salt adjuvants can create a strong immune response.

Previously, the immune response of a liquid vaccine formulation containing a recombinant ricin toxin antigen and aluminum salt adjuvant has been proven to produce an immune response in mice, but the liquid form of the vaccine is unstable and cannot be stored for long periods of time [4]. Through the process of freeze drying the vaccine will be able to be stabilized. Freeze drying a vaccine containing an aluminum salt adjuvant can create particles larger than the initial size particles. Particles of a large size may not be able to produce a strong immune response. In this animal study we would like to look at the how to size of particles present in the vaccines and the amount of time and temperature at which the vaccine is stored affects the immune response in mice. The vaccine in a liquid formulation (not freeze dried) contains small particles and has previously been proven to produce a strong immune response. We would like to vary the size of particles in the vaccine formulation and determine if the size of particles has an effect on the strength of the immune response.

[1] U.S. Food and Drug Administration. Complete List of Vaccines Licensed for Immunization and Distribution in the US. FDA. 6/3/2010 <http://www.fda.gov/BiologicsBloodVaccines/Vaccines/ApprovedProducts/ucm093833.htm>.

[2] O'Hagen, Derek T. and Ennio De Gregorio. "The path to a successful vaccine adjuvant – 'The long and winding road'. " *Drug Discovery Today* 14.11/12 (2009): 541-551.

[3] Diminsky, Dvorah, Neomi Moav, Marian Gorecki and Yechezkel Barenholz. "Physical, chemical and immunological stability of CHO-derived hepatitis B surface antigen (HBsAg) Particles." *Vaccine* 18 (2000): 3-17.

[4] Peek, Laura J., Robert N. Brey and C. Russell Middaugh. "A rapid, three-step process for the preformulation of a recombinant ricin toxin A-chain vaccine." *Journal of Pharmaceutical Sciences* 96.1 (2007): 44-60.

II. SPECIFIC DETAILS ON ANIMAL USE AND CARE

1. Species (common name, genus and species)	Maximum number of animals used during project	Approximate number on hand at any time
a. Mouse, Swiss Webster	390	110
b.		
c.		
d.		

2. How long is your experience in working with this species?

During the Summer of 2008 I interned at IDEXX Laboratories and was responsible for IP, SC, and IV injections on mice as well as bleeding of the mice. Spring 2010 I attended the Animal Care and Use training conducted by the University of Colorado. I have also shadowed a senior PhD graduate student in her work with the mice at the University of Colorado.

3. Source of Animals* (CU, Jackson Labs, etc.)?

Taconic

> ***Animal transport and some biologically-derived products from an off-campus site may endanger the health of animal colonies on campus. Such transport requires prior approval by the veterinarian.** All antibodies which are produced *under contract* must be obtained from NIH-approved labs, and their animal welfare assurance number/USDA registration number must be submitted to Animal Resources. See section entitled "Animal Procurement" in the CU *Training and Reference Manual for the Care and Use of Laboratory Animals* or contact the Animal Resources Office at 492-8187 for more details. If your laboratory does not have a manual, contact Animal Resources to obtain a copy.

4. Buildings and rooms for housing:

a. Indicate where the animals will be housed.
Biology Building, WM Keck Foundation Laboratory, Transgenic facility.

b. Will you need to return rodents to the central animal facilities after taking them out for less than 24 hours.
No

b. If animals must be removed from housing area indicated above for more than 24 hours, give location and justification.
Animals will not be removed.

5. Buildings and rooms for procedures:

a. Provide the buildings and room numbers where experimental procedures will occur:
MCD Biology Building, WM Keck Foundation Laboratory, Transgenic facility A2B29

b. Will you conduct procedures at another institution? No
If yes, please explain.

Note: Ensure that you have prior approval before conducting work in a new location.

6. Do you plan to remove rodents from the central animal facilities (e.g. Psychology, MCDB, IBG, IPHY) for procedures and return them? No
If yes, please explain.

Note: before transporting animals to and from the animal facilities you must have approval from the animal facility manager to verify that the standard operating procedure is followed.

7. Person(s) responsible for daily animal care: Gail Ackerman

8. Who will conduct the non-surgical procedures? Kimberly Hassett

9. Will surgery be done? ___Yes _X_No. If "no", go to Item 15.

10. Will this project involve recovery from surgical anesthesia? ___Yes ___No

11. If the animal is to survive after surgery, appropriate surgical techniques and equipment must be used. For cats, rabbits and larger animals, this requires sterile surgical procedures (i.e., sterile instruments, gloves, drapes, etc.) done in a dedicated surgical facility. Surgical procedures on smaller rodents and other vertebrates do not require a special surgical facility, but should be done using sterile instruments and aseptic procedures. Indicate room where surgical procedure(s) will be done and how instruments are to be sterilized.

Room:

Sterilization method (e.g. steam autoclave):

12. Who will do the surgical procedures?

13. If you plan to use anesthetics, analgesics or tranquilizers to alleviate pain or distress, please supply the following*: (Use of analgesia is required during the post-operative recovery period after major survival surgical procedures--One analgesic is Buprenorphine (0.01 - 0.05 mg/kg for rats or 0.05 - 0.1 mg/kg for mice) injected either subcutaneously or IP, given once at completion of surgery. This can be repeated every 8-12 hours if required. **If analgesics are not to be used, provide detailed justification in Item III and check Category D)**

Route of

Species	Drug(s)	Dose Rate	Administration
a. Swiss Webster mice	isofluorane	3-5%	inhalation
b.			
c.			
d.			

* See section entitled "Anesthesia, Analgesia and Tranquilization" in the CU *Training and Reference Manual for the Care and Use of Laboratory Animals.*

14. Please outline type of postsurgical care and its frequency/duration.

15. Who is responsible for monitoring post-operative care or post-procedural care (e.g. surgery, or injection of tumor cells)?
Kimberly Hassett

16. Please provide any expected outcome from the procedures that could compromise the health of the animal such as illness, injury or abnormal behavior and how these outcomes will be managed (e.g. frequent monitoring, consultation with the veterinarian, euthanize animals if a tumor reaches a certain size, etc.)
Note: The Institutional Veterinarian should be notified of any unexpected deaths, illness, distress or other deviations from normal in animals.
No procedures are expected to compromise the health of the mice

17. Euthanasia of animals. For a discussion of acceptable methods of euthanasia, see report of the Panel on Euthanasia of the American Veterinary Medical Association, Journal of the American Veterinary Medical Association, Vol. 281, No. 5, 670-696. Also, see the section entitled "Methods of Euthanasia" in the CU *Training and Reference Manual for the Care and Use of Laboratory Animals.*

a. Briefly describe the method(s) of euthanasia to be used (CO_2 given to effect, cervical dislocation, etc.).

Species	Drug	Dose Rate	Route of Administration
a. Mouse, Swiss Webster	CO_2	1 liter per minute x 15 minutes	inhalation
b.			
c.			
d.			

b. If your methods of euthanasia differ from the above report, (i.e., not using tranquilizers or anesthetics before decapitation) describe why such a method is required.

c. Who will perform euthanasia?
Kimberly Hassett

III. SPECIFIC EXPERIMENTAL PROCEDURES INVOLVING ANIMALS

For each species, describe the general sequence of animal use procedures, including surgery, in the order in which they will be performed. Include only those experimental procedures which employ living animals.

All mice used for the experiment will be allowed at least one week to acclimate before any procedures are conducted. During the study, each Swiss Webster mouse will be bled three times and injected with a vaccine formulation twice. Before the initial injection mice will be bled and then on day 0 injected with a vaccine formulation. The initial bleeding is necessary so that each mouse can be its own baseline. 21 days later the mice will be bled and injected with a booster vaccine formulation. 35 days after the initial injection the mice will be bleed one last time.

Before bleeding procedures the mice will be anesthetized using is isofluorane inhalant. The mice will have their noses held in a tube with a steady flow of 5% isofluorane until they have taken at least 10 breaths and gone limp at which point the flow will be reduced to 3-4% isofluorane. During the bleeding and injection process the heart rate of the mice will be monitored visually and the mouse nose will be positioned towards the isofluorane to insure that the mice stay anesthetized during the procedures. Before procedures are conducted on the mice, a toe pinch will be given to the mice to make sure that they are adequately anesthetized.

Blood will be drawn from the retro-orbital venous sinus of the mice. A drop of proparacaine will be put on the eye from which blood will be drawn and then blood will be collected using 50μL capillary tubes. Approximately 100-200μL of blood will be drawn during each bleeding. To make sure that mouse's health is not compromised by removing blood, less than 20% of the mouse's blood will be removed during a bleeding. Mice will be weighed before the bleeding and it will be assumed that 7% of the mouse weight is blood. After collection of blood, sterile gauze will be used to gently blot the eye, a drop of proparacaine will be applied, and the eye will be gently held shut for 1-2 minutes. The serum removed from the mice will be analyzed for antibody content.

The two injections will be given to the mice following bleeding. The injections will be given subcutaneously on the back of the neck. The injections will be given s.c. Previous studies with this protein have been given both i.m. and s.c. and we have chosen to do s.c. injections because they are less invasive and easier to do properly. The injection volume will 50 uL, and the dose will contain 10 ug of protein. Vaccine formulation will contain recombinant ricin toxin antigen (rRTA) that has been expressed in *E. Coli*, the protein has been genetically modified with two point mutations to be non-toxic, and purified to be endotoxin free. Different experimental and control injections are listed below. After injection, mice will be placed back in their cage.

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

*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 will be used. All samples will contain the disaccharide trehalose.
** Alhydrogel is an aluminum hydroxide vaccine adjuvant.

During the experiment, mice will have free access to drinking water and food.

An initial study will be conducted with negative controls, positive control and experimental groups 1-4 at time 0 (no storage time). Follow-up studies will be completed only with experimental groups 1-4. In these studies vaccine formulations will be stored at two temperatures for four time points over six months and will be injected into mice. Each group will contain 10 mice.

# of Time 0 Mice
7 groups of mice x 10 mice/group = 70 mice

# of Additional Time Point Mice
2 storage temperatures x 4 time points/temperature x 4 groups of mice/time point x 10 mice/group = 320 mice

IV. DISCOMFORT-PAIN CATEGORIES / JUDICIOUS USE OF ANIMALS / SAFETY

The answers to the following questions are necessary for the Institutional Animal Care and Use Committee to fulfill its obligations under the Public Health Service Policy. Investigators and course directors are requested to categorize their use of vertebrate animals based on the discomfort or pain involved. Consideration should be given to methods that result in a lesser degree of unavoidable pain or discomfort, and the use of the smallest number of animals consistent with meeting the scientific or educational objectives. It is also necessary to provide assurance that animals and personnel working with animals are not unduly exposed to a hazardous environment.

1. Please check ALL of the discomfort/pain categories that apply to your research project.

_X_ **CATEGORY A**: Project involves little or **NO** discomfort or pain.
- _X_ Subcutaneous, intraperitoneal or intramuscular injections of somewhat harmless substances
- _X_ Blood withdrawal in small amounts (less than 20% of total blood volume)
- _X_ Antibody and monoclonal antibody production
- __ Studies of embryonated eggs
- __ Behavioral testing or observation that does not involve restraint or exposure to a noxious stimulus
- __ Use of euthanasia that results in rapid loss of consciousness and death (e.g., to obtain a tissue sample)
- __ Other:

__ **CATEGORY B**: Procedures that involve **SOME** discomfort or pain.
- __ Ethanol diet
- __ Chronic drug treatment causing addiction
- __ Implantation of chronic cannula, subcutaneous implants, catheters or electrodes under anesthesia
- __ Surgical procedures under general anesthesia that could result in a functional deficit or postoperative pain limited to the immediate postoperative period
- __ Physiological or behavioral studies on conscious animals involving short-term stressful restraint (<4 hrs) which includes the stress associated with live-capture of animals in the wild

__Exposure to a noxious stimulus from which escape is still possible
__Abnormal behavior resulting from excessive crowding (e.g., increased aggression) or social isolation.
__Studies conducted on completely anesthetized animals that do not regain consciousness
__Deprivation of food and/or water for about 12 to 24 hours
__A procedure not listed above that may result in some discomfort or pain--please describe in a phrase or sentence:

__**CATEGORY C**: Procedures that may result in **SIGNIFICANT** discomfort or pain.
__Drug treatment that is apt to cause seizures, etc.
__Major surgical procedures under anesthesia that could result in substantial postoperative pain, discomfort, or permanent functional or behavioral deficit
__Exposure to a noxious stimulus from which escape is not possible
__Prolonged physical restraint lasting longer than 4 hours
__Imposition of stress as indicated by significantly abnormal behavior (excessive fighting, self-mutilation)
__Prolonged deprivation of food and/or water resulting in a 20% loss in body weight
__A procedure not listed above with risk of significant pain or discomfort--please describe in a phrase or sentence:

__**CATEGORY D**: Procedures that produce pain in which, for experimental reasons, anesthetics, or analgesics cannot be used. Example: Toxicity testing with death as an end point. **(Must be <u>thoroughly</u> described and scientifically justified in the previous Item III.)**

2. Judicious Use of Animals

a. Could mathematical models, computer simulation or <u>in vitro</u> biological systems be used as alternatives to the use of animals in this project? Explain. See section entitled "Replacement" in the CU *Training and Reference Manual for the Care and Use of Laboratory Animals.*

Due to the complex nature of how the immune system functions, computer simulations and in vitro biological systems cannot be used.

b. Justify the use of the animal species listed. Describe the biological characteristics of the animal that are essential to the project.

Mice are an appropriate model for this study because they are relatively inexpensive and have immune systems capable of producing a response to the vaccine formulations being tested. The serum that is drawn for the mice will be used to detect levels of antibody produced against the vaccine antigen.

Swiss Webster mice are being used because previous studies have been conducted in them and by having our study also in Swiss Webster mice we will be able to easily compare our results to previous work and not have an additional variable of type of mouse change.

c. Justify the number of animal used. Describe the size and number of experimental groups and the number of animals needed for procedure development. Specifically state why fewer animals cannot be used. See section entitled "Reduction of Numbers of Animals Used" in the CU *Training and Reference Manual for the Care and Use of Laboratory Animals.*

To be able to have groups of mice where a standard deviation can be created even if , 10 mice will be used in each group. The following experimental and control groups are required to adequately test the hypothesis of the experiment. Since the liquid formulation is known to create an immune response it will be used as a positive control. The negative controls will be freeze dried alhydrogel in buffer. The experimental groups are needed to create a variety of particle sizes. The experimental groups will be repeated after being stored at two temperatures and over five different time points to see how the storage

time and temperature effect the immune response.

# of Time 0 Mice

7 groups of mice x 10 mice/group = 70 mice

# of Additional Time Points Mice

2 storage temperatures x 4 time points/temperature x 4 groups of mice/time point x 10 mice/group = 320 mice

Total= 390 mice

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

d. Are any State or Federal permits required (e.g., permits to obtain wild animals, importation permits for animal tissue, etc.)?

_X_No ___Yes If "Yes," submit copies of all permits with the application.

e. If this is a field study, please submit your standard operating procedure for the field that includes safety procedures for personnel and decontamination of study equipment between study sites if applicable.

The following Items 2f and 2g pertain only to projects involving animals covered by the Animal Welfare Act (cats, dogs, rabbits, guinea pigs, hamsters, gerbils, non-human primates and other mammals normally found in a wild state, e.g., deer mice or ground squirrels).

f. For projects that may cause more than momentary or slight pain to the animals involved (Categories B, C, or D), provide a written narrative describing the methods and sources (e.g., biological abstracts, Index Medicus, Current Research Information (CRIS), Animal Welfare Information Center, etc.) used to determine that less distressful alternatives were not available.

g. Provide written assurance that the proposed activities do not unnecessarily duplicate previous experiments. USDA requires that the investigator make a reasonable good faith effort in determining that a proposed experiment is not unnecessarily duplicative. For this assurance, you may choose to cite information from the introduction, significance or bibliographic sections of your grant proposal or any other material that may be relevant to this question.

Although a liquid formulation of the recombinant ricin toxin antigen vaccine has already been created and tested in mice [1], the processing step of freeze drying to stabilize the vaccine has potential to drastically alter the immunogenicity of the vaccine and needs to be tested in again in mice. Amber Clausi et al. has done previous studies that looked at the immunogenicity of freeze dried vaccines using the model proteins lysozyme and alkaline phosphatase, but not the recombinant ricin toxin antigen [2,3]. The vaccines tested by Clausi et al. used different vaccine antigens, buffers and slightly different freeze drying processing techniques which could all affect the immune response of the vaccine. Since the immunogenicity of each vaccine is very closely related to its components and processing, the purposed

activities in this protocol do not unnecessarily duplicate previous experiments.

[1] Peek, Laura J., Robert N. Brey and C. Russell Middaugh. "A rapid, three-step process for the preformulation of a recombinant ricin toxin A-chain vaccine." *Journal of Pharmaceutical Sciences* 96.1 (2007): 44-60.

[2] Clausi, Amber, Andrea Morin, John F. Carpenter and Theodore W. Randolph. "Influence of protein conformation and adjuvant aggregation on the effectiveness of aluminum hydroxide adjuvant in a model alkaline phosphatase vaccine." *Journal of Pharmaceutical Sciences.* 98.1 (2009): 114-121.

[3] Clausi, Amber, Jessica Cummiskey, Scott Merkley, John F. Carpenter, LaToya Jones Braun and Theodore W. Randolph. "Influence of particle size and antigen binding on effectiveness of aluminum salt adjuvants in a model lysozyme vaccine." *Journal of Pharmaceutical Sciences.* 97.12 (2008): 5252-5262.

3. Safety of Animals and the Personnel Working with Animals.

IACUC - EH&S Training, Compliance, & Safety Checklist

Required Training and Compliance Review -

Animal Care & Use: The basic animal care and use training is required for anyone working with laboratory animals on campus. All research involving the use of animals, **to include transgenic animals**, must be reviewed and approved by the Institutional Animal Care & Use Committee (IACUC) prior to the commencement of the research.

Have you and everyone who will be working with the animals described in this application attended the CU Training Program on Animal Use and Care?* X Yes __ No

- If No, If "no", you must either: 1) attach a detailed description of your past training and clearly establish that it is equivalent to CU's program or 2) register and attend a training session.

Failure to comply with either option may result in non-consideration of your protocol. Contact the Animal Resources Office for the training schedule at 303 492-8187, http://www.colorado.edu/VCResearch/integrity/animalcare/index.html

Every investigator needs to recognize that if you pursue activities for which you do not have current IACUC approval, that the CU administration and the IACUC are empowered to shut down all research activities in your laboratory, either temporarily or permanently dependent upon the gravity of the violation(s). Noncompliance is a very serious issue and one that could put all animal research at the University of Colorado in serious jeopardy. Such activities have a very real probability of the Office of Laboratory Animal Welfare halting all animal research across the entire university, so noncompliance by investigators is not tolerated.

Do you acknowledge that you have read and understand the previous statement regarding NON-COMPLIANCE:

X Yes, I acknowledge that I have read and understand this section.

Hazardous Waste: It is the policy of the University of Colorado at Boulder that all persons (faculty, student, and staff) involved with the generation, handling and management of Hazardous Waste on Campus shall be properly trained, including those who supervise personnel who generate/manage waste.

Will you or anyone in your lab be generating, handling, or managing hazardous waste? __Yes _X_ No

1.) If Yes, has everyone received the initial Hazardous Waste Generator training and the annual refresher training? __Yes __ No
2) If No, please contact EH&S at 492-6025.

Institutional Biosafety Committee (IBC) Application, Bloodborne Pathogen Training, and Biological Material Shipping Training: It is the policy of the University of Colorado at Boulder that research involving human blood, blood products, reagents

derived from blood, tissue, saliva, sputum, etc. or other potentially infectious materials from humans or animals be reviewed and approved by the IBC committee and complete bloodborne pathogen training from EH&S. If your research includes the shipping or receipt of these types of materials, then biological material shipping training from EH&S is required.

Does your research involve the collection or use of human blood, blood products, reagents derived from blood, human specimens such as tissue, saliva, sputum, etc. or other potentially infectious materials from humans or animals? __ Yes _X_ No

1.) If Yes, has an Institutional Biosafety Committee (IBC) Application been submitted and Approved? __ Yes __ No
- If No, please contact EH&S at 492-6025.
- If Yes, please list date of submission or approval: _____

IBC Application has been: Submitted ☐ Approved ☐

2.) If Yes, has everyone completed bloodborne pathogen training from EH&S?* __Yes __No
- If No, please contact EH&S at 492-6025.

Does your research involve the shipping or receipt of human blood, blood products, reagents derived from blood, human specimens such as tissue, saliva, sputum, etc. or other potentially infectious materials from humans or animals? __ Yes _X_ No

1.) If Yes, has everyone received the biological material shipping training from EH&S? __ Yes __ *No
*If No, please contact EH&S at 492-6025.

Use of Human Subjects or Human Tissues: All research involving human participants that is conducted by UCB faculty, staff or students must receive some level of review by the Institutional Review Board.

Does your research involve collection, receipt, or use of human specimens? __ Yes _X_ No

1.) If Yes, has the project been reviewed and approved by the Institutional Review Board?* __ Yes __ No
*If No, please contact the Institutional Review Board at 735-3702;
http://www.colorado.edu/VCResearch/integrity/humanresearch/index.html

Radioactive Materials: It is the policy of the University of Colorado at Boulder that that all persons (faculty, student, and staff) involved with the use of radioactive materials is authorized and properly trained.

Will radioactive materials be used as part of the research listed in this Application? __ Yes _X_ No

1.) If yes, are you licensed for the possession and use of each radioisotope? __ Yes __ No
* If No, please contact EH&S-Health Physics at 492-6523.

2.) Have all persons involved with the use of radioactive materials received training from the Radiation Safety/Health Physics group at EH&S? __ Yes __ No

* If No, please contact EH&S-Health Physics at 492-6523.

X-ray Machines: It is the policy of the University of Colorado at Boulder that that all persons (faculty, student, and staff) involved with the use of X-ray Machines is authorized and properly trained.

Will X-ray Machines be used as part of the research listed in this Application? __ Yes _X_ No

1.) If yes, are you licensed for the possession and use of each machine? __ Yes __ *No
* If No, please contact EH&S-Health Physics at 492-6523.
2.) Have all persons involved with the use of X-ray Machines received training from the Radiation Safety/Health Physics group at EH&S? __ Yes __ *No
* If No, please contact EH&S-Health Physics at 492-6523.

Safety Review –

It is ultimately the principal investigator's responsibility to ensure all lab and animal husbandry personnel are made thoroughly aware of the potential hazards of any chemical, biological, or radiological materials, hazardous gases (i.e. anesthetics) or procedures being used in their research or laboratories. If you are unsure of the risk involved, contact Environmental Health and Safety (EH&S) at 492-6025 for more information.

Will the use of any of the chemical, biological, or radiological materials, hazardous gases (i.e. anesthetics) or procedures listed in this application pose a direct hazard to health or safety of the animal colony or personnel working with animals? _X_ Yes __ No

If yes, please list the hazards, explain how they will be mitigated, and provide an MSDS is available:

Isofluorane will be used to anesthetize the mice. To reduce hazard, a low flow rate of isofluorane will be used and the room where procedures are conducted will be well ventilated.

The isofluorane will be delivered to the mice in a well ventilated surgery room. When the mice are first administered the isofluorane in a box to initial put them to sleep, the isoflurane goes into the box through one tube and is then captured going out the box by another tube with a filter. When the mice are removed from the box for bleeding and injections their nose will be positioned in a cone receiving isofluorane in one side and then a stream of isofluorane will be removed out the other side with a filter. A small amount of the isofluorane could potentially escape the system in the cone where the mouse nose is positioned in which having the room well ventilated is important but the majority of the isofluorane will the captured in filters.

_____ Based on the the article by Ellen S. Vitetta, et. Al. (PNAS, Feb 14, 2006, Vol 103, No. 7), 10 microgram of this protein did elicit an immunogenic response in healthy adults. Therefore, lab personnel must be made aware of the potential hazard if self-injected and that they should seek immediate follow-up medical care if they experience any reaction to this protein. An individual immunogenic response can vary greatly based on their sensitivity and possible previous exposure history.

V. PROTOCOL APPROVAL

1. Approval for Scientific/Instructional Merit

Before starting any project, it must first be reviewed and approved for scientific or educational merit, either externally (see "a" below) or internally (see "b" below). The IACUC does not review projects for scientific merit, except where it bears upon humane care/use of animals.

a. This project will be reviewed and approved for scientific/instructional merit by:

Federal Agency (specify NIH, NSF, FDA, etc.)_____

Non-governmental agency (specify)_____

b. For all other projects, the chair's signature is required prior to submission of the protocol to the IACUC. Even preliminary studies require this approval.

Approval by the Chair:

I assure that this project has been reviewed and approved for scientific / instructional merit by:

✓ Chair's own review

___Chair's expert designate's review_____(Name)

___Other (describe)

_____ Date 3/3/11

Signature of Department Chair or Official Designate

2. Check One of the Following and then Sign under P.I./Course Director

Animals Used for Research

___ I certify that the foregoing statements are true and that the protocol is essentially the same as found in the grant application or program/project. The Institutional Animal Care and Use Committee (IACUC) will be notified of any changes in the proposed project prior to proceeding with any animal experimentation. No work may commence until approved by the IACUC. Any persons performing procedures on animals involved in this project will be appropriately trained and will be made aware of this animal protocol.

Animals Used for Teaching

___ I certify that the information in this application is essentially the same as contained in the course outline and a copy of the laboratory exercises using animals is on file with the IACUC Office. No work may commence until approved by the IACUC. Any persons performing procedures on animals involved in this course will be appropriately trained and will be made aware of this animal protocol.

IACUC *Adverse Event* Statement:

The investigator will notify the IACUC (303-492-8187) of any unexpected results that adversely affect the welfare of the animals. Investigator will report any unanticipated pain or distress, morbidity or mortality to the attending veterinarian and the IACUC.

If there is an adverse event involving the health and/or safety of any research or animal handling personnel, the investigator will contact the IACUC and EH&S (303 492-7072), and complete a University Risk Management *Employee's Injury Report Form* (https://urm.colorado.edu/docs/forms/incident_report_form.asp), within 48 hours of the event. Investigator will also provide the IACUC (UCB 345) and EH&S (UCB 413) with a copy of this completed form.

Signature of Principal Investigator/Course Director — Date: 3-3-11

Signature of Advisor/ responsible faculty (If applicable) — Date

Chair, Institutional Animal Care and Use Committee — Date: 3-16-11

D.V.M.
Director, Department of Animal Resources — Date: 3-16-11

Institutional Animal Care and Use Committee, University of Colorado-Boulder
IACUC Administrator Email: richard.husser@colorado.edu Campus Box: 563 UCB Ph: 303-492-8187

APPROVED ADDENDUM

Submitting the addendum:

1) Submit DIRECTLY to the IACUC Administrator and not the department representative.
2) **Email the completed Word version of the Addendum to the IACUC Administrator, Richard Husser** richard.husser@colorado.edu
3) **For principal investigators who are not CU Boulder Faculty, the advisor must be copied on the email to the IACUC Administrator.**

Addendum #1

Date: 5/15/12
Principal Investigator: Theodore Randolph
Responsible Faculty (for anyone who is not a UCB faculty member):
Department: Chemical and Biological Engineering
Title of protocol (required): Evaluating the immunogenicity of freeze dried recombinant ricin toxin antigen (rRTA) vaccines containing a range of particle sizes
Protocol Number: 1103.07
Date of Protocol Approval: 5/20/11

Please answer all questions below unless stated otherwise.

1. Briefly list the reason(s) for this addendum. **For a change in the principal investigator of the protocol, please contact the IACUC Administrator for instructions**. **For a change in animal numbers ONLY, go directly to #7-9.**
   6 mouse blood samples were broken in the centrifuge and we would like to collect blood through a terminal bleed for these 6 mice so that a sample can be obtained. This is the last step of this protocol (week 5) in the series of week 0 – bleed/1st vaccine injection, week 3 – bleed/2nd vaccine injection, week 5 – bleed.

2. **TIER 1: Administrative changes (Estimated review time: 1-3 days).** Are you changing one or more of the following aspects of the protocol? If yes, please explain the reason for each change (a-d).
   a. Protocol title change: No
   b. Funding source: No
   c. Location of experiments or animal care facility (contact the facility manager where you will be transferring animals to and get approval before submitting the addendum) No
   d. Addition or removal of personnel to a protocol (**Personnel must be properly trained prior to conducting animal research which includes IACUC training and individual hands-on training**): No

3. Are you making any changes other than, or in addition to, those in 2 above? Yes
   If not, go to #7-9.

4. **TIER 2: Minor scientific changes (Changes that will most likely result in minimal or no increase in pain and distress to the animal).** If you are changing any of the following aspects of the protocol, explain reasons for each (a-e). **Estimated review time: 3-10 days (If you are not submitting Tier 3 changes (5), go to question 6-9)**
   a. Sex, age, or strain of species already approved for the protocol (excluding immunodeficient or transgenic animals - see below): No
   b. Drugs or methods used to induce anesthesia, analgesia, or euthanasia (include any disposal/safety issues): No
   c. Field studies requiring brief capture, sample/data collection, &/or tagging: No
   d. Addition of innocuous, non-invasive procedures such as behavioral procedures or giving palatable food to non-deprived animals: No

e. Terminal procedures conducted under surgical plane of anesthesia: Yes
Under anesthesia of isofluorane I would like to perform a cardiac puncture to the 6 mice missing serum samples. After the procedure the mice will be euthanized.

5. **TIER 3: Major scientific changes (A**ll other changes).
Estimated review time: 10-14 days unless the addendum has to be sent to the full committee

Examples:
- Change in time course (duration and frequency) of procedure or drug delivery
- Change in species; addition of transgenic or immunocompromised animals
- Adding new procedures or combining previously approved procedures
- Addition of new drugs or drug doses
- Change in site of drug administration (e.g., changing brain cannulation site; change from systemic to microinjected)
- Adding procedures, not previously approved, that involve unrelieved pain and distress
- Adding sampling of body fluids or tissues, where animals are conscious or will regain consciousness
- Change in surgical plans (minor to major survival surgery, multiple survival surgeries, additional procedures)

Describe the changes below for Item 5:
Adding new procedures to previously approved procedures: Under anesthesia I would like to perform a cardiac puncture on the mouse and then euthanize the mouse for only the 6 mice missing serum samples.

6. Provide scientific justification for the changes. If applicable, cite references and previous experience to provide support for the changes.
6 of the mice are missing serum for the last time point of the study and to have a more complete set of data for each group of mice, the last serum sample would be needed.

7. Increase in animal numbers:
   a. What is the total number of each species added to the protocol by this addendum? 0

   b. Provide a justification for additional animals, including the numbers needed to be added in each group of the experimental design.

8. Animal health:
   a. Do you expect animals to experience more clinical illness, pain, or distress, or any other health related issues as a result of the procedures proposed in this amendment?
   If yes or you are not sure, please explain and include whether there is a need to increase monitoring of the animals due to these changes.

   No, since the animals will be under anesthesia when the procedure occurs and then directly euthanized no increased pain, distress or illness is expected.

   b. Explain how these outcomes will be managed to minimize the pain and distress for the animals. Note: The Institutional Veterinarian should be notified of any unexpected deaths, illness, distress or other deviations from normal in animal. Contact: Albert Petkus, DVM at 303-492-3411 or albert.petkus@colorado.edu

   The animals will be kept under anesthesia for the cardiac puncture and then euthanized so that the mice should not wake up.

9. Do you expect there to be any other concerns resulting from this addendum that are not listed above, such as: personnel safety, biosafety (includes rDNA experiments; introduction of biological agent or cell lines in-vivo or in-vitro; producing or using transgenic animals), additional disposal, additional hazardous/radioactive materials (including human or non-human animal tissues or blood), or animal transportation? NO

Please explain and address how you will manage these concerns. For changes in personnel safety, biosafety, hazardous/radioactive materials or disposal, contact Denise Donnelly at 303-492-6025 or EHSBIO@colorado.edu

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For IACUC use only:
Review method: DMR
Addendum approval Date: 5/16/2012

IACUC Signature for Approval

Form last updated: 02/04/2011

This addendum, once approved, will expire with the protocol.

9/20/2013 Version

University of Colorado at Boulder
Institutional Animal Care and Use Committee
VERIFICATION OF EUTHANASIA AFTER USING GAS OR INJECTABLE FORMS OF EUTHANASIA IN RODENTS

Protocol Number: 1103.07
Addendum 2
Principal Investigator: Theodore Randolph
If applicable, Responsible Faculty:
Protocol Title: Evaluating the immunogenicity of freeze dried recombinant ricin toxin antigen (rRTA) vaccines containing a range of particle sizes

It is standard veterinary practice to perform a secondary method of euthanasia when using gas or injectable anesthetics in order to ensure death. The Guide for the Care and Use of Laboratory Animals (8th Edition) states that death must be confirmed in an animal after euthanasia, and that a secondary method of euthanasia can be used to ensure death. The AVMA Guidelines for the Euthanasia of Animals (2013 Edition) states that a secondary method of euthanasia can be performed after an overdose of inhaled anesthesia as part of a two-step euthanasia process. Please fill out this addendum to state in your protocol which secondary methods of euthanasia you utilize after rodents are euthanized via inhalation or injection. If helpful, please see the IACUC's Standard Operating Procedure #19 Carbon Dioxide Euthanasia.

Acceptable secondary physical methods of euthanasia for rodents are:

i. Decapitation using very sharp scissors or guillotine
ii. Cardiac perfusion
iii. Removal of vital organs (e.g. heart, lungs, brain)
iv. Bilateral Thoracotomy
v. Cutting the major blood vessels to induce exsanguination (e.g. aorta, vena cava)
vi. Cervical dislocation on adult rodents weighing less than 200 grams. Cervical dislocation is an inappropriate method to use on rats larger than 200 grams; and on neonates at any time prior to 21 days of age.

1) For the protocol number listed above, what is the species, primary method of euthanasia (carbon dioxide, injection with Euthanasia Solution, overdose with isoflurane, etc.) and secondary method of euthanasia you use?
We euthanize mice weighing less than 200 grams with carbon dioxide and our secondary method is cervical dislocation.

2) Can you ensure, once this addendum form is approved, that all rodents that are euthanized also receive a secondary physical method of euthanasia (as described in #1) by a person trained in these procedures? You will need to communicate this change, if it is a change, to all your research personnel trained to perform euthanasia.
Yes

If your lab needs training to conduct proper secondary methods of euthanasia, please contact the OAR Veterinary Technician Toni Mufford (toni.mufford@colorado.edu) to schedule a training session.
Per regulatory requirements, failure to comply with this policy may result in notification of your funding agency (e.g. NIH) and regulatory agencies (e.g. OLAW, USDA) that your research has violated federal and/or local policies regarding the humane use of animals. This notification may affect continuous funding of your animal-related research. Further, depending on the violation, you may be required to take additional training and/or your privilege to conduct animal research at UC Boulder might be temporarily suspended or even completely revoked.

PLEASE LEAVE BLANK-FOR IACUC OFFICE USE ONLY
Review Method: DMR
Addendum Approval Date: 10/29/2013
Cornella Dotweiler
IACUC Signature for Approval

Appendix B

Animal protocol 1209.02

This appendix contains University of Colorado IACUC animal protocol 1209.02 and protocol appendices 1-5. Protocol 1902.02 was used for animal studies with the anthrax and HPV vaccines.

University of Colorado at Boulder
Institutional Animal Care and Use Committee
APPLICATION TO USE VERTEBRATE ANIMALS FOR RESEARCH OR TEACHING*

Reviewed by IACUC Department Representative _____

Protocol No. 1209.02
(assigned by Animal Resources)

Date of Receipt 8/31/2012

APPROVED

IACUC Approval Date 10/9/2012

IACUC Expiration Date 10/9/2015

Date: August 14, 2012

Department: Chemical and Biological Engineering

Principal Investigator: Theodore Randolph

Phone: 303-492-4776

Fax: 303-492-4341

E-Mail: theodore.randolph@colorado.edu

Faculty Responsible for Project (if appropriate):

Project (Course) Title: Evaluating the immunogenicity of freeze dried anthrax vaccines using a variety of adjuvants

Proposed Start Date of Project: October 2012 Projected End Date of Project: October 2014
NOTE: The proposed start date and projected end date do not indicate the actual approval period of the protocol.

Funding source: NIH grant through Soligenix Inc
Grant number: UO1-A1-08-2210 (Project #1546546)

Important: Please submit an electronic copy of the grant, contract, or proposal along with the electronic Word version of the protocol via email to richard.husser@colorado.edu

This is a:

_X_ New Application

___ Renewal/Continuation application – Please note: **All** addendums connected with this re-application are expired along with the original protocol.

***See the last page for the submission checklist and details. For further information and for a list of the protocol submission deadlines visit the Animal Resources website at:**
http://www.colorado.edu/VCResearch/integrity/animalcare/index.html

I. ABSTRACT

The ability to store vaccines at temperatures above what refrigerators and freezers can provide is very important when there are breaks in the cold chain, the vaccine is stockpiled at ambient temperature or the vaccine needs to be administered in locations with very limited refrigeration. Unfortunately, most vaccines must be refrigerated or frozen in order to maintain the necessary stability to be safe and efficacious. Lyophilization is frequently used to extend the stability of therapeutic protein products (Carpenter, 1997) and shows potential in being able to extend the shelf life of vaccines at higher temperatures as well. In the lyophilized state, degradation reactions are minimal due to the glassy state that forms with glass-forming excipients and the low water content present in the formulation.

In addition to having increased stability, it is also necessary to have immunogenic vaccines. Many antigens, such as recombinant proteins, have low immunogenicity on their own and require an adjuvant for an appropriate immune response, (O'Hagen, 2009) making adjuvants necessary components of many vaccines. In this study a variety of adjuvants such as aluminum salts and TLR 4 agonists will be freeze dried and the immune responses will be compared to determine the adjuvant or adjuvant combination that produces the highest immune response as well as the earliest onset immune response. The vaccines prepared will also be incubated over time to determine if they can be stored.

Carpenter, John F., Michael J. Pikal, Byeong S. Chang and Theodore W. Randolph. "Rational design of stable lyophilized protein formulations: Some practical advice." *Pharmaceutical Research* 14.8 (1997): 969-975.

O'Hagen, Derek T. and Ennio De Gregorio. "The path to a successful vaccine adjuvant – 'The long and winding road'. " *Drug Discovery Today* 14.11/12 (2009): 541-551.

II. SPECIFIC DETAILS ON ANIMAL USE AND CARE

1. Species (common name, genus and species)	Maximum number of animals used during project	Approximate number on hand at any time
a. Mouse, BALB/c	380	100
b.		
c.		
d.		

2. How long is your experience in working with this species?

During the Summer of 2008 I interned at IDEXX Laboratories and was responsible for IP, SC, and IV injections on mice as well as bleeding of the mice. Spring 2010 I attended the Animal Care and Use training conducted by the University of Colorado and shadowed a senior PhD graduate student in her work with the mice at the University of Colorado. During 2011-2012 I conducted an animal study using mice at the University of Colorado (protocol #1103.07)

3. Source of Animals* (CU, Jackson Labs, etc.)?
Taconic

*Animal transport and some biologically-derived products from an off-campus site may endanger the health of animal colonies on campus. Such transport requires prior approval by the veterinarian. All antibodies which are produced *under contract* must be obtained from NIH-approved labs, and their animal welfare assurance number/USDA registration number must be submitted to Animal Resources. See section entitled "Animal Procurement" in the CU *Training and Reference Manual for the Care and Use of Laboratory Animals* or contact the Animal Resources Office at 492-8187 for more details. If your laboratory does not have a manual, contact Animal Resources to obtain a copy.

4. Buildings and rooms for housing:

a. Indicate where the animals will be housed.
Biology Building, WM Keck Foundation Laboratory, Transgenic facility.

b. Will you need to return rodents to the central animal facilities after taking them out for less than 24 hours.
No

b. If animals must be removed from housing area indicated above for more than 24 hours, give location and justification.
Animals will not be removed

5. Buildings and rooms for procedures:

a. Provide the buildings and room numbers where experimental procedures will occur:
MCD Biology Building, WM Keck Foundation Laboratory, Transgenic facility A2B29

b. Will you conduct procedures at another institution?
If yes, please explain.
Note: Ensure that you have prior approval before conducting work in a new location.
No

6. Do you plan to remove rodents from the central animal facilities (e.g. Psychology, MCDB, IBG, IPHY) for procedures and return them?
If yes, please explain.
No animals will be removed from the facility

Note: before transporting animals to and from the animal facilities you must have approval from the animal facility manager to verify that the standard operating procedure is followed.

7. Person(s) responsible for daily animal care: Robin Richeson

8. Who will conduct the non-surgical procedures? Kimberly Hassett, Megan Cousins, Lilia Rabia

9. Will surgery be done? ___Yes _X_No. If "no", go to Item 15.

10. Will this project involve recovery from surgical anesthesia? ___Yes _X_No

11. If the animal is to survive after surgery, appropriate surgical techniques and equipment must be used. For cats, rabbits and larger animals, this requires sterile surgical procedures (i.e., sterile instruments, gloves, drapes, etc.) done in a dedicated surgical facility. Surgical procedures on smaller rodents and other vertebrates do not require a special surgical facility, but should be done using sterile instruments and aseptic procedures. Indicate room where surgical procedure(s) will be done and how instruments are to be sterilized.

Needles come individually packaged and sterile.

Room:

Sterilization method (e.g. steam autoclave):

12. Who will do the surgical procedures? Kimberly Hassett, Megan Cousins, Lilia Rabia

13. If you plan to use anesthetics, analgesics or tranquilizers to alleviate pain or distress, please supply the following*: (Use of analgesia is required during the post-operative recovery period after major survival surgical procedures--One analgesic is Buprenorphine (0.01 - 0.05 mg/kg for rats or 0.05 - 0.1 mg/kg for mice) injected either subcutaneously or IP, given once at completion of surgery. This can be repeated every 8-12 hours if required. **If analgesics are not to be used, provide detailed justification in Item III and check Category D)**

Species	Drug(s)	Dose Rate	Route of Administration
a. Mouse, BALB/c	Isofluorane	3-5%	inhalation
b.			
c.			
d.			

* See section entitled "Anesthesia, Analgesia and Tranquilization" in the CU *Training and Reference Manual for the Care and Use of Laboratory Animals*.

14. Please outline type of postsurgical care and its frequency/duration.
After bleeding and injecting mice, mice will be placed on a warm plate to help recovery.

15. Who is responsible for monitoring post-operative care or post-procedural care (e.g. surgery, or injection of tumor cells)?
Kimberly Hassett, Lilia Rabia, Megan Cousins

16. Please provide any expected outcome from the procedures that could compromise the health of the animal such as illness, injury or abnormal behavior and how these outcomes will be managed (e.g. frequent monitoring, consultation with the veterinarian, euthanize animals if a tumor reaches a certain size, etc.)
Note: The Institutional Veterinarian should be notified of any unexpected deaths, illness, distress or other deviations from normal in animals.
No procedures are expected to compromise the health of the mice

17. Euthanasia of animals. For a discussion of acceptable methods of euthanasia, see report of the Panel on Euthanasia of the American Veterinary Medical Association, Journal of the American Veterinary Medical Association, Vol. 281, No. 5, 670-696. Also, see the section entitled "Methods of Euthanasia" in the CU *Training and Reference Manual for the Care and Use of Laboratory Animals*.

a. Briefly describe the method(s) of euthanasia to be used (CO_2 given to effect, cervical dislocation, etc.).

Species	Drug	Dose Rate	Route of Administration
a. Balb/c	CO_2	1 liter/minute x 15 minues	inhalation
b.			
c.			
d.			

b. If your methods of euthanasia differ from the above report, (i.e., not using tranquilizers or anesthetics before decapitation) describe why such a method is required.

c. Who will perform euthanasia?
Kimberly Hassett

III. SPECIFIC EXPERIMENTAL PROCEDURES INVOLVING ANIMALS

All mice used for the experiment will be allowed at least one week to acclimate before any procedures are conducted. During the experiment, mice will have free access to drinking water and food. During the study, each BALB/c mouse will be bled three times and injected with a vaccine formulation twice. Before the initial injection mice will be bled and then on day 0 injected with a vaccine formulation. The initial bleeding is necessary so that each mouse can be its own baseline. 14 days later the mice will be bled and injected with a booster vaccine formulation. 28 days after the initial injection the mice will be bleed one last time.

Before procedures the mice will be anesthetized using is isofluorane inhalant. The mice will have their noses held in a tube with a steady flow of 5% isofluorane until they have taken at least 10 breaths and gone limp at which point the flow will be reduced to 3-4% isofluorane. During the bleeding and injection process the heart rate of the mice will be monitored visually and the mouse nose will be positioned towards the isofluorane to insure that the mice stay anesthetized during the procedures. Before procedures are conducted on the mice, a toe pinch will be given to the mice to make sure that they are adequately anesthetized.

Blood will be drawn from the retro-orbital venous sinus of the mice. A drop of proparacaine will be put on the eye from which blood will be drawn and then blood will be collected using 50μL capillary tubes. Approximately 200μL of blood will be drawn during each bleeding. To make sure that mouse's health is not compromised by removing blood, less than 20% of the mouse's blood will be removed during a bleeding. Mice will be weighed before the bleeding and it will be assumed that 7% of the mouse weight is blood. After collection of blood, sterile gauze will be used to gently blot the eye, a drop of proparacaine will be applied, and the eye will be gently held shut for 1-2 minutes. The serum removed from the mice will be analyzed for antibody content.

The two injections will be given to the mice following bleeding. The injections will be given subcutaneously in the back of the neck. Injections will be 50μL. Vaccine formulation will contain Dominant Negative Inhibitor (DNI) that has been genetically modified with two point mutations from the anthrax subunit protein protective antigen (PA) to be non-toxic, and purified to be endotoxin free. Different experimental and control injections are listed below. After injection, mice will be placed back in their cage.

Group	Contents
Negative Control 1	Freeze dried adjuvant 1*
Negative Control 2	Freeze dried adjuvant 2*
Positive Control 1	Liquid formulation of DNI (low dose) with adjuvant 1
Positive Control 2	Liquid formulation of DNI (high dose) with adjuvant 1
Positive Control 3	Liquid formulation of DNI (low dose) with adjuvant 2
Positive Control 4	Liquid formulation of DNI (high dose) with adjuvant 2
Positive Control 5	Liquid formulation of DNI (low dose) with adjuvant 1 and adjuvant 2
Positive Control 6	Liquid formulation of DNI (high dose) with adjuvant 1 and adjuvant 2
Experimental Group 1	Freeze dried DNI (low dose) with adjuvant 1
Experimental Group 2	Freeze dried DNI (high dose) with adjuvant 1
Experimental Group 3	Freeze dried DNI (low dose) with adjuvant 2
Experimental Group 4	Freeze dried DNI (high dose) with adjuvant 2
Experimental Group 5	Freeze dried DNI (low dose) with adjuvant 1 and adjuvant 2
Experimental Group 6	Freeze dried DNI (high dose) with adjuvant 1 and adjuvant 2

*Adjuvant 1 and adjuvant 2 are aluminum hydroxide and a TLR 4 agonist such as Glycopyranoside Lipid A

-The experimental groups will be repeated after five different length storage time points

IV. DISCOMFORT-PAIN CATEGORIES / JUDICIOUS USE OF ANIMALS / SAFETY

The answers to the following questions are necessary for the Institutional Animal Care and Use Committee to fulfill its obligations under the Public Health Service Policy. Investigators and course directors are requested to categorize their use of vertebrate animals based on the discomfort or pain involved. Consideration should be given to methods that result in a lesser degree of unavoidable pain or discomfort, and the use of the smallest number of animals consistent with meeting the scientific or educational objectives. It is also necessary to provide assurance that animals and personnel working with animals are not unduly exposed to a hazardous environment.

1. Please check ALL of the discomfort/pain categories that apply to your research project.

__X__ CATEGORY A: Project involves little or **NO** discomfort or pain.
- __X__ Subcutaneous, intraperitoneal or intramuscular injections of somewhat harmless substances
- __X__ Blood withdrawal in small amounts (less than 20% of total blood volume)
- __X__ Antibody and monoclonal antibody production
- ___ Studies of embryonated eggs
- ___ Behavioral testing or observation that does not involve restraint or exposure to a noxious stimulus
- ___ Use of euthanasia that results in rapid loss of consciousness and death (e.g., to obtain a tissue sample)
- ___ Other:

___ CATEGORY B: Procedures that involve **SOME** discomfort or pain.
- ___ Ethanol diet
- ___ Chronic drug treatment causing addiction
- ___ Implantation of chronic cannula, subcutaneous implants, catheters or electrodes under anesthesia
- ___ Surgical procedures under general anesthesia that could result in a functional deficit or postoperative pain limited to the immediate postoperative period
- ___ Physiological or behavioral studies on conscious animals involving short-term stressful restraint (<4 hrs) which includes the stress associated with live-capture of animals in the wild
- ___ Exposure to a noxious stimulus from which escape is still possible
- ___ Abnormal behavior resulting from excessive crowding (e.g., increased aggression) or social isolation.
- ___ Studies conducted on completely anesthetized animals that do not regain consciousness
- ___ Deprivation of food and/or water for about 12 to 24 hours
- ___ A procedure not listed above that may result in some discomfort or pain--please describe in a phrase or sentence:

___ CATEGORY C: Procedures that may result in **SIGNIFICANT** discomfort or pain.
- ___ Drug treatment or other treatment that is apt to cause seizures, etc.
- ___ Major surgical procedures under anesthesia that could result in substantial postoperative pain, discomfort, or permanent functional or behavioral deficit
- ___ Prolonged physical restraint lasting longer than 4 hours
- ___ Imposition of stress as indicated by significantly abnormal behavior (excessive fighting, self-mutilation)
- ___ Prolonged deprivation of food and/or water resulting in a 20% loss in body weight
- ___ A procedure not listed above with risk of significant pain or discomfort--please describe in a phrase or sentence:
- ___ Exposure to a noxious stimulus from which escape is not possible

___ **CATEGORY D**: Procedures that produce pain in which, for experimental reasons, anesthetics, or analgesics cannot be used.
Example: Toxicity testing with death as an end point. **(Must be <u>thoroughly</u> described and scientifically justified in the previous Item III.)**

2. Judicious Use of Animals

a. Could mathematical models, computer simulation or in vitro biological systems be used as alternatives to the use of animals in this project? Explain. See section entitled "Replacement" in the CU *Training and Reference Manual for the Care and Use of Laboratory Animals*.

Due to the complex nature of how the immune system functions, computer simulations and in vitro biological systems cannot be used.

b. Justify the use of the animal species listed. Describe the biological characteristics of the animal that are essential to the project.

Mice are an appropriate model for this study because they are relatively inexpensive and have immune systems capable of producing a response to the vaccine formulations being tested. The serum that is drawn for the mice will be used to detect levels of antibody produced against the vaccine antigen.

c. Justify the number of animal used. Describe the size and number of experimental groups and the number of animals needed for procedure development. Specifically state why fewer animals cannot be used. See section entitled "Reduction of Numbers of Animals Used" in the CU *Training and Reference Manual for the Care and Use of Laboratory Animals*.

In order to find statistical differences between the groups, 10 mice will be used in each group.

The negative and positive control mice will each be tested once
(2 negative control groups + 6 positive control groups) X (10 mice/group) = 80 mice

The experimental mice will be tested without any storage time and then with 4 storage times
(6 experimental groups) x (10 mice/group) x (5 storage time points) = 300 mice

Total mice: 80 + 300 = 380 mice

d. Are any State or Federal permits required (e.g., permits to obtain wild animals, importation permits for animal tissue, etc.)?

_X_No ___Yes If "Yes," submit copies of all permits with the application.
e. If this is a field study, please submit your standard operating procedure for the field that includes safety procedures for personnel and decontamination of study equipment between study sites if

applicable.

The following Items 2f and 2g pertain only to projects involving animals covered by the Animal Welfare Act (cats, dogs, rabbits, guinea pigs, hamsters, gerbils, non-human primates and other mammals normally found in a wild state, e.g., deer mice or ground squirrels).

f. For projects that may cause more than momentary or slight pain to the animals involved (Categories B, C, or D), provide a written narrative describing the methods and sources (e.g., biological abstracts, Index Medicus, Current Research Information (CRIS), Animal Welfare Information Center, etc.) used to determine that less distressful alternatives were not available.

g. Provide written assurance that the proposed activities do not unnecessarily duplicate previous experiments. USDA requires that the investigator make a reasonable good faith effort in determining that a proposed experiment is not unnecessarily duplicative. For this assurance, you may choose to cite information from the introduction, significance or bibliographic sections of your grant proposal or any other material that may be relevant to this question.

Work conducted by Ivins et al suggests that a vaccine containing protective antigen (PA), which is very similar in structure and function to dominant negative inhibitor (DNI) is effective in guinea pigs when freeze dried in the presence of aluminum hydroxide adjuvant or is freeze dried from the an emulsion containing the TLR 4 agonist monophosphoryl lipid A (Ivins, 1995). Since DNI has advantages over the PA antigen, it may be a better choice for a vaccine antigen (Cao, 2009). TLR 4 agonists are capable of being formulated different ways such as aqueous, with co-solvents, adsorbed to aluminum adjuvants, in emulsions or in liposomes (Fox, 2010). Since the particular antigen and adjuvants that we are purposing to use have not been freeze dried together before, we are not duplicating the work of others. Since slightly changes in protein and adjuvant structure and form have the ability to alter to immune response, the purposed additional animal studies are needed to determine the immunogenicity of freeze dried DNI vaccines containing adjuvants.

Fox, Christopher B., Martin Friede, Steven G. Reed and Gregory C. Ireton. "Synthetic and natural TLR4 agonists as safe and effective vaccine adjuvants." Endotoxins: Structure, Function and Recognition. Springer Science and Business Media (2010): 303-321.

Ivins, Bruce, Patricia Fellows, Louise Pitt, James Estep, Joseph Farchaus, Arthur Friedlander and Paul Gibbs. "Experimental anthrax vaccines: Efficacy of adjuvants combined with protective antigen against an aerosol *Bacillus anthracis* spore challenge in guinea pigs." 13.18 *Vaccine* (1995): 1779-1784.

Cao, Sha, Aizhen Guo, Ziduo Liu, Yadi Tan, Gaobing Wu, Chengcai Zhang, Yaxing Zhao and Huanchun Chen. "Investigation of new dominant-negative inhibitors of anthrax protective antigen mutants for use in therapy and vaccination." 77.10 *Infection and Immunity* (2009): 4679-4687.

3. Safety of Animals and the Personnel Working with Animals.

IACUC - EH&S Training, Compliance, & Safety Checklist

Required Training and Compliance Review -

Animal Care & Use: The basic animal care and use training is required for anyone working with laboratory animals on campus. All research involving the use of animals, **to include transgenic animals**, must be reviewed and approved by the Institutional Animal Care & Use Committee (IACUC) prior to the commencement of the research.

Have you and everyone who will be working with the animals described in this application attended the CU Training Program on Animal Use and Care?* _X_ Yes __ No

- If No, If "no", you must either: 1) attach a detailed description of your past training and clearly establish that it is equivalent to CU's program or 2) register and attend a training session.

Failure to comply with either option may result in non-consideration of your protocol. Contact the Animal Resources Office for the training schedule at 303 492-8187, http://www.colorado.edu/VCResearch/integrity/animalcare/index.html

Every investigator needs to recognize that if you pursue activities for which you do not have current IACUC approval, that the CU administration and the IACUC are empowered to shut down all research activities in your laboratory, either temporarily or permanently dependent upon the gravity of the violation(s). Noncompliance is a very serious issue and one that could put all animal research at the University of Colorado in serious jeopardy. Such activities have a very real probability of the Office of Laboratory Animal Welfare halting all animal research across the entire university, so noncompliance by investigators is not tolerated.

Do you acknowledge that you have read and understand the previous statement regarding NON-COMPLIANCE:

☒ Yes, I acknowledge that I have read and understand this section.

Hazardous Waste: It is the policy of the University of Colorado at Boulder that all persons (faculty, student, and staff) involved with the generation, handling and management of Hazardous Waste on Campus shall be properly trained, including those who supervise personnel who generate/manage waste.

Will you or anyone in your lab be generating, handling, or managing hazardous waste? _X_ Yes __ No

1.) If Yes, has everyone received the initial Hazardous Waste Generator training and the annual refresher training? _X_ Yes __ No
2) If No, please contact EH&S at 492-6025.

Institutional Biosafety Committee (IBC) Application, Bloodborne Pathogen Training, and Biological Material Shipping Training: It is the policy of the University of Colorado at Boulder that research involving human blood, blood products, reagents derived from blood, tissue, saliva, sputum, etc. or other potentially infectious materials from humans or animals be reviewed and approved by the IBC committee and complete bloodborne pathogen training from EH&S. If your research includes the shipping or receipt of these types of materials, then biological material shipping training from EH&S is required.

Does your research involve the collection or use of human blood, blood products, reagents derived from blood, human specimens such as tissue, saliva, sputum, etc. or other potentially infectious materials from humans or animals? __ Yes _X_ No

1.) If Yes, has an Institutional Biosafety Committee (IBC) Application been submitted and Approved? __ Yes __ No
- If No, please contact EH&S at 492-6025.
- If Yes, please list date of submission or approval: _____

IBC Application has been: Submitted ☐ Approved ☐

2.) If Yes, has everyone completed bloodborne pathogen training from EH&S?* __Yes __No
- If No, please contact EH&S at 492-6025.

Does your research involve the shipping or receipt of human blood, blood products, reagents derived from blood, human specimens such as tissue, saliva, sputum, etc. or other potentially infectious materials from humans or animals? __ Yes _X_ No

1.) If Yes, has everyone received the biological material shipping training from EH&S? __ Yes __ *No
*If No, please contact EH&S at 492-6025.

Use of Human Subjects or Human Tissues: All research involving human participants that is conducted by UCB faculty, staff or students must receive some level of review by the Institutional Review Board.

Does your research involve collection, receipt, or use of human specimens? __ Yes _X_ No

1.) If Yes, has the project been reviewed and approved by the Institutional Review Board?* __ Yes __ No
*If No, please contact the Institutional Review Board at 735-3702;
http://www.colorado.edu/VCResearch/integrity/humanresearch/index.html

Radioactive Materials: It is the policy of the University of Colorado at Boulder that that all persons (faculty, student, and staff) involved with the use of radioactive materials is authorized and properly trained.

Will radioactive materials be used as part of the research listed in this Application? __ Yes _X_ No

1.) If yes, are you licensed for the possession and use of each radioisotope? __ Yes __ No
* If No, please contact EH&S-Health Physics at 492-6523.

2.) Have all persons involved with the use of radioactive materials received training from the Radiation Safety/Health Physics group at EH&S? __ Yes __ No

* If No, please contact EH&S-Health Physics at 492-6523.

X-ray Machines: It is the policy of the University of Colorado at Boulder that that all persons (faculty, student, and staff) involved with the use of X-ray Machines is authorized and properly trained.

Will X-ray Machines be used as part of the research listed in this Application? __ Yes _X_ No

1.) If yes, are you licensed for the possession and use of each machine? __ Yes __ *No
* If No, please contact EH&S-Health Physics at 492-6523.
2.) Have all persons involved with the use of X-ray Machines received training from the Radiation Safety/Health Physics group at EH&S? __ Yes __ *No
* If No, please contact EH&S-Health Physics at 492-6523.

Safety Review –
It is ultimately the principal investigator's responsibility to ensure all lab and animal husbandry personnel are made thoroughly aware of the potential hazards of any chemical, biological, or radiological materials, hazardous gases (i.e. anesthetics) or procedures being used in their research or laboratories. If you are unsure of the risk involved, contact Environmental Health and Safety (EH&S) at 492-6025 for more information.

Will the use of any of the chemical, biological, or radiological materials, hazardous gases (i.e. anesthetics) or procedures listed in this application pose a direct hazard to health or safety of the animal colony or personnel working with animals? _X_ Yes __ No

If yes, please list the hazards, explain how they will be mitigated, and provide an MSDS is available:

Isofluorane will be used to anesthetize the mice. To reduce hazard, filters are placed at the outlet of isofluorane tubes to prevent release of isofluorane to the room. In addition a low flow rate of isofluorane will be used and the room where procedures are conducted will be well ventilated.

V. PROTOCOL APPROVAL

1. Approval for Scientific/Instructional Merit

Before starting any project, it must first be reviewed and approved for scientific or educational merit, either externally (see "a" below) or internally (see "b" below). The IACUC does not review projects for scientific merit, except where it bears upon humane care/use of animals.

a. This project will be reviewed and approved for scientific/instructional merit by:

Federal Agency (specify NIH, NSF, FDA, etc.)_____

Non-governmental agency (specify)_____

b. For all other projects, the chair's signature is required prior to submission of the protocol to the IACUC. Even preliminary studies require this approval.

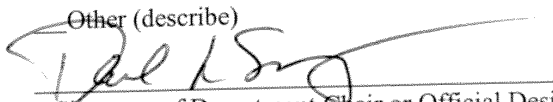
Approval by the Chair:

I assure that this project has been reviewed and approved for scientific / instructional merit by:

X Chair's own review

___Chair's expert designate's review ~~Dan Schwartz~~

___~~Other~~ (describe)



Signature of Department Chair or Official Designate Date 8/23/12

2. Check One of the Following and then Sign under P.I./Course Director

Animals Used for Research

X I certify that the foregoing statements are true and that the protocol is essentially the same as found in the grant application or program/project. The Institutional Animal Care and Use Committee (IACUC) will be notified of any changes in the proposed project prior to proceeding with any animal experimentation. No work may commence until approved by the IACUC. Any persons performing

procedures on animals involved in this project will be appropriately trained and will be made aware of this animal protocol.

Animals Used for Teaching

___ I certify that the information in this application is essentially the same as contained in the course outline and a copy of the laboratory exercises using animals is on file with the IACUC Office. No work may commence until approved by the IACUC. Any persons performing procedures on animals involved in this course will be appropriately trained and will be made aware of this animal protocol.

IACUC *Adverse Event* Statement:

The investigator will notify the IACUC (303-492-8187) of any unexpected results that adversely affect the welfare of the animals. Investigator will report any unanticipated pain or distress, morbidity or mortality to the attending veterinarian and the IACUC.

If there is an adverse event involving the health and/or safety of any research or animal handling personnel, the investigator will contact the IACUC and EH&S (303 492-7072), and complete a University Risk Management *Employee's Injury Report Form* (https://urm.colorado.edu/docs/forms/incident_report_form.asp), within 48 hours of the event. Investigator will also provide the IACUC (UCB 345) and EH&S (UCB 413) with a copy of this completed form.

Signature of Principal Investigator/Course Director — Date: 8-20-2012

Signature of Advisor/ responsible faculty (If applicable) — Date

Chair, Institutional Animal Care and Use Committee — Date: 9/19/2012

D.V.M.
Director, Department of Animal Resources — Date: 9/19/12

Institutional Animal Care and Use Committee, University of Colorado-Boulder
IACUC Administrator Email: richard.husser@colorado.edu Campus Box: 563 UCB Ph: 303-492-8187

APPROVED ADDENDUM

Submitting the addendum:

1) Submit DIRECTLY to the IACUC Administrator and not the department representative.
2) **Email the completed Word version of the Addendum to the IACUC Administrator, Richard Husser** richard.husser@colorado.edu
3) **For principal investigators who are not CU Boulder Faculty, the advisor must be copied on the email to the IACUC Administrator.**

Addendum #1

Date: 3/19/13
Principal Investigator: Theodore Randolph
Responsible Faculty (for anyone who is not a UCB faculty member):
Department: Chemical and Biological Engineering
Title of protocol (required): Evaluating the immunogenicity of freeze dried anthrax vaccines using a variety of adjuvants
Protocol Number: 1209.02
Date of Protocol Approval: 10/9/12

Please answer all questions below unless stated otherwise.

1. Briefly list the reason(s) for this addendum. **For a change in the principal investigator of the protocol, please contact the IACUC Administrator for instructions**. **For a change in animal numbers ONLY, go directly to #7-9.**

   We want to add an addendum to our current protocol so that additional groups of animals can be added using the same methods as the current protocol. In addition to testing freeze dried vaccines stored at an elevated temperature we would like to test liquid vaccines stored at an elevated temperature for comparison. In addition we would like to test what happens when the liquid vaccine is frozen and thawed.

2. **TIER 1: Administrative changes (Estimated review time: 1-3 days).** Are you changing one or more of the following aspects of the protocol? If yes, please explain the reason for each change (a-d).
   a. Protocol title change:
   b. Funding source (submit electronic copy of any new grant or proposal):
   c. Location of experiments or animal care facility (contact the facility manager where you will be transferring animals to and get approval before submitting the addendum; excludes field research)
   d. Addition or removal of personnel to a protocol (**Personnel must be properly trained prior to conducting animal research which includes IACUC training and individual hands-on training**):

   SAME AS PARENT PROTOCOL

3. Are you making any changes other than, or in addition to, those in 2 above?
   YES

4. **TIER 2: Minor scientific changes (Changes that will most likely result in minimal or no increase in pain and distress to the animal).** If you are changing any of the following aspects of the protocol, explain reasons for each (a-e). **Estimated review time: 3-10 days (If you are not submitting Tier 3 changes (5), go to question 6-9)**
   a. Sex, age, or strain of species already approved for the protocol (excluding immunodeficient or transgenic animals - see below):
   b. Drugs or methods used to induce anesthesia, analgesia, or euthanasia (include any disposal/safety issues):

c. Field studies requiring brief capture, sample/data collection, &/or tagging:
d. Addition of innocuous, non-invasive procedures such as behavioral procedures or giving palatable food to non-deprived animals:
e. Terminal procedures conducted under surgical plane of anesthesia:
f. Location of field research (provide any required permits by email)

SAME AS PARENT PROTOCOL

5. **TIER 3: Major scientific changes (A**ll other changes).
Estimated review time: 10-14 days unless the addendum has to be sent to the full committee

Examples:
- Change in time course (duration and frequency) of procedure or drug delivery
- Change in species; addition of transgenic or immunocompromised animals
- Adding new procedures or combining previously approved procedures
- Addition of new drugs or drug doses
- Change in site of drug administration (e.g., changing brain cannulation site; change from systemic to microinjected)
- Adding procedures, not previously approved, that involve unrelieved pain and distress
- Adding sampling of body fluids or tissues, where animals are conscious or will regain consciousness
- Change in surgical plans (minor to major survival surgery, multiple survival surgeries, additional procedures)

Describe the changes below for Item 5:

SAME AS PARENT PROTOCOL

6. Provide scientific justification for the changes. If applicable, cite references and previous experience to provide support for the changes.

7. Increase in animal numbers:
a. What is the total number of each species added to the protocol by this addendum?

100 mice

Justification of 10 mice per group:

Sample Size for t-test using SigmaPlot:

Data source: Data 1 in Titer Values

Sample Size	10
Difference in Means	2,000,000
	This is the difference in titer between liquid positive control with alum +TLR 4 adjuvant and liquid positive control with only alum adjuvant
Standard Deviation	1,400,000
	This is the average standard deviation of groups run for the parent protocol so far
Power	0.850
Alpha	0.05

b. Provide a justification for additional animals, including the numbers needed to be added in each group of the experimental design.

Vaccines have the potential to go bad during transport and storage by being exposed to temperatures both higher and lower than the recommended storage temperature. We would like to add a group to show what happens when liquid vaccines are stored at a high temperature and groups that show what happens when vaccines are stored at temperatures lower than recommended that have experienced freeze thaw cycles. Since some vaccines

during the shipping process experience very little freeze thawing and other vaccine experience many freeze thaw cycles we would like to have both a low and high freeze thaw condition. Stabilizers can help protect vaccines from damage that occurs during freeze-thawing so we would also like to add groups that contain stabilizers to see the how much more effective the vaccine is when stabilizers are present. The stabilizer used will be the sugar trehalose. By adding these groups, a strong argument can be made that the freeze dried vaccines are more effective than a liquid vaccine during storage and transport. 10 mice will be used in each group since that is consistent with studies in the original protocol and necessary for detecting statistical differences as shown above.

New Group	# of Mice Needed
Liquid Vaccine with High Temperature Storage	10
Liquid Vaccine with Low Freeze Thaw	10
Liquid Vaccine with High Freeze Thaw	10
Liquid Vaccine Containing Stabilizer (trehalose) with Low Freeze Thaw	10
Liquid Vaccine Containing Stabilizer (trehalose) with High Freeze Thaw	10

Each new group will be done with a vaccine containing alum adjuvant and a vaccine containing alum with a TLR 4 agonist adjuvant (Glycopyranoside lipid A). 2 x 50 = 100 mice

8. Animal health:
   a. Do you expect animals to experience more clinical illness, pain, or distress, or any other health related issues as a result of the procedures proposed in this amendment?
   If yes or you are not sure, please explain and include whether there is a need to increase monitoring of the animals due to these changes.
   NO

   b. Explain how these outcomes will be managed to minimize the pain and distress for the animals. Note: The Institutional Veterinarian should be notified of any unexpected deaths, illness, distress or other deviations from normal in animal. Contact: Albert Petkus, DVM at 303-492-3411 or albert.petkus@colorado.edu
   No additional pain or distress for the animals

9. Do you expect there to be any other concerns resulting from this addendum that are not listed above, such as: personnel safety, biosafety (includes rDNA experiments; introduction of biological agent or cell lines in-vivo or in-vitro; producing or using transgenic animals), additional disposal, additional hazardous/radioactive materials (including human or non-human animal tissues or blood), animal transportation; new field sites or species requiring federal or state permits? NO

   Please explain and address how you will manage these concerns. For changes in personnel safety, biosafety, hazardous/radioactive materials or disposal, contact Holly Gates-Mayer at 303-492-8683 or EHSBIO@colorado.edu

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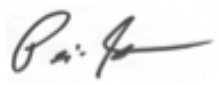
For IACUC use only:
Review method: DMR
Addendum approval Date: 3/26/2013



IACUC Signature for Approval

This addendum, once approved, will expire with the protocol.

Institutional Animal Care and Use Committee, University of Colorado-Boulder
IACUC Administrator Email: richard.husser@colorado.edu Campus Box: 563 UCB Ph: 303-492-8187

APPROVED ADDENDUM

Submitting the addendum:

1) Submit DIRECTLY to the IACUC Administrator and not the department representative.
2) **Email the completed Word version of the Addendum to the IACUC Administrator, Richard Husser** richard.husser@colorado.edu
3) **For principal investigators who are not CU Boulder Faculty, the advisor must be copied on the email to the IACUC Administrator.**

Addendum #2

Date: 4/19/13
Principal Investigator: Theodore Randolph
Responsible Faculty (for anyone who is not a UCB faculty member):
Department: Chemical and Biological Engineering
Title of protocol (required): Evaluating the immunogenicity of freeze dried anthrax vaccines using a variety of adjuvants
Protocol Number: 1209.02
Date of Protocol Approval: 10/9/12

Please answer all questions below unless stated otherwise.

1. Briefly list the reason(s) for this addendum. **For a change in the principal investigator of the protocol, please contact the IACUC Administrator for instructions**. **For a change in animal numbers ONLY, go directly to #7-9.**
   In addition to anthrax vaccines, we would like to apply the same freeze drying methods and adjuvants to another vaccine antigen to prove that our methods are not vaccine specific.

   Changes:
   -Addition of funding source
   -Addition of another vaccine antigen
   -Change of route of administration for new vaccine antigen
   -Addition of mice for new vaccine antigen testing

2. **TIER 1: Administrative changes (Estimated review time: 1-3 days).** Are you changing one or more of the following aspects of the protocol? If yes, please explain the reason for each change (a-d).
   Yes
   a. Protocol title change:
      No
   b. Funding source (submit electronic copy of any new grant or proposal):
      Yes – see attached grant proposal that will fund this addendum (University of Colorado Innovative Seed Grant Program (IGP)). Funding source on parent protocol is still in used.
   c. Location of experiments or animal care facility (contact the facility manager where you will be transferring animals to and get approval before submitting the addendum; excludes field research)
      No
   d. Addition or removal of personnel to a protocol (**Personnel must be properly trained prior to conducting animal research which includes IACUC training and individual hands-on training**):
      No

3. Are you making any changes other than, or in addition to, those in 2 above?
   If not, go to #7-9.
   YES

4. **TIER 2: Minor scientific changes (Changes that will most likely result in minimal or no increase in pain and distress to the animal).** If you are changing any of the following aspects of the protocol, explain reasons for each (a-e).
Estimated review time: 3-10 days (If you are not submitting Tier 3 changes (5), go to question 6-9)
   a. Sex, age, or strain of species already approved for the protocol (excluding immunodeficient or transgenic animals - see below):
   b. Drugs or methods used to induce anesthesia, analgesia, or euthanasia (include any disposal/safety issues):
   c. Field studies requiring brief capture, sample/data collection, &/or tagging:
   d. Addition of innocuous, non-invasive procedures such as behavioral procedures or giving palatable food to non-deprived animals:
   e. Terminal procedures conducted under surgical plane of anesthesia:
   f. Location of field research (provide any required permits by email)

Same as parent protocol including euthanasia of carbon dioxide asphyxiation followed by cervical dislocation.

5. **TIER 3: Major scientific changes (A**ll other changes).
Estimated review time: 10-14 days unless the addendum has to be sent to the full committee

Examples:
- Change in time course (duration and frequency) of procedure or drug delivery
- Change in species; addition of transgenic or immunocompromised animals
- Adding new procedures or combining previously approved procedures
- Addition of new drugs or drug doses
- Change in site of drug administration (e.g., changing brain cannulation site; change from systemic to microinjected)
- Adding procedures, not previously approved, that involve unrelieved pain and distress
- Adding sampling of body fluids or tissues, where animals are conscious or will regain consciousness
- Change in surgical plans (minor to major survival surgery, multiple survival surgeries, additional procedures)

Describe the changes below for Item 5:
Mice added in this addendum will be used for testing a human papillomavirus (HPV) vaccine to prove that the freeze drying technique used to create the original anthrax vaccines can be generalized to other vaccines and will allow other vaccines to be stored at elevated temperatures. The human papillomavirus vaccines will contain highly purified low endotoxin protein antigen HPV capsomeres at a range of doses. We expect similar results as those we have seen with the freeze dried dominant negative inhibitor vaccine (protocol 1209.02) and recombinant ricin toxin antigen vaccine (protocol 1103.07). Since both of the previous vaccines have looked at using non-toxic derivatives of toxic proteins that a person could be exposed to, we would like to look at another type of vaccine, one that uses part of the virus protein coat as the antigen to protect against exposure to a virus. The vaccine will contain no adjuvant, aluminum hydroxide adjuvant or aluminum hydroxide adjuvant with Glycopyranoside lipid A. The vaccine will be administered in a similar fashion to the anthrax vaccine in the parent protocol except administration route. The administration route for this vaccine will be an intramuscular (i.m.) injection.

6. Provide scientific justification for the changes. If applicable, cite references and previous experience to provide support for the changes.
These changes are being added to protocol 1209.02 to broad the application of freeze dried vaccine technique by looking at another vaccine antigen. To be consistent research being done in collaboration with the Garcea lab, vaccines will be administered i.m.

7. Increase in animal numbers:
   a. What is the total number of each species added to the protocol by this addendum?
   460 balb/c mice

   b. Provide a justification for additional animals, including the numbers needed to be added in each group of the experimental design.

New Group	# of Mice Needed
Protein – Low dose	10

Protein – Medium dose	10
Protein – High dose	10
Protein + Alum – Low dose	10
Protein + Alum – Medium dose	10
Protein + Alum – High dose	10
Protein + Alum + GLA – Low dose	10
Protein + Alum + GLA – Medium dose	10
Protein + Alum + GLA – High dose	10
Commercially available HPV vaccine	10

*Each new group will be tested as an initial liquid formulation and as a freeze dried formulation stored at elevated temperature over time at four time points. The dose range of the vaccines will be from 1-10 µg protein. For each vaccine, Protein, Protein+Alum and Protein+Alum+GLA, three doses will be given. There will be a low dose (~2 µg), medium dose (~5 µg) and a high dose (~8 µg). For each group 10 mice will be used.

Total mice = 9 groups x 10 mice/group x 5 storage points (1 liquid and 4 lyophilized) + 1 group positive control x 10 mice/group = 460

Protocol 1209.02 Addendum 1 justifies the use of 10 mice per group, see justification below.

Justification of 10 mice per group:

Sample Size for t-test using SigmaPlot:

Data source: Data 1 in Titer Values

Sample Size 10
Difference in Means 2,000,000
This is the difference in titer between liquid positive control with alum +TLR 4 adjuvant and liquid positive control with only alum adjuvant for the dominant negative inhibitor vaccine used in the parent protocol
Standard Deviation 1,400,000
This is the average standard deviation of groups run for the parent protocol so far
Power 0.850
Alpha 0.05

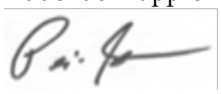
8. Animal health:
   a. Do you expect animals to experience more clinical illness, pain, or distress, or any other health related issues as a result of the procedures proposed in this amendment?
   If yes or you are not sure, please explain and include whether there is a need to increase monitoring of the animals due to these changes.
   No

   b. Explain how these outcomes will be managed to minimize the pain and distress for the animals. Note: The Institutional Veterinarian should be notified of any unexpected deaths, illness, distress or other deviations from normal in animal. Contact: UCB.Veterinarian@colorado.edu

9. Do you expect there to be any other concerns resulting from this addendum that are not listed above, such as: personnel safety, biosafety (includes rDNA experiments; introduction of biological agent or cell lines in-vivo or in-vitro; producing or using transgenic animals), additional disposal, additional hazardous/radioactive materials (including human or non-human animal tissues or blood), animal transportation; new field sites or species requiring federal or state permits? No

   Please explain and address how you will manage these concerns. For changes in personnel safety, biosafety, hazardous/radioactive materials or disposal, contact Holly Gates-Mayer at 303-492-8683 or EHSBIO@colorado.edu

For IACUC use only:
Review method: DMR
Addendum approval Date: 4/30/2013



IACUC Signature for Approval

This addendum, once approved, will expire with the protocol.

Institutional Animal Care and Use Committee, University of Colorado-Boulder
IACUC Administrator Email: richard.husser@colorado.edu Campus Box: 563 UCB Ph: 303-492-8187

APPROVED ADDENDUM

Submitting the addendum:

1) Submit DIRECTLY to the IACUC Administrator and not the department representative.
2) **Email the completed Word version of the Addendum to the IACUC Administrator, Richard Husser** richard.husser@colorado.edu
3) **For principal investigators who are not CU Boulder Faculty, the advisor must be copied on the email to the IACUC Administrator.**

Addendum #3

Date: 5/23/13
Principal Investigator: Theodore Randolph
Responsible Faculty (for anyone who is not a UCB faculty member):
Department: Chemical and Biological Engineering
Title of protocol (required): Evaluating the immunogenicity of freeze dried anthrax vaccines using a variety of adjuvants
Protocol Number: 1209.02
Date of Protocol Approval: 10/9/12

Please answer all questions below unless stated otherwise.

1. Briefly list the reason(s) for this addendum. **For a change in the principal investigator of the protocol, please contact the IACUC Administrator for instructions**. **For a change in animal numbers ONLY, go directly to #7-9.**

   We want to add an addendum to our current protocol so that the bleed and injection schedule can be set to two injections with three bleed time points instead of having specific days for each injection/bleed time point. This will allow for different antigens to be injected at slightly difference frequencies.

2. **TIER 1: Administrative changes (Estimated review time: 1-3 days).** Are you changing one or more of the following aspects of the protocol? If yes, please explain the reason for each change (a-d).
   a. Protocol title change: NO
   b. Funding source (submit electronic copy of any new grant or proposal): NO
   c. Location of experiments or animal care facility (contact the facility manager where you will be transferring animals to and get approval before submitting the addendum; excludes field research) NO
   d. Addition or removal of personnel to a protocol (**Personnel must be properly trained prior to conducting animal research which includes IACUC training and individual hands-on training**): NO

SAME AS PARENT PROTOCOL

3. Are you making any changes other than, or in addition to, those in 2 above?
   YES

4. **TIER 2: Minor scientific changes (Changes that will most likely result in minimal or no increase in pain and distress to the animal).** If you are changing any of the following aspects of the protocol, explain reasons for each (a-e). **Estimated review time: 3-10 days (If you are not submitting Tier 3 changes (5), go to question 6-9)**
   a. Sex, age, or strain of species already approved for the protocol (excluding immunodeficient or transgenic animals - see below): NO
   b. Drugs or methods used to induce anesthesia, analgesia, or euthanasia (include any disposal/safety issues): NO

c. Field studies requiring brief capture, sample/data collection, &/or tagging: NO
d. Addition of innocuous, non-invasive procedures such as behavioral procedures or giving palatable food to non-deprived animals: NO
e. Terminal procedures conducted under surgical plane of anesthesia: NO
f. Location of field research (provide any required permits by email) NO

SAME AS PARENT PROTOCOL

5. **TIER 3: Major scientific changes (A**ll other changes).
Estimated review time: 10-14 days unless the addendum has to be sent to the full committee

Examples:
- Change in time course (duration and frequency) of procedure or drug delivery
- Change in species; addition of transgenic or immunocompromised animals
- Adding new procedures or combining previously approved procedures
- Addition of new drugs or drug doses
- Change in site of drug administration (e.g., changing brain cannulation site; change from systemic to microinjected)
- Adding procedures, not previously approved, that involve unrelieved pain and distress
- Adding sampling of body fluids or tissues, where animals are conscious or will regain consciousness
- Change in surgical plans (minor to major survival surgery, multiple survival surgeries, additional procedures)

Describe the changes below for Item 5:

We want to change the injection and bleed schedule so that it can accommodate both protein antigens on the protocol. To be consistent with previous work, the different vaccine antigens need slightly different injection and bleed schedules. Currently the mice are injected with two doses of vaccine two weeks apart and the mice are bleed before each injection as well as two weeks after the final injection. We would like to change the schedule to be such that the mice will be injected twice and bleed three times during the study.

6. Provide scientific justification for the changes. If applicable, cite references and previous experience to provide support for the changes.

   To be consistent with previous work, the dominant negative inhibitor (DNI) vaccine antigen needs to be injected twice with two weeks in between injections and the HPV vaccine antigen needs to be injected with three weeks in between injections. The mice will be bled before each injection and one time after the last injection. The number of injections and bleeds will be the same as before.

7. Increase in animal numbers:
   a. What is the total number of each species added to the protocol by this addendum?

      0

   b. Provide a justification for additional animals, including the numbers needed to be added in each group of the experimental design.

8. Animal health:
   a. Do you expect animals to experience more clinical illness, pain, or distress, or any other health related issues as a result of the procedures proposed in this amendment?
      If yes or you are not sure, please explain and include whether there is a need to increase monitoring of the animals due to these changes.
      NO. It is unlikely that this change in the series of bleeds will have any effect on the health of the animal. The use of inhaled anesthetics is unlikely to affect the animal but occasional idiosyncratic adverse reactions to the anesthetic have been described.

   b. Explain how these outcomes will be managed to minimize the pain and distress for the animals. Note: The

Institutional Veterinarian should be notified of any unexpected deaths, illness, distress or other deviations from normal in animal. Contact: Albert Petkus, DVM at 303-492-3411 or albert.petkus@colorado.edu
No additional pain or distress for the animals

9. Do you expect there to be any other concerns resulting from this addendum that are not listed above, such as: personnel safety, biosafety (includes rDNA experiments; introduction of biological agent or cell lines in-vivo or in-vitro; producing or using transgenic animals), additional disposal, additional hazardous/radioactive materials (including human or non-human animal tissues or blood), animal transportation; new field sites or species requiring federal or state permits? NO

   Please explain and address how you will manage these concerns. For changes in personnel safety, biosafety, hazardous/radioactive materials or disposal, contact Holly Gates-Mayer at 303-492-8683 or EHSBIO@colorado.edu

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For IACUC use only:
Review method: DMR
Addendum approval Date: 6/3/2013

IACUC Signature for Approval

This addendum, once approved, will expire with the protocol.

7/22/2013 Version

University of Colorado at Boulder
Institutional Animal Care and Use Committee
APPROVED ADDENDUM

Instructions for submitting the addendum:

1) Submit DIRECTLY to the IACUC and not the department representative.
2) For adding personnel do not add to this addendum. Submit a separate personnel addendum to iacucoffice@colorado.edu
3) Email the completed Word version of the Addendum to iacucoffice@colorado.edu
4) For principal investigators who are not CU Boulder Faculty, the advisor must be copied on the email to the IACUC Office.

Protocol Number: 1209.02
Addendum Number: 4
Today's Date: 9/18/12
Principal Investigator: Theodore Randolph
If applicable, Responsible Faculty:
Department: Chemical and Biological Engineering
Protocol Title: Evaluating the immunogenicity of freeze dried anthrax vaccines using a variety of adjuvants
Funding Source: NIH grant (U01-08-2210) through Soligenix and University of Colorado Innovative Seed Grant Program
Protocol Expiration Date: 10/9/15

Note: This form cannot be used to add personnel (use the Personnel Addendum Form), nor can it be used to change the Principal Investigator of an animal protocol. Please contact the IACUC Office if you need to change the name of the PI on a protocol.

Which types of changes in the protocol are being proposed through this addendum? Check all that apply.

TIER 1: Administrative Changes

	Change in protocol title
	Change in funding source (submit electronic copy of new grant)
X	Change in location of animals (contact facility manager if applicable)
	Change in animal source (vet must approve imports prior to shipment)

TIER 2: Minor Scientific Changes (No or minimal increase in pain or distress)

	Change in sex, age, or strain of a species already approved in the protocol
	Change in anesthesia or analgesia (include description of disposal/ safety issues)
	Change in euthanasia method/ terminal procedures
	Addition/deletion of an innocuous, non-invasive procedure
	Change in location of field research (email us any required permits)
X	Change in number of animals

TIER 3: Major Scientific Changes (all other changes or increase in pain/distress)

	Change in animal species
X	Change in time course of procedure or drug delivery (frequency and duration)
	Change in an invasive or surgical procedure, combining procedures
	Addition/deletion of procedure
	Change in surgical plans (pre-op, post-op, minor to major, multiple major, etc.)
	Change in care or use of a Special Care Form
	Change in hazardous substance use
	Addition/removal of a drug/drug dose/route of administration
	Other (describe briefly immediately below)

1. Provide scientific justification for all the changes proposed above. If applicable, cite references and previous experience to provide support for the changes.

1 – Location of animals. All research on this protocol will take place in the JSCBB BioFrontiers Vivarium. Our lab has

moved to the JSCBB building and it will be significantly more convenient for us to do our animal studies in the JSCBB building than on main campus. We discussed the possibility of these studies with the facility manager in JSCBB and she said that there is plenty of room for our studies to be switched to this location.

2 – Increase in animals. We would like to increase the number of doses of HPV vaccine that we test, in order to do this, we must increase the number of animals needed.

3 – Increase injection volume for HPV vaccine studies. We want all injections to be given in a volume of 100 uL (50 uL to each hind leg). During training on IM injections with the campus veterinarian, it was recommended not to exceed 50 uL injections for one leg. Smaller volume doses of vaccine can cause a loss in protein especially with low doses since there is a dead volume in the syringe needle which gets changed with every mouse. The previous low dose tested (Protocol 1209.02 Addendum 2) did not show any immune response even with the positive control Gardasil which is known to produce an immune response at the 2 ug dose tested. We believe that this loss was because the injection volume was too small. We used lower volume injections for lower doses. To avoid this problem, we would like to dilute all doses to injection volumes of 100 uL. To minimize loss of the protein dose, we would like to give the vaccine to the animals in a larger volume (100 uL) so that the percent of protein lost in each injection can be similar regardless of the dose.

2. If you are requesting an increase in animal numbers, what is the total number of each species added to this protocol by this addendum?

   140 mice

3. Reduction Strategies: If you are requesting an increase in animal numbers, provide scientific justification for why an increase in animal numbers above is required. Describe the size and number of experimental groups and the number of animals needed for procedure development. This is required in the Guide for the Care and Use of Laboratory Animals, Eighth Edition: "Whenever possible, the number of animals and experimental group sizes should be statistically justified" (page 25). You can provide any of the following as justification if appropriate to your study: 1) power analysis, 2) citation of scientific literature with an explanation of why that paper relates to your study (in layman's terms), 3) results of a pilot study, 4) a number is explicitly required by the FDA or other federal agency, 5) animal numbers are based on N per experiment, variables, etc. Please refer to the IACUC's SOP #9 page 4 for more information. If it is appropriate to do a power analysis or some other statistical method to justify animal numbers, do so here. Include the parameters you used. If there are a lot of experiments/animals used on the protocol it is helpful to provide a summary table.

   Vaccines: Protein*, Alum+Protein, Alum+GLA+Protein, Gardasil, Cerarix
   Doses: Range 1-10 ug (proposed doses 1, 3, 5, 7, 9 ug)
   Storage time points: 0, 1 and 2-6 months

   Total Mice Added = 5 vaccines x 5 doses x 3 storage points x 10 mice per group = 750 mice

   Since the protocol still has 610 mice remaining, we only need to have 140 mice added to this addendum to complete this study. Many of the mice previously proposed for studies were not used.

   Vaccines will be tested initially (0 storage time) and after 1 and 2+ months storage at an elevated temperature to determine the stability of the vaccine during storage.

   *Protein will be a HPV capsomere protein. The capsomere protein has been purified to be endotoxin free.
   Gardasil and Cervarix are HPV vaccines that are currently FDA approved vaccines for use in humans.
   Vaccines will be made using high purity chemicals and tested to be low endotoxin.
   Lyophilized vaccines will be reconstituted in sterile water for injection.
   Vaccines diluted to create different doses will be diluted in the same buffer used the make the vaccines.

Protocol 1209.02 Addendum 1 justifies the use of 10 mice per group, see justification below.

Justification of 10 mice per group:

Sample Size for t-test using SigmaPlot:

Data source: Data 1 in Titer Values

Sample Size	10
Difference in Means	2,000,000
	This is the difference in titer between liquid positive control with alum +TLR 4 adjuvant and liquid positive control with only alum adjuvant for the dominant negative inhibitor vaccine used in the parent protocol
Standard Deviation	1,400,000
	This is the average standard deviation of groups run for the parent protocol so far
Power	0.850
Alpha	0.05

4. Do you expect animals to experience more clinical illness, pain, or distress, or any other health related issues as a result of the procedures proposed in this amendment (if yes or unsure, please explain and include whether there is a need to increase monitoring due to these proposed changes)?

   They may be a slight increase in pain to the mice, since both hind legs are injected with vaccine. Because IM injections can cause soreness in the muscles, we will want to check mice after injection to make sure that they are walking normally and can access their food. Since the mice are only injected twice with over two weeks between injections allowing for a full recovery, we do not expect this change to cause a significant increase in animal pain or stress.

5. List any potential adverse events for aspects added with this addendum (e.g. procedures, genotypes, etc) and explain how these outcomes will be managed to minimize the pain and distress for the animals.

Mice may experience increased soreness in their hind leg muscles. Mice will be given plenty of time between the two injections to fully recover.

6. Do you expect there to be any other concerns resulting from this addendum that are not listed above, such as: personnel safety, biosafety (includes rDNA experiments; introduction of biological agent or cell lines in-vivo or in-vitro; producing or using transgenic animals), additional disposal, additional hazardous/radioactive materials (including human or non-human animal tissues or blood), animal transportation; new field sites or species requiring federal or state permits?

   No

7. If "Yes" to number 6 above, please explain and address how you will manage these concerns.

IMPORTANT NOTES:
The Institutional Veterinarian should be notified of any unexpected deaths, illness, distress or other deviations from normal in animals. UCB.Veterinarian@colorado.edu or 303-815-8036

For changes in personnel safety, biosafety, hazardous/radioactive materials or disposal, contact Holly Gates-Mayer at 303-492-8683 or EHSBIO@colorado.edu

PLEASE LEAVE BLANK-FOR IACUC OFFICE USE ONLY

Review Method: DMR

Addendum Approval Date: 9/24/2013

Cornella Dehweiler

IACUC Signature for Approval

9/20/2013 Version

University of Colorado at Boulder
Institutional Animal Care and Use Committee
VERIFICATION OF EUTHANASIA AFTER USING GAS OR INJECTABLE FORMS OF EUTHANASIA IN RODENTS

Protocol Number: 1209.02
Addendum 5
Principal Investigator: Theodore Randolph
If applicable, Responsible Faculty:
Protocol Title: Evaluating the immunogenicity of freeze dried anthrax vaccines using a variety of adjuvants

It is standard veterinary practice to perform a secondary method of euthanasia when using gas or injectable anesthetics in order to ensure death. The Guide for the Care and Use of Laboratory Animals (8th Edition) states that death must be confirmed in an animal after euthanasia, and that a secondary method of euthanasia can be used to ensure death. The AVMA Guidelines for the Euthanasia of Animals (2013 Edition) states that a secondary method of euthanasia can be performed after an overdose of inhaled anesthesia as part of a two-step euthanasia process. Please fill out this addendum to state in your protocol which secondary methods of euthanasia you utilize after rodents are euthanized via inhalation or injection. If helpful, please see the IACUC's Standard Operating Procedure #19 Carbon Dioxide Euthanasia.

Acceptable secondary physical methods of euthanasia for rodents are:

i. Decapitation using very sharp scissors or guillotine
ii. Cardiac perfusion
iii. Removal of vital organs (e.g. heart, lungs, brain)
iv. Bilateral Thoracotomy
v. Cutting the major blood vessels to induce exsanguination (e.g. aorta, vena cava)
vi. Cervical dislocation on adult rodents weighing less than 200 grams. Cervical dislocation is an inappropriate method to use on rats larger than 200 grams; and on neonates at any time prior to 21 days of age.

1) For the protocol number listed above, what is the species, primary method of euthanasia (carbon dioxide, injection with Euthanasia Solution, overdose with isoflurane, etc.) and secondary method of euthanasia you use?
We euthanize mice weighing less than 200 grams with carbon dioxide and our secondary method is cervical dislocation.

2) Can you ensure, once this addendum form is approved, that all rodents that are euthanized also receive a secondary physical method of euthanasia (as described in #1) by a person trained in these procedures? You will need to communicate this change, if it is a change, to all your research personnel trained to perform euthanasia.
Yes

If your lab needs training to conduct proper secondary methods of euthanasia, please contact the OAR Veterinary Technician Toni Mufford (toni.mufford@colorado.edu) to schedule a training session.

Per regulatory requirements, failure to comply with this policy may result in notification of your funding agency (e.g. NIH) and regulatory agencies (e.g. OLAW, USDA) that your research has violated federal and/or local policies regarding the humane use of animals. This notification may affect continuous funding of your animal-related research. Further, depending on the violation, you may be required to take additional training and/or your privilege to conduct animal research at UC Boulder might be temporarily suspended or even completely revoked.

PLEASE LEAVE BLANK-FOR IACUC OFFICE USE ONLY
Review Method: DMR
Addendum Approval Date: 10/29/2013
Cornella Dotweiler
IACUC Signature for Approval

Appendix C

Lyophilization optimization

Many parameters can be varied during the lyophilization process such as freezing rate, drying time, annealing steps, excipients, buffer concentration and numerous more. This appendix will focus on varying the freezing rate, buffer concentration, and type of buffer.

C.1 Freezing rate

Formulations can be frozen at many different rates during the freezing stage of lyophilization. The freezing rate can be varied by exposing formulations to a range of temperatures. For these studies, aluminum hydroxide placebo formulations were exposed to liquid nitrogen, -10 °C pre-cooled lyophilizer shelves or room temperature lyophilizer shelves. Formulations were exposed to liquid nitrogen in one of two ways, the first by dipping vials filled with 1 mL of formulation into liquid nitrogen (LN2 Dip) such that liquid nitrogen only comes into contact with the glass vial and does not get inside the vial or the second method of adding vaccine formulation dropwise to a vial containing liquid nitrogen (LN2 Spray). By adding vaccine formulation dropwise to liquid nitrogen, a larger surface area of vaccine formulation has been exposed to a colder temperature allowing for even more rapid freezing to occur. With both the liquid nitrogen dip and spray method, the initial aluminum hydroxide particle size distribution can be maintained if greater than 4% trehalose is added to the formulation as can be seen in Figure C.1. When no trehalose was added to the formulation, the particle size distribution drastically shifted towards larger particle sizes. Similar results were seen in a sodium succinate buffer system [42].

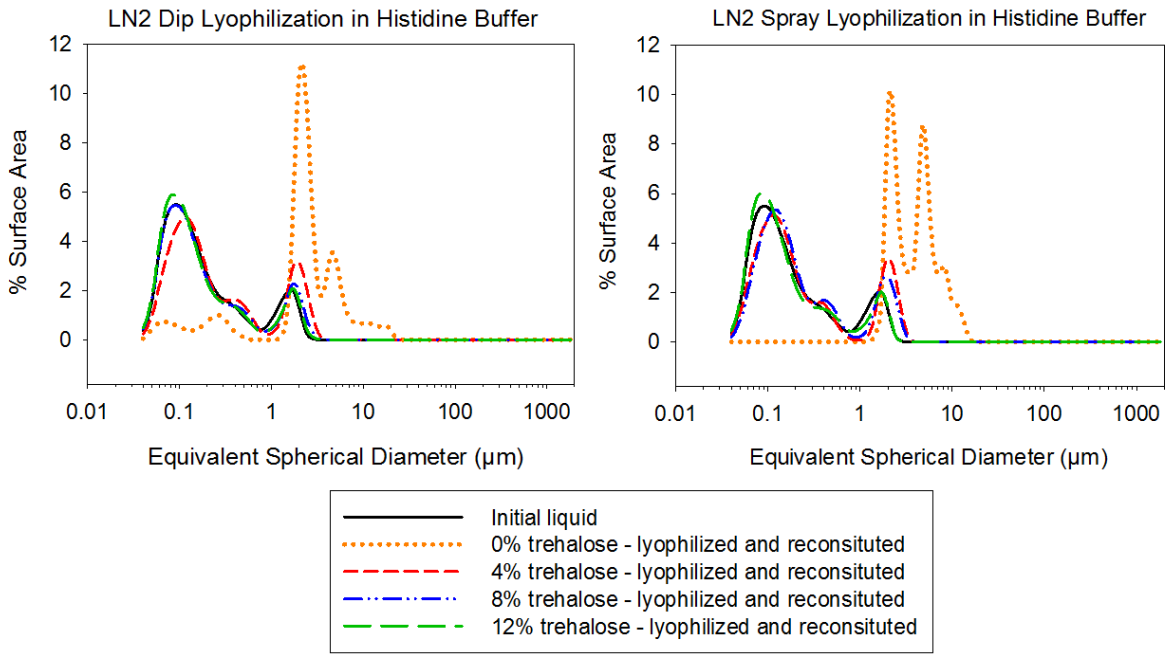

Figure C.1: Aluminum hydroxide particle size distribution after lyophilization and reconstitution with liquid nitrogen freezing

Slower freezing rates were explored by varying initial lyophilizer shelf temperature during freezing. The lyophilizer shelf was set to -10 °C (Pre-cooled shelves) or room temperature ~25 °C (Room temperature shelves) before vials were loaded on the shelves. By having the vials set on pre-cooled shelves, they were able to completely freeze in a shorter amount of time. With the slower rate of freezing, more trehalose was needed to preserve the particle size distribution as seen in Figure C.2.

C.2 Settling of aluminum hydroxide formulations before freezing

In addition to the rate of freezing, the amount of time the vaccine formulation settles before freezing can impact the particle size distribution after lyophilization and reconstitution. Adjuvants such as aluminum salts which are more dense than the formulation will settle at the bottom of the vial over time as can be seen in Figure C.3.

When aluminum salt adjuvants freeze, they can be pushed together as ice crystals form, potentially causing irreversible aggregation [201]. If aluminum adjuvant particles have settled before freezing, the particles are in closer proximity to each other before freeze concentration occurs making it even more likely for irreversible aggregation to occur. As settling time before freezing on pre-cooled shelves increases, aluminum hydroxide particle size increases as can be seen in Figure C.4.

During freezing, ice crystals will form until the remaining liquid has reach a maximally freeze concentrated state. With an increasing concentration of an excipient, such as the glass-forming agent trehalose, the formulation components will concentration less, allowing for less interactions between aluminum adjuvant particles and therefore less aggregation. When no trehalose was added to formulations, a drastic increase in particle size was seen after lyophilization and reconstitution. However, when 8 w/v% trehalose was added to formulations, adjuvant particle aggregation was inhibited as long as formulations were placed on pre-cooled shelves after less than 30 minutes of settling time (Figure C.4).

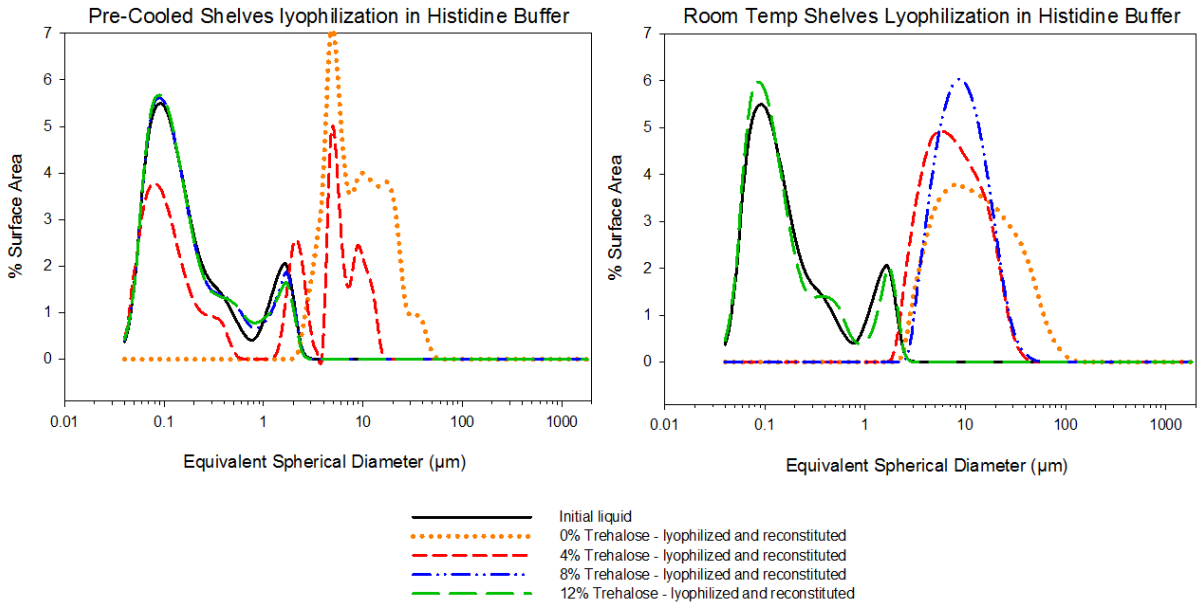

Figure C.2: Aluminum hydroxide particle size distribution after lyophilization and reconstitution with pre-cooled and room temperature shelf freezing

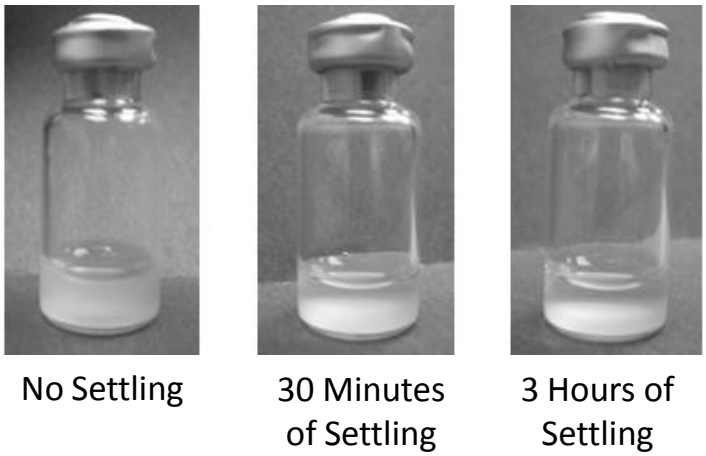

Figure C.3: Aluminum hydroxide particles settling

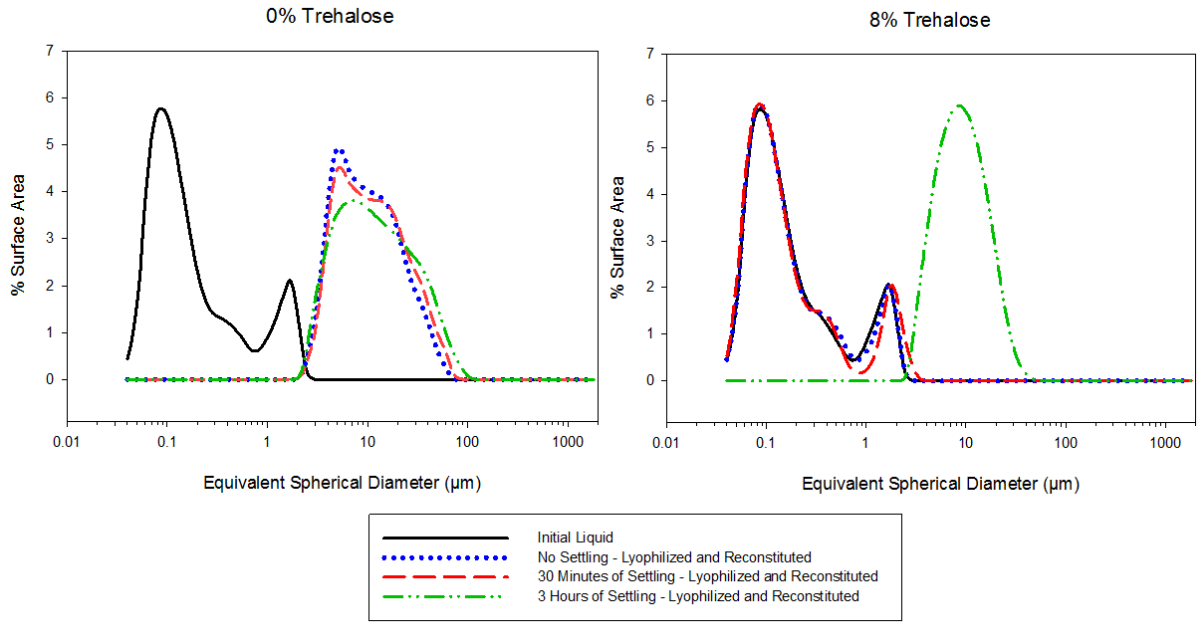

Figure C.4: Aluminum hydroxide particle size distribution after settling before freezing and lyophilization

C.3 Buffer concentration

Buffer concentration can affect the quality of lyophilized cake. If the buffer concentration drastically lowers the glass transition temperature to beyond a point where it is feasible to conduct primary drying (i.e. a temperature below the glass transition temperature that would take an unreasonable amount of time for drying to occur), drying will have to take place at a temperature above the formulation glass transition temperature, which can cause lyophilized cakes to collapse. Cake collapse has to potential to leave the protein intact but may require more effort to reconstitute. Figure C.5 shows an example of how increasing the ammonium acetate buffer concentration from 0 to 1 M with a constant trehalose concentration of 8 w/v% can affect the lyophilized cake quality.

C.4 Buffer type

Different buffers have been shown to form different lyophilized cakes. Without trehalose, buffer alone may not be able to form a pharmaceutically elegant cake. Glycine is commonly used as a bulking agent and can form a cake without the assistance of trehalose, whereas arginine, histidine and sodium succinate have trouble forming a full cake. Ammonium acetate is volatile is is not able to form a cake. The addition of trehalose in the same concentration, allows all of these buffers to form a nice cake reguardless of their previous behavior (Figure C.6).

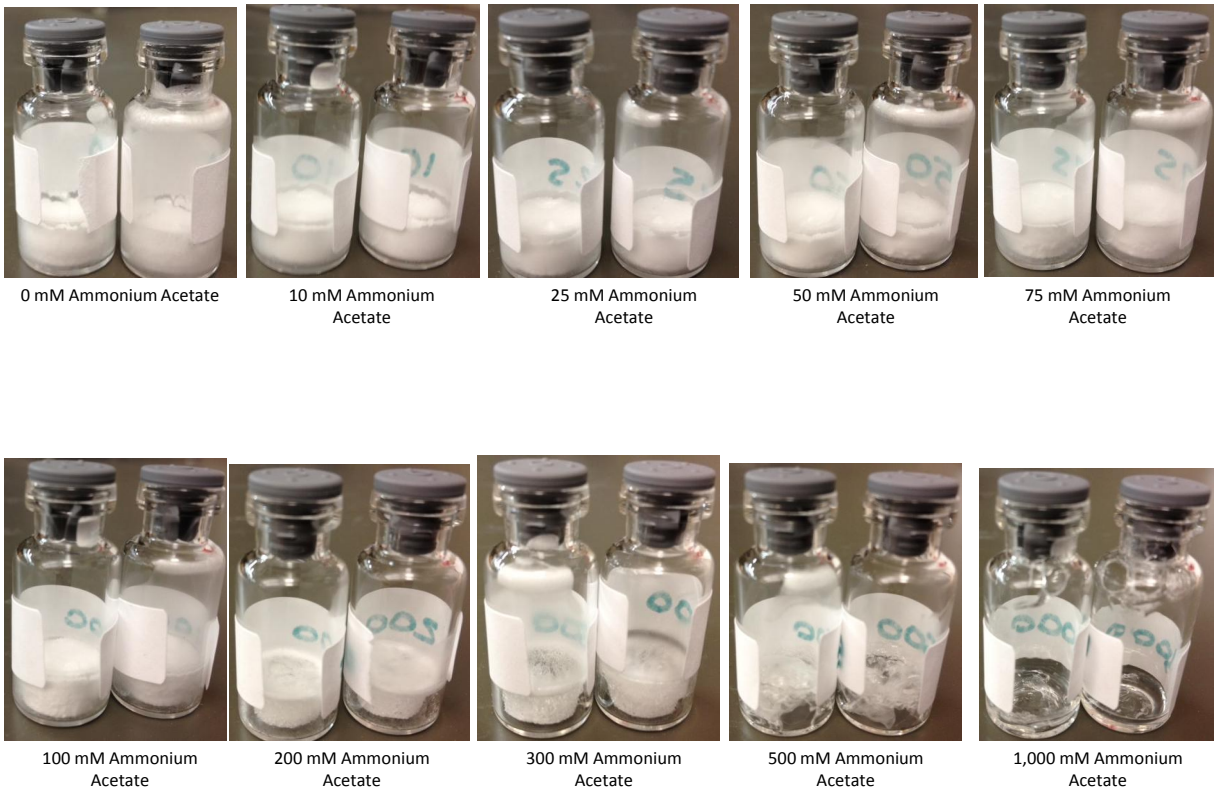

Figure C.5: Lyophilized cakes with varying ammonium acetate concentration

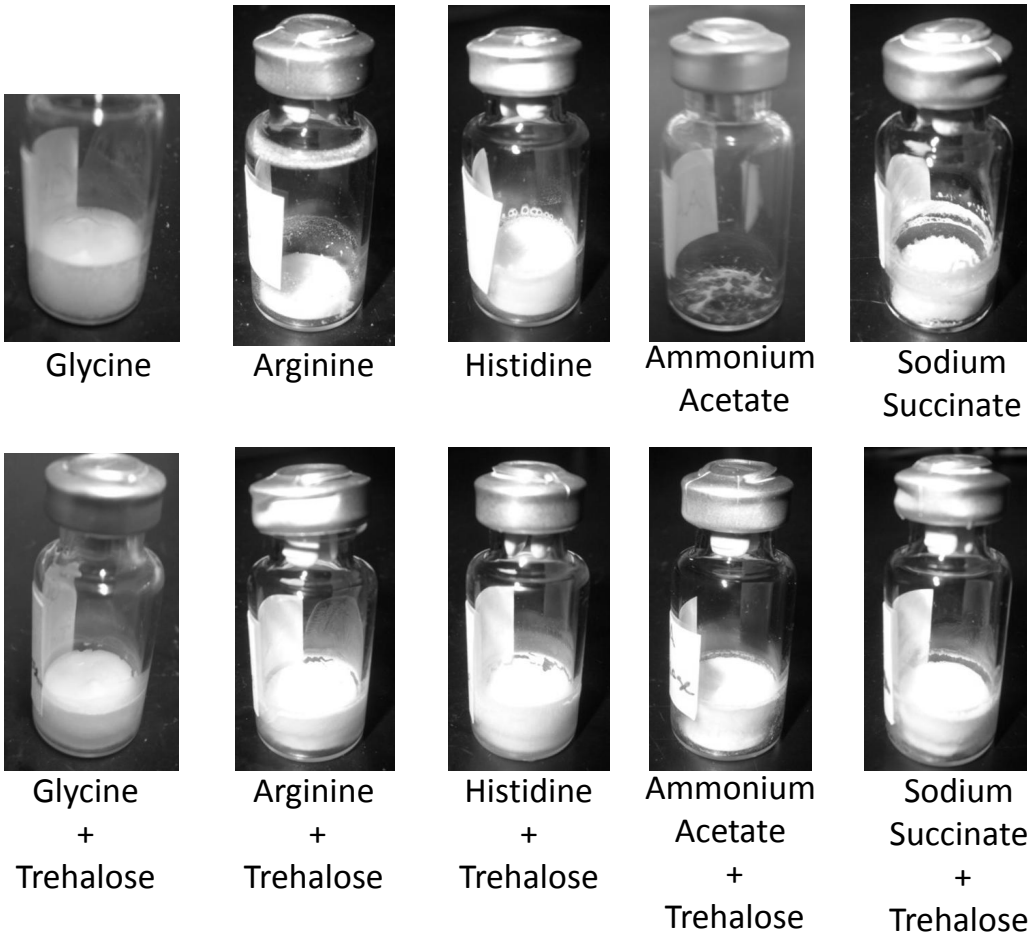

Figure C.6: Lyophilized cakes of varying buffers with and without trehalose

Appendix D

Freezing and thawing DNI vaccine formulations containing salt

To see the effects of freeze-thaw cycles in the absence of the stabilizer trehalose, freeze-thaw studies were conducted with the DNI vaccine in an isotonic sodium chloride formulation. Vaccines were analyzed for particle size and concentration, protein structure by melting temperature, deamidation by capillary isoelectric focusing, adsorption of protein to adjuvant, and immunogenicity by total anti-DNI and neutralizing antibodies.

D.1 Vaccine formulation

All vaccines were formulated to contain 10 mM ammonium acetate pH 7 with 0.2 mg/mL DNI and 0.5 mg/mL Al from Alhydrogel. For isotonicity, 139 mM sodium chloride was added. In addition to aluminum hydroxide, 0.05 mg/mL GLA was added as a second adjuvant to half of the vaccine formulations. GLA was prepared at 1 mg/mL by suspending lyophilized GLA in a 0.5% triethanolamine pH 7 solution using probe sonication [13]. To create the vaccine formulations containing GLA, suspended GLA was added to Alhydrogel suspensions, vortexed for 5 seconds and then rotated end over end for 30 minutes at 4 °C. 0.2 mg/mL DNI protein antigen was added to buffered adjuvant solutions and rotated end over end for 30 minutes to allow protein to adsorb completely to adjuvant particles.

D.2 Freeze-thaw study

Freeze-thaw stability was examined for liquid vaccine formulations. Formulations were cycled between -20 °C and 4 °C, leaving formulations at each temperature for one day to permit complete freezing or thawing. Vaccines experienced 0, 1, or 5 freeze-thaw cycles.

D.3 Vaccine characterization methods

Vaccine formulations were characterized after 0, 1 and 5 freeze-thaw cycles. Particles greater than five microns were monitored by microflow image analysis using a FlowCAM. Particles were also visualized by differential interference contrast microscopy. Protein structure was monitored by melting temperature measured by fluorescence spectroscopy. Deamidation was monitored by capillary isoelectric focusing. Protein adsorption was monitored by centrifugation of protein adsorbed to adjuvant and measurement of protein concentration in the supernatant. Total anti-DNI antibodies were measured by ELISA and neutralzing antibodies were measured by a cell based assay. For more detailed methods see 3.

D.4 Vaccine particles

Initially, all formulations appeared similar regardless of adjuvant or excipient present (Figure D.1). When sodium chloride is present in the formulation instead of trehalose, which was presented early, more particle aggregation is seen after one freeze-thaw cycle. After five freeze-thaw cycles, significant aggregation was seen in all formulations.

Particles after 0, 1, or 5 freeze-thaw cycles were quantified by microflow imaging. After one freeze-thaw cycle, there was a significant increase in larger particle sizes. After additional freeze-thaw cycles, a decrease in small particles can be seen as larger particles are formed (Figure D.2).

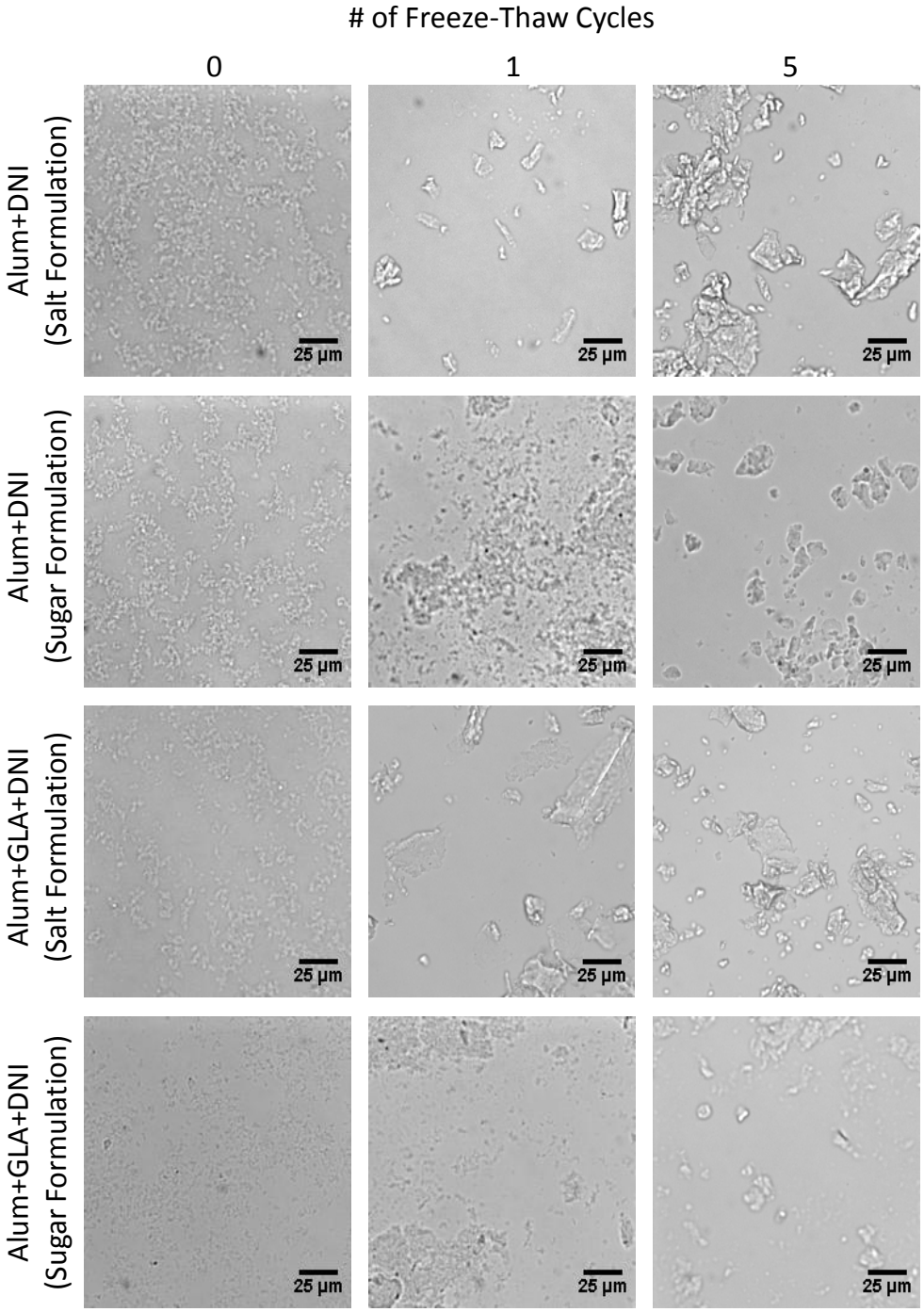

Figure D.1: Aluminum hydroxide adjuvant particles aggregate during freezing and thawing as seen by differential interference contrast microscopy images after 0, 1, and 5 freeze-thaw cycles. More particle aggregation is observed with increasing the number of freeze-thaw cycles and when sodium chloride is present in the formulation.

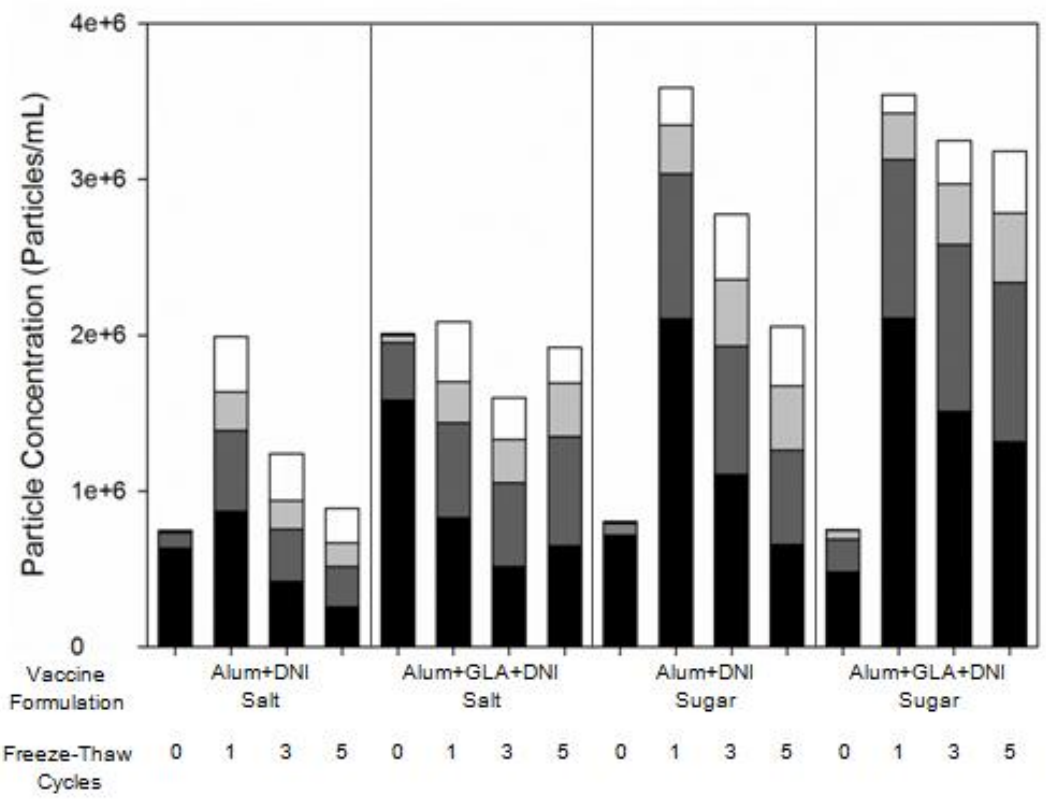

Figure D.2: Aluminum hydroxide particle size and concentration after 0, 1, 3, and 5 freeze-thaw cycles. After more freeze-thaw cycles occur, a decrease in 2-5 μm particles is detected and an increase in larger particles is seen. Particles 5-10 μm (black), 10-20 μm (dark gray), 20-30 μm (light gray), 30+ μm (white).

D.5 DNI protein structure

After 0, 1, or 5 freeze-thaw cycles no changes in protein structure were detected. The DNI protein had a melting temperature 40-50 °C regardless of adjuvant present, excipient (trehalose or sodium chloride), or number of freeze-thaw cycles. The number of deamidated residues also did not increase after freezing and thawing formulations.

D.6 DNI adsorption to adjuvant

After formulations containing DNI adsorbed to aluminum hydroxide particles in trehalose solutions were pelleted by centrifugation and resuspended in PBS for 1 hour at 37 °C, ∼20% of the DNI desorbed. In contrast no DNI desorbed from formulations prepared by adsorbing the DNI from sodium chloride solutions.

D.7 Immunogenicity of frozen and thawed vaccines

Freezing and thawing vaccine formulations, drastically reduced their immunogenicity, especially when sodium chloride was present in the formulation instead of trehalose (Figure D.3). When sodium chloride was present in the formulation, a large fraction of mice did not respond to the vaccine after one injection with the Alum+DNI and the Alum+GLA+DNI vaccines. After two injections, most mice showed anti-DNI antibodies but very few responded with neutralizing antibodies. With five freeze-thaw cycles, the antibodies titers were further reduced with sodium chloride in the formulations. When trehalose was included in the formulation, freezing and thawing the vaccine had less effect on the immunogenicity.

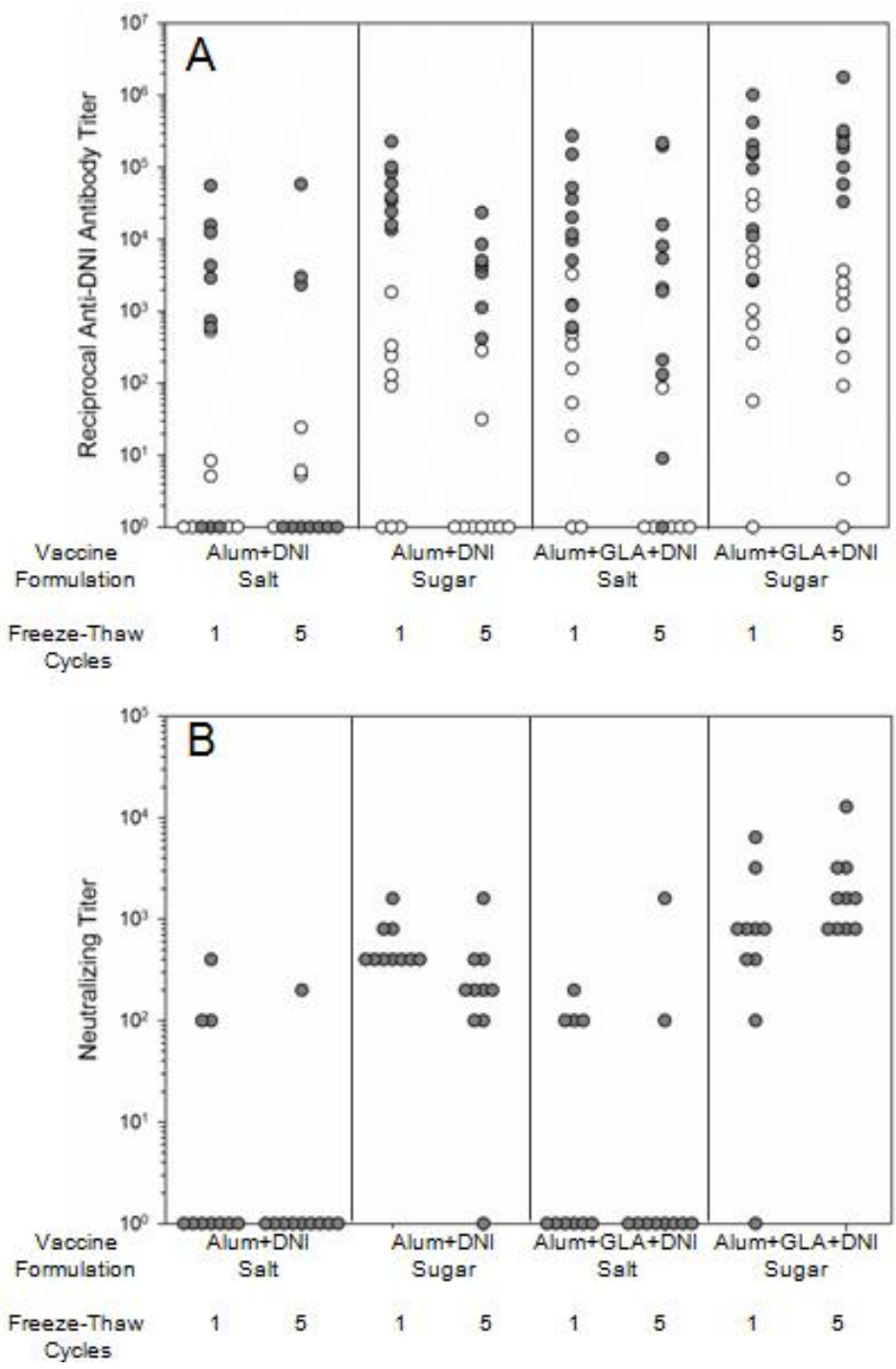

Figure D.3: Total anti-DNI antibody titers (A) and neutralizing antibody titers (B) after one vaccine injection (white circles) and after two vaccine injections (gray circles) for liquid vaccine after 0, 1, and 5 freeze-thaw cycles. Reduced immunogenicity is detected with 5 freeze-thaw cycles after one injection.

Appendix E

Front face fluorescence

E.1 Fluorescence of proteins

Intrinsic fluorescence can be used to monitor the tertiary structure of proteins. When excited, naturally fluorescent amino acid residues such as tryptophan, tyrosine or phenylalanine adsorb a photon and have an electron go to a higher energy state. After excitation, an electron returns to a lower energy state releasing energy which can be viewed as an emission spectra. The maximum absorpsion of the three aromatic amino acids is 280, 270, and 258 nm for tryptophan, tryosine, and phenylalanine respectively. Since tryptophan has the highest absorption coefficient and a good quantum yield, it can be used for intrinsic fluorescence studies. By exciting at a wavelength between 295 and 300 nm, the emission spectra of only tryptophan can be monitored [89]. The intensity of the emission spectra is a dependent on many parameters such as protein concentration, number of amino acids capable of fluorescing, protein conformation, temperature, etc.

E.2 Cuvette geometry

When only protein is present in the solution, the angle of incidence can be 90°. However, when particles are present in the formulations this geometry will no longer be effective. The front face fluorescence geometry with an angle of incidence of 53° works well with protein solutions containing particles. Vaccine formulations that contain adjuvants, such as aluminum salt adjuvants can effectively be analyzed with this geometry. Protein can even be associated with the particles in this geometry. By having the cuvette rotated in the holder, scattering and specular reflection

signals can be avoided [16].

E.3 Fluorescence quenching

To measure the accessibility of tryptophan resides, fluorescence quenching can be preformed. For this experiment, emission spectra are collected by exciting protein molecules at a set wavelength and collecting the emission spectra over a range of wavelengths. Molecules such as acrylamide or iodine can be used as quenchers. When the quencher is able to access tryptophan residues, the fluorescence intensity will decrease. Figure E.1 shows how the flouresnce spectra change as an increasing concentration of acrylamide is added the a solution of HPV 16 L1 capsomere.

From the quenching spectra, the maximum fluorescence intensity can be monitored. The Stern-Volmer Constant can be calculated once the fluorescence intensity is monitored for a range of quencher concentrations. The Stern-Volmer equation uses the ratio of fluorescence intensity without quencher present, Fo, to fluorescence intensity with quencher present, F, equaling one plus the Stern-Volmer constant, K_{SV}, multiplied by the quencher concentration, $[Q]$. The Stern-Volmer equation is as follows: $Fo/F = 1 + K_{SV}[Q]$ [16]. The Stern-Volmer constant is normally calculated with the linear portion of the plot. An example of a Stern-Volmer plot is shown in Figure E.2.

The Stern-Volmer constant can give a general idea of the relative environment of trytophan residues. A low Stern-Volmer constant means that the tryptophan residues are hard to access, which suggests to a more folded protein structure. A high Stern-Volmer constant means that the tryptophan resides are easily accessable, suggesting an unfolded protein. The highest Stern-Volmer constant which can be achieved is that of free tryptophan residues.

Quenching measurements give an average environment of all the tryptophan resides. Even if the Stern-Volmer constant suggests that the protein of interest is folded, the position of some tryptophan residues may have changed. By finding the Stern-Volmer constant of native and unfolded protein, protein samples undergoing different stresses can be compared to being more like the native or unfolded states.

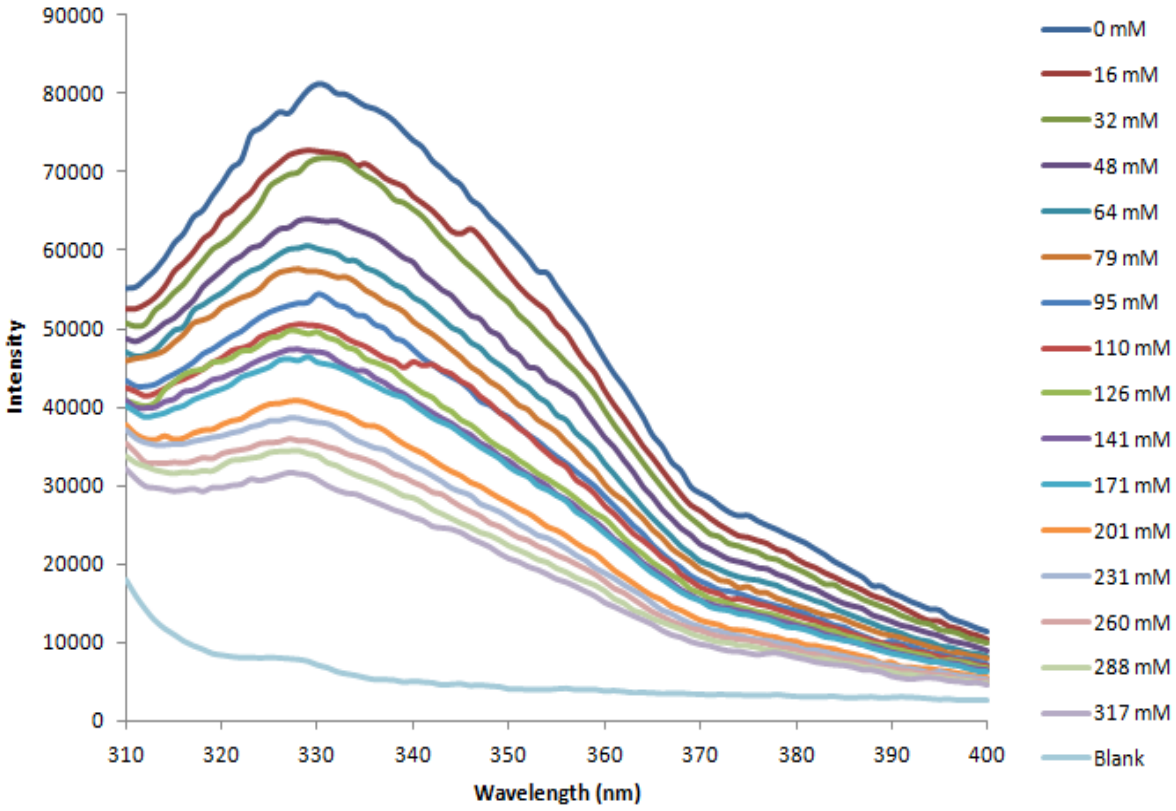

Figure E.1: Acrylamide quenching example spectra. As acrylamide is added to the solution of HPV 16 L1 capsomere, the fluorescence intensity decreases

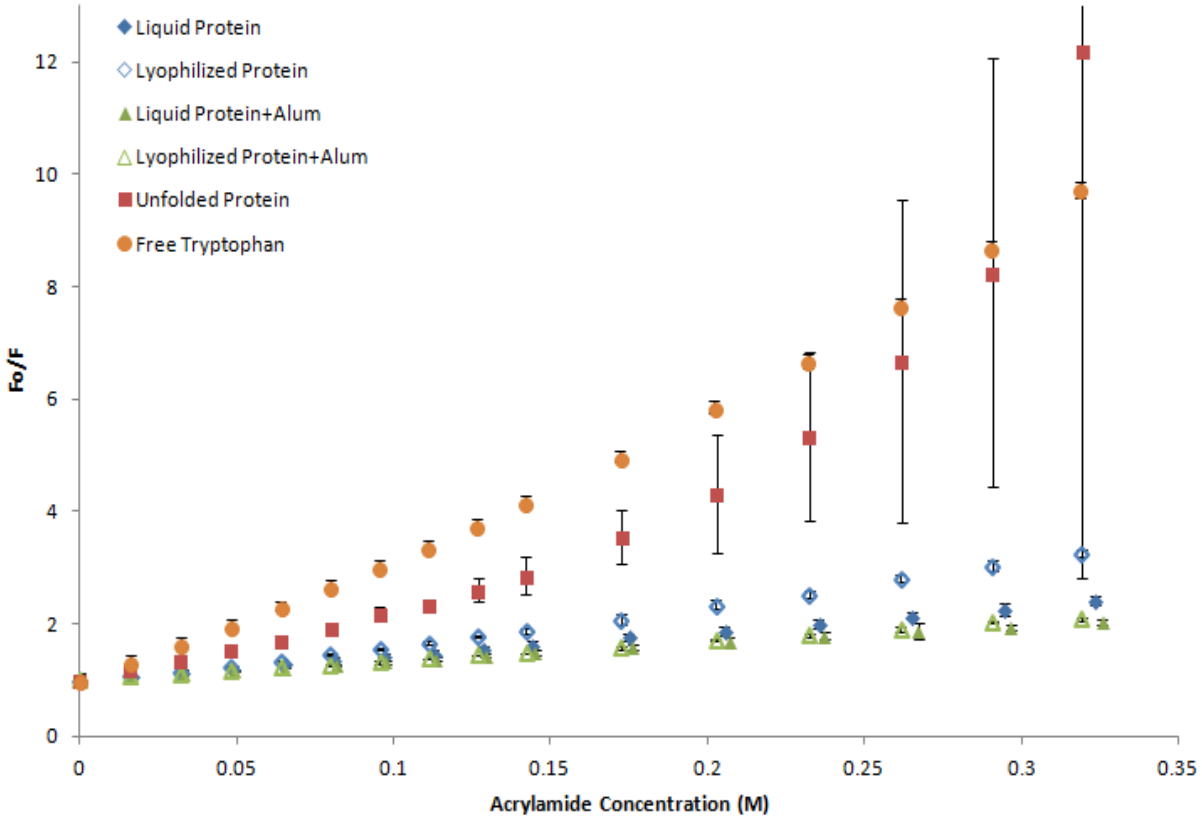

Figure E.2: Stern-Volmer plot example

Appendix F

Antibody titer

F.1 Enzyme linked immunosorbent assay (ELISA)

Enzyme linked immunosorbent assay (ELISA) described here allows for comparisons on the amount of antibodies produced by mice after vaccine immunizations. After ELISA experiments have been run, antibody titers can be calculated. A higher antibody titer indicates a higher level of antibodies produced. The first step in determining the antibody titer is the conduct an indirect ELISA. In an indirect ELISA, the protein injected into mice is coated onto the 96-well ELISA plate and incubated overnight. Residual coating solution is washed away and blocking solution containing bovine serum albumin (BSA) or the milk protein casein is added to plates to fill in any areas not coated with the antigen of interest. Residual blocking solution is washed away and mouse serum of varying dilutions is added to the plate and incubated. After washing away residual serum, a secondary antibody recognizing the primary antibody labeled with horse radish peroxidase (HRP) is added. If the animal study was conducted in mice, an anti-mouse antibody would be required for the secondary antibody. Residual secondary antibody is washed away and TMB is added to the plates. After a significant color change has occurred, sulfuric acid can be added to stop the reaction. At this point the plates can be read at 450 nm. A general outline of this method can be seen in Figure F.1. For more specific details on the ELISA protocol used in this thesis, please see the materials and methods sections of Chapter 2, 3, and 4.

An ELISA should be run at a range of dilutions of mouse serum such that a high signal and a signal similar to the background can be seen for each sample. The starting dilution for each mouse

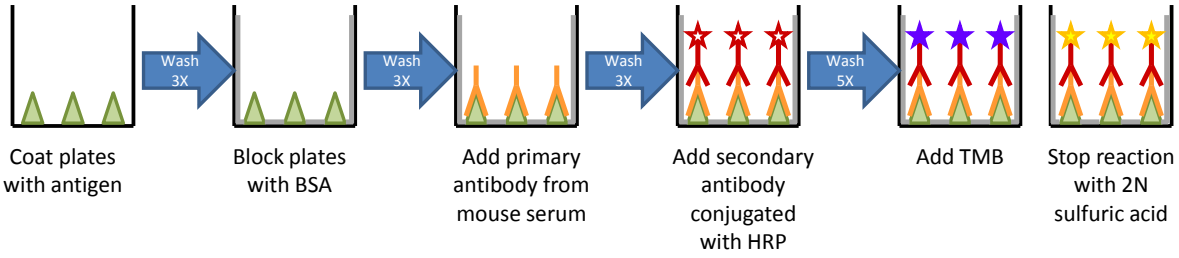

Figure F.1: ELISA experimental setup

serum sample may not be the same. Mouse serum from a mouse that has been injection more times, with a higher dose of antigen or with a stronger adjuvant may require a higher dilution. When optimizing ELISA conditions, the background noise seen on week 0 serum (serum exposed to no antigen) should be relatively low. Example ELISA dilution curves for mice injected with HPV 16 L1 capsomere protein (7, 5, 3, or 1 μg) with aluminum hydroxide adjuvant can be seen in Figure F.2.

In order to calculate endpoint titers, a cut off value must be selected. The cut off value should always be higher than the week 0 mouse serum as any value above the cutoff value will be seen as a positive response. If the week 0 mouse serum has a relatively constant value, a cutoff value of several times the average background value can be used. If the week 0 mouse serum has values that vary based on the mouse, each mouse can have an individual cut off value based on several times its own week 0 value. The reciprocal endpoint titer, is the highest dilution of mouse serum which has a positive response to the antigen of interest, also the dilution of mouse serum which has a value of the cutoff. See Figure F.3 for a graphical representation of this value.

Since all the ELISA dilution curves may not cross the cutoff value and the curve may not cross the at a dilution run, the reciprocal end point titer can be calculated. First, the ELISA dilution curve can be fit to a four parameter logistic fit. The equation is as follows: $OD450Value = min + \frac{(max-min)}{1+(\frac{SerumDilution}{EC50})-Hillslope}$. To find the reciprocal endpoint titer, the OD450 Value can be set to the cutoff value and the SerumDilution can be solved for.

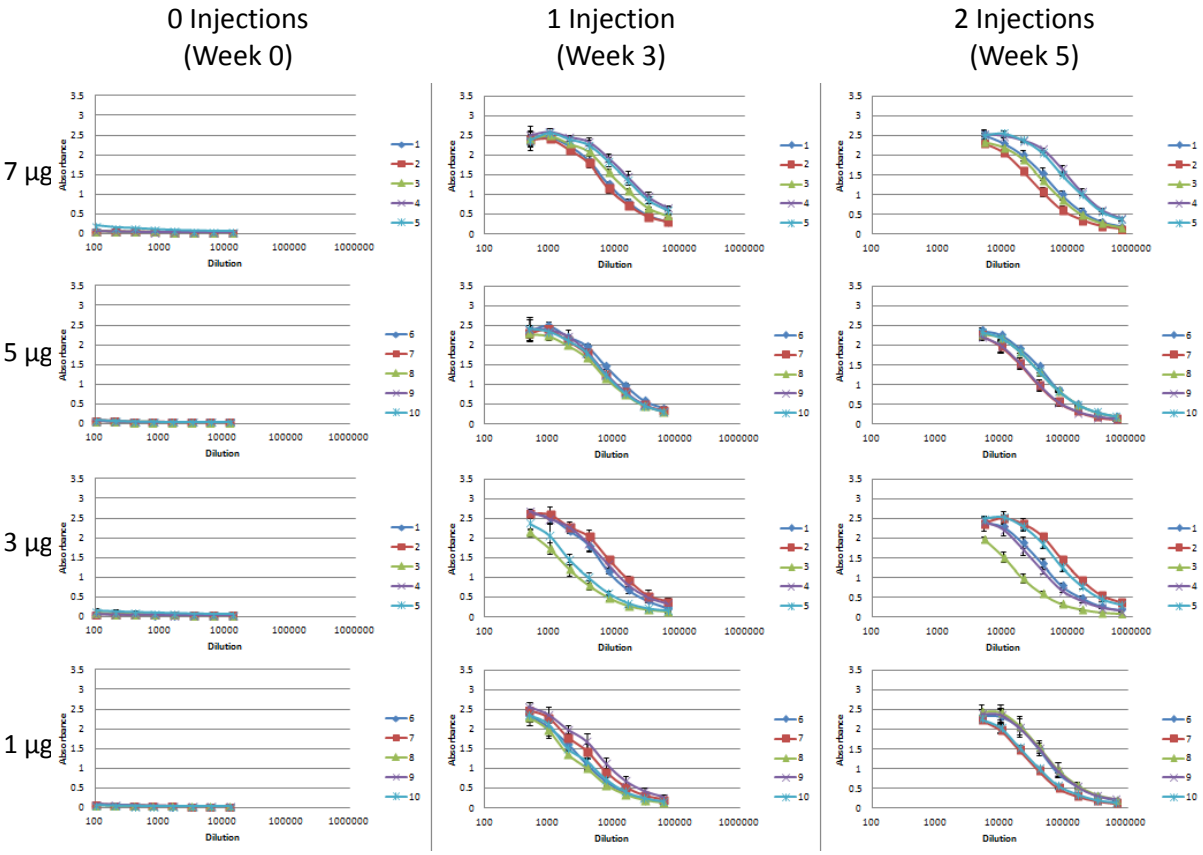

Figure F.2: ELISA dilution curves

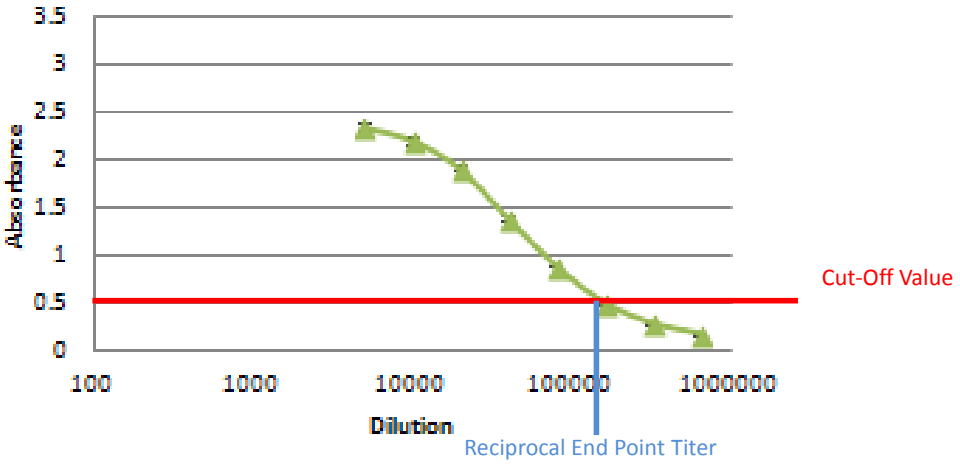

Figure F.3: ELISA reciprocal end point titer and cutoff values