Synthetic nanoparticle-based vaccines against respiratory pathogens

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Synthetic nanoparticle-based vaccines against respiratory pathogens

by

Kathleen Alaine Ross

A dissertation submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Major: Chemical Engineering

Program of Study Committee:
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Ames, Iowa
2013

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CHAPTER 1: Introduction

Acute respiratory infections (ARIs) remain the leading cause of both morbidity and mortality throughout the world, causing approximately 4.25 million worldwide deaths each year and contributing to 6% of the world’s disability and death.\textsuperscript{1,2} These infections affect a wide range of the population, including healthy adults as well as immunocompromised individuals, children, and the elderly.\textsuperscript{1} The past ten years has seen a rise in the emergence of ARIs including the SARS outbreak of 2002-2003, the surge in multi-drug resistant tuberculosis in 2008, and the 2009 H1N1 influenza pandemic (Table 1.1).

Influenza is a key respiratory pathogen that contributes up to 500,000 deaths annually.\textsuperscript{2} Influenza A, specifically, can be more problematic when compared to influenza B and C due to its increased mutation rate.\textsuperscript{1} While all influenza viruses go through antigenic drift, or slow changes in epitope presentation due to point mutations and error-prone RNA polymerases, influenza A is the only class capable of antigenic shift, or re-assortment of the virus.\textsuperscript{1,2} The reservoir of influenza A is waterfowl; however, the viruses have been known to infect humans, swine, and other mammals.\textsuperscript{1,2} Re-assortment of the virus, typically in swine susceptible to both avian and human influenza strains, leads to antigenic shift and possibly creates influenza pandemics in a naïve human population.

Avian influenza A H5N1 is rapidly gaining the potential to be the next influenza pandemic threat.\textsuperscript{3-5} Human cases of H5N1 have proven to be approximately 60% fatal with a growing number of strains becoming resistant to medical treatments such as oseltamivir (Tamiflu).\textsuperscript{6,7} Due to the severe pathology associated with H5N1 influenza and its resistance to treatment, it has been referred to as highly pathogenic avian influenza (HPAI).

Currently, the infection of HPAI is mainly spread from avian species to humans and rarely from human to human. However, due to the HPAI strain’s ability to achieve viral mutations at an extremely rapid rate and ability to be weaponized, many scientists believe that human-to-
human transmission of the virus is imminent, unleashing a possible influenza pandemic across the globe.\textsuperscript{3-5} If such a pandemic occurs, scientists predict that the H5N1 influenza will cause an estimated 50 million deaths and a ten-year lifespan reduction in the U.S. alone.\textsuperscript{6} Therefore, there is a strong need for new research developments for both the prevention and treatment of HPAI.

While the treatment of influenza is important, prevention of the disease through vaccination is critical especially in the event of a pandemic. The overall goal of the research described in this thesis is to create a safe, efficacious vaccine against H5N1 avian influenza. This was achieved by encapsulating H5N1 hemagglutinin (HA) antigen into a novel delivery platform composed of polyanhydride nanoparticles. Polyanhydrides have been known to possess many beneficial characteristics as vaccine adjuvants/delivery vehicles, including sustained antigen release, antigen stabilization, immune cell activation, and immunomodulation to achieve protection against infection.\textsuperscript{8,9} The thesis describes both \textit{in vitro} and \textit{in vivo} studies that are focused on the development of a next generation nanoparticle-based vaccine against HPAI H5N1.

### Table 1.1: ARI occurrences in the past decade\textsuperscript{1,10-13}

<table>
<thead>
<tr>
<th>Respiratory Infection</th>
<th>Annual Occurrence Cases/year (deaths/year)</th>
<th>Symptoms</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pneumonia</td>
<td>156 million (1.6 million)</td>
<td>Cough, fever, rapid breathing, chest pain</td>
</tr>
<tr>
<td>Influenza</td>
<td>Up to 2 billion (up to 500,000)</td>
<td>Cough, fever, runny nose, body aches</td>
</tr>
<tr>
<td>Respiratory Syncytial Virus</td>
<td>3.4 million (up to 199,000)</td>
<td>Cough, fever, sneezing, wheezing</td>
</tr>
<tr>
<td>Tuberculosis</td>
<td>9 million (2 million)</td>
<td>Cough (with blood), fever, sweating, chest pain</td>
</tr>
</tbody>
</table>
REFERENCES


CHAPTER 2: Literature Review

As previously discussed, H5N1 avian influenza has the potential to become the next influenza pandemic and thus, research efforts to prevent influenza are critical. Often times, alternative vaccine approaches utilizing viral proteins are safer; however these proteins are poorly immunogenic and require the use of adjuvants to boost immunity. This chapter will begin by focusing on the different adjuvant strategies that have been explored in vaccine design and development, including the novel polyanhydride nanoparticle adjuvant/delivery platform used in this work (Section 2.1). Section 2.2 will discuss intranasal vaccination, which is an important factor in mucosal immunity and the route of immunization for this work. Section 2.3 will cover the molecular characteristics of H5N1 and the protective and pathogenic immune responses that have been observed to date. Finally, Section 2.4 will describe the current research in the field of influenza vaccination, covering both traditional/seasonal vaccines and the novel approaches used for developing pandemic vaccines.

2.1. VACCINE ADJUVANTS

2.1.1. Introduction

Traditional vaccines, such as killed or live attenuated vaccines, are fairly immunogenic on their own.\(^1\) Killed vaccines consist of entire pathogens inactivated by heat or chemicals. In these vaccines, the pathogen cannot replicate and often has a prolonged shelf life.\(^2\) However, killed vaccines have several drawbacks including inflammation at the injection site, the need for multiple doses or “boosters” to achieve protection, and a lack of cell-mediated immunity. Live attenuated vaccines, although more immunogenic, also have several pitfalls with the most serious being replication and disease in immunocompromised patients. In a goal to increase safety of vaccination, many researchers have turned to DNA or subunit vaccines. These vaccines encode for or contain a purified protein of the pathogen, or simply put, one small piece
of the pathogen.\textsuperscript{1-4} While side effects due to chemical inactivators, whole, or live pathogens are eliminated with purified proteins, subunit vaccines are often poorly immunogenic requiring the use of adjuvants to achieve protection.\textsuperscript{2,4,5}

Adjuvants are materials that non-specifically enhance immune responses. When coupled with an antigen, adjuvants can enhance the immune response towards the desired protein and increase the efficacy of the vaccine.\textsuperscript{2,6,7} Adjuvants accomplish this enhancement in immunogenicity by performing three main functions: creating an antigen depot for sustained release, targeting the antigen towards antigen presenting cells (APCs), and modulating the type of immune responses initiated.\textsuperscript{2,6} Aside from these three functions, adjuvants can provide other attributes, including antigen stabilization, targeting of cellular organelles or vesicles, and enhancing memory recall.\textsuperscript{3,8}

Although adjuvants have been utilized for many decades, there are relatively few that have been approved for human use. For example, aluminum salts, often termed Alum, are one of two adjuvants approved for use in the United States.\textsuperscript{2,3,9} Surprisingly, even after over 70 years of use, the adjuvanticity mechanism of Alum is just beginning to be understood. Alum forms a depot by adsorbing antigen to charged aluminum particles, which in turn promotes uptake via phagocytosis.\textsuperscript{1,10} Although studies have also suggested that Alum activates the inflammasome, it is a relatively weak adjuvant, producing only humoral responses.\textsuperscript{3,11} A more potent adjuvant, MF59 (approved in Europe), is an oil-in-water emulsion that forms small droplets readily internalized by APCs.\textsuperscript{4,9} Although the mechanism of MF59 is largely unclear, it is known to increase cross reactivity and antibody titers in seasonal influenza vaccines.\textsuperscript{1,5} MF59 has also been known to increase early CD4\textsuperscript{+} T cell responses and to induce cytokines at the injection site.\textsuperscript{3} Another adjuvant, monophosphoryl lipid A (MPLA), has been recently approved for use with Alum in a human papilloma virus vaccine.\textsuperscript{3,9} MPLA is a non-toxic derivative of lipopolysaccharide (LPS), a Toll-like receptor (TLR)-4 ligand and provides immune stimulation through the production of the cytokines, IL-1\textbeta, IL-12, and IFN-\gamma.\textsuperscript{1,2}
Although Alum, MF59, and MPLA have been shown to be useful in enhancing vaccine efficacy, they are by no means suitable for all vaccine applications. For example, while Alum has been shown to boost humoral antibody responses, it fails to stimulate cell-mediated immunity, an important factor in vaccines against intracellular pathogens. Thus, it is important for vaccine research to shift from a “one-adjuvant-fits-all” philosophy to an approach of rationally designing appropriate adjuvants to match the desired antigen. The size and stability of the antigen, route of administration, target cells, and desired immune response must be considered when developing new adjuvants. To fulfill these requirements, there has been a lot of attention devoted to the development of novel adjuvants. Despite the large number of excipients and immunostimulatory materials being examined, such as TLR-9 agonists and virus-like particles, this section will focus on biodegradable polymeric particles for use as vaccine adjuvants and delivery vehicles. These particles have been extensively studied as carriers for drugs, proteins, and vaccines, and present some compelling advantages to other adjuvant candidates. Table 2.1 summarizes the various classes of biodegradable polymers that have been studies and the performance of these materials is discussed below.

### 2.1.2. Poly(esters)

Poly(esters) are class of biodegradable polymers that have been extensively studied for drug delivery applications. These materials are typically composed of lactic acid, glycolic acid, copolymers thereof (PLGA), and poly(ɛ-caprolactone) (PCL). Poly(esters) can be easily formed into most shapes and sizes, leading to applications in degradable sutures, bone implants and screws, tissue scaffolds, and drug delivery. Poly(esters) are also FDA-approved for a number of human applications due to their lengthy safety record.

PLGA hydrolyzes into well tolerated degradation products: lactic and glycolic acids. Lactic and glycolic acid are naturally found in vivo, and easily metabolized and eliminated via the citric acid cycle. Studies have also shown that lactic and glycolic acid produced from
degraded poly(esters) have no effect on cell function and result in minimal systemic toxicity.\textsuperscript{13,14} Although the degradation of poly(esters) may be favorable in terms of biocompatibility, their mechanism of hydrolysis, bulk erosion, can attribute to challenges when encapsulating sensitive cargos like protein antigens.

The bulk erosion mechanism, in which water diffusion occurs more rapidly than cleavage of the polymer chain, allows water penetration throughout the material before payload release, affecting both the release and stability of encapsulated proteins.\textsuperscript{15} Typically, the release kinetics of the cargo is dictated by the erosion kinetics of the polymer. In the case of poly(esters) such as PLGA, degradation of the polymer has been successful at sustaining release up to four months, and longer for the more hydrophobic PCL.\textsuperscript{12,14} Release from poly(esters) is also typically characterized by an initial “burst,” or large release of cargo, which signifies less control over both rate of release and sustained release.\textsuperscript{16} Although the rate of degradation can be controlled by both polymer molecular weight and copolymer composition, poly(esters) may not be suitable for long-term vaccine and drug delivery.

While bulk erosion may be successful in short-term applications, the process can be detrimental to the payload stability. As mentioned previously, bulk erosion allows for interactions between water and proteins before release. Often times, water can create instability by aggregating proteins, but what may be more detrimental is the acidic microenvironment created by polymer degradation products.\textsuperscript{12,14} Both lactic and glycolic acid are fairly acidic (pH ~2-3) and can cause degradation and denaturation of encapsulated proteins, thus affecting the activity and epitope availability of protein antigens.\textsuperscript{12,14} Additives such as amino acids and bases have been proposed for co-encapsulation to improve aggregation and pH respectively.\textsuperscript{12} However, the encapsulation of these components may be undesirable in terms of safety or other interactions.

Despite the challenges in bulk erosion, poly(esters) have been successfully used in many drug delivery platforms. PCL, for example, lacks the extreme pH of other poly(esters) and has been successful at stabilizing the structure and immunogenicity of hemagglutinin (HA), an
influenza antigen.\textsuperscript{17} Encapsulation into poly(esters) also protects antigens from proteolytic cleavage, as well as endosomal degradation. Other adjuvant properties exemplified by poly(esters) include enhanced delivery to APCs, internalization, endosomal escape, and cross presentation.\textsuperscript{13-18} Though some studies have noted that the immune response to poly(esters) is similar to that of Alum, it may not be as useful in vaccine platforms and dependent on the type of antigen. For example, during a study encapsulating the model antigen ovalbumin (OVA), it was found that PLGA microparticles were not stimulatory enough for primary immunizations, but suitable for booster immunizations.\textsuperscript{19} Other experiments, on the other hand, have shown protective immunity by encapsulating tetanus toxoid.\textsuperscript{13} However, despite this variability, poly(esters) are good candidates for surface modification for targeting (APC\textarrowright vaccine, cancer cell\textarrowright drug delivery) and co-encapsulation of innate stimulators such as TLR ligands.\textsuperscript{14,15,20}

Despite these challenges, poly(esters) remain one of the front runners in the field of biodegradable materials. Their enhanced immunogenicity is well suited for acid-stabilized antigens to survive the denaturing microenvironment created by bulk erosion. Likewise, their extensive safety profile and FDA approval allow for the successful application of poly(esters) to numerous applications ranging from degradable sutures to cancer vaccines.

\section*{2.1.3. Acid-Catalyzed Polymers}

Although poly(esters) such as PLGA are one of the most widely studied biodegradable polymers, it was recognized in the early 1970s that there was room for improvement in drug delivery vehicles.\textsuperscript{21} A new class of biodegradable materials, termed acid-catalyzed polymers, was developed to enable controlled drug release kinetics, targeting of intracellular components such as endosomes, and stabilization of payloads. Examples of acid-catalyzed polymers include poly(ketals), poly(acetals), poly(urethanes), and poly(ortho esters). The last category will be further discussed in this section.
Poly(ortho esters), one of the most well-known acid-catalyzed polymers, were developed in the early 1970s out of a need for biodegradable materials other than PLA, PGA, and their copolymer, PLGA. While PLGA was making strides in terms of its applicability in sutures and other surgical techniques, it was recognized that PLGA may not be the ideal polymer for drug delivery vehicles. For example, the bulk erosion mechanism of PLGA allows for little control over release kinetics and can create microenvironments with a pH as low as 1.5, a challenge for acid-sensitive payloads such as proteins or DNA. Thus, poly(ortho esters) were developed in an effort to sustain drug release via surface erosion as well as to protect encapsulated cargos.

Poly(ortho esters) were developed in four major classes, viz. POE I, II, III, and IV. The first attempts at synthesis involved a transesterification process under high vacuum and high temperature conditions. However, these reactions required long periods of time and resulted in relatively low molecular weights. Often, these reactions allowed for little control over molecular weight and the process was abandoned for commercial scale. POE IV synthesis has since transitioned to addition polymerization of diols to cyclic diketene acetals. These poly(ortho esters) integrate glycolic or lactic acid segments into the polymer backbone for additional control over hydrolysis, and therefore, drug release. Further control over release kinetics have been observed in copolymerization of poly(ortho esters) with poly(ethylene glycol), poly(acetal), and amides.

In contrast to poly(esters) such as PLGA, acid-catalyzed polymers tend to degrade through surface erosion instead of bulk erosion, increasing control over cargo release kinetics (Figure 2.1). Hydrolysis, for example in POE IV, begins with the hydrolysis of lactic or glycolic acid components within the polymer backbone, leaving behind a carboxylic end group. This reduces the pH of the local environment and thus, catalyzes the ortho ester hydrolysis. This acid-induced mechanism, while allowing for sustained release, can also target the payload vehicle. For example, antigen presentation through MHC I molecules is an important factor when activating cell mediated immune cells such as cytotoxic T lymphocytes (CTLs). Acid-
labile delivery vehicles, such as microparticles encapsulating a vaccine antigen, can provide protein protection at neutral pH where very little polymer degradation occurs. However, once internalized into mildly acidic endosomes the polymer quickly erodes, swiftly releasing the antigen. In fact, in a vaccine platform composed of CpG DNA encapsulated in an acetal-cross-linked hydrogel, less than 10% of the DNA was released at neutral pH after several hours. Upon placing the same construct in a mild acidic buffer, 100% of the encapsulated DNA was released after two hours. In another study, acid-degradable polyurethane formulations most sensitive to degradation at pH 5 enhanced MHC I presentation and CTL activation. Aside from targeting intracellular compartments, the acid-induced degradation of poly(acetals) and poly(ketals) has also been applied for cancer treatment. In many cases, the tumor pH is more acidic than the surrounding tissue, resulting in drug containment during circulation at neutral pH, and release locally at the tumor site.

Although surface erosion permits beneficial control over the release of proteins and other small molecules, degradation products can lead to detrimental effects on payload stability or the surrounding tissue. In the case of PLGA, degradation products induce a highly acidic microenvironment that can quickly disrupt protein stability. On the other hand, acid-catalyzed polyurethane degrades into diols and acetone that do not cause detrimental acidic environments and have similar cytotoxicity to the FDA-approved PLGA. Other acid-catalyzed polymers such as poly(ortho ester amides), poly(acetals), and poly(ketals) have shown no significant effects on microenvironment pH, as well as excellent compatibility with fibroblasts in vitro, stability of encapsulated DNA, and have been used in orthopedic implants.

Acid-degradable microparticles have shown increased phagocytosis by macrophages, up to 10 times more than soluble protein alone in 24 hours. These materials can be used for targeting of endosomes and enhancement of cell-mediated immunity through MHC I and CTL activation after particle vaccination. These immune enhancing properties are critical in designing polymer adjuvants for efficacious vaccines. Acid-catalyzed polymers have also been
extensively studied as thermogels for parenteral drug delivery. Many thermogels, such as Pluronic block copolymers and PLGA-g-PEG copolymers, are either non-degradable (Pluronic) or lack controlled release (PLGA-g-PEG).\textsuperscript{23,24} In contrast, poly(ortho ester amides) have shown fully reversible soluble-gel transitions in water and are completely degradable.\textsuperscript{23} Likewise, thermogels consisting of graft copolymers with PEG, poly(acetal) and/or poly(ortho ester) have shown controllable erosion kinetics from very short (a few days) to long (100 days) time periods, permitting thermogel drug release to be adjusted for each therapeutic application.\textsuperscript{15}

Acid catalyzed polymers, mainly poly(ortho esters), are considered to be more biologically inert than other classes of polymers, such as poly(esters) and polyanhydrides.\textsuperscript{25} Although this is very beneficial in cases of drug delivery or implants, vaccine adjuvants may need to exhibit more immunostimulatory properties such as mild inflammation. In this case, polyanhydrides may be excellent alternative candidates for both sustained released via surface erosion and adjuvanticity in vaccine delivery applications.

\subsection*{2.1.4. Polyanhydrides}

Polyanhydrides are a unique class of biodegradable polymers possessing qualities well suited for both vaccine and drug delivery. Although PLGA remains one of the most extensively studied polymers for drug delivery, its bulk eroding mechanism can be unfavorable for the delivery and stabilization of sensitive payloads by allowing interactions with water before release. Polyanhydrides, however, eliminate many of these challenges via a surface erosion mechanism which protects antigens and pharmaceuticals while controlling release.

Polyanhydrides are composed of diacid monomers linked by anhydride bonds. While the anhydride bonds are hydrolytically labile, the diacid monomers themselves are hydrophobic and exclude water from penetrating into the bulk of the material contributing to the surface erosion mechanism.\textsuperscript{29,30} Although release kinetics can be affected by polymer-payload interactions, release of proteins from polyanhydrides is highly correlated to the surface degradation of the
polymer. This surface erosion, and consequently release of payload, is easily tailored by varying the copolymer composition of polyanhydrides, allowing the manipulation of release from several days to years.\textsuperscript{29,31,32} For example, a copolymer composed of the two polyanhydrides, 1,6-bis (\(p\)-carboxyphenoxy) hexane (CPH) and sebacic acid (SA), will contain CPH-CPH bonds that undergo hydrolysis relatively slowly, SA-SA bonds that hydrolyze more quickly, and CPH-SA bonds. By synthesizing CPH:SA copolymers that are SA-rich or CPH-rich, degradation times of these copolymers can be tuned to weeks, months, or anything in between.\textsuperscript{29}

Surface erosion is an important factor not only for sustained release, but for the stability of the encapsulated antigen or drug. Exposure to water in bulk eroding polymers can lead to instability of proteins due to, for example, water-induced aggregation.\textsuperscript{33} Bulk erosion also confines unreleased protein in the presence of polymer degradation products. These degradation products, for example, lactic and glycolic acid in PLGA, create a highly acidic microenvironment that causes protein degradation and denaturation.\textsuperscript{33,34} Proteins encapsulated in surface eroding polyanhydrides are only exposed to water at the uppermost surface and proteins are not exposed to water or degradation products until release. The degradation products of polyanhydrides, which are dicarboxylic acids, are much less acidic, maintaining a microenvironment close to neutral pH.\textsuperscript{30,31} Although it is possible that the hydrophobicity of polyanhydrides can lead to protein aggregation, polyanhydrides can be designed to create more amphiphilic, favorable environments for protein stability. One such example, 1,8-bis (\(p\)-carboxyphenoxy)-3,6-dioxaoctane (CPTEG), was developed in the Narasimhan laboratory by incorporating oligomeric ethylene glycols into the backbone of CPH.\textsuperscript{33} The CPTEG and CPH monomers can be copolymerized to result in an amphiphilic polyanhydride that has been shown to sustain the release of stable antigens.\textsuperscript{33}

The degradation products of polyanhydrides have been shown to be biocompatible.\textsuperscript{30,31} As the polymer degrades, aromatic monomers are simply eliminated with no further need for metabolism. Aliphatic monomers, on the other hand, typically take part in the \(\beta\)-oxidation
pathway. The monomers are further eliminated through exhaled carbon dioxide, urine, or feces, while any small, insoluble fragments are cleared by macrophages and other inflammatory cells.\textsuperscript{29-31} Increased recruitment of these inflammatory cells is often correlated with injection site reactions such as pain and swelling; however, Huntimer et al. have recently demonstrated that even 10-fold doses of polyanhydride nanoparticles are less inflammatory than traditional adjuvants such as Alum or MPLA.\textsuperscript{35} In contrast with Alum, polyanhydride nanoparticles displayed minimal cell infiltration and resulted in normal kidney and liver function.\textsuperscript{35}

The promising biocompatibility of polyanhydrides has led to their use in many drug delivery applications. One of the best successes of polyanhydrides is the FDA-approved Gliadel\textsuperscript{®} wafer, a poly(1,3-bis(p-carboxyphenoxy)propane-co-sebacic acid) (CPP:SA) wafer encapsulating the nitrosourea oncolytic agent, carmustine. After tumor resection, the wafer is placed directly into the surgical cavity sustaining carmustine release.\textsuperscript{36} The CPP:SA formulation has also been used in the form of microspheres to deliver insulin for diabetic patients.\textsuperscript{29} In contrast to a typical regimen of multiple insulin injections per day, a single dose of CPP:SA microspheres released insulin for up to 35 days and maintained basal insulin levels \textit{in vivo}.\textsuperscript{29}

Aside from drug delivery, polyanhydrides have been investigated in vaccine delivery applications. Studies by Kipper et al. have shown that polyanhydride microparticles possess immunomodulatory capabilities, allowing the stimulation and targeting of specific arms of the immune system—which is beneficial for designing customized vaccine platforms for both extracellular and intracellular pathogens.\textsuperscript{37} The hydrophobicity of polyanhydrides is theorized to provide foreign danger signals to the immune system, leading to polyanhydride particles being more readily phagocytized by APCs, which is an important mechanism for vaccine efficacy.\textsuperscript{38} Recent studies have shown that polyanhydride microparticles possess dose sparing capabilities, producing antibody titers similar to 64-fold higher doses of antigen alone.\textsuperscript{39} Polyanhydride particles can also be surface modified to achieve pathogen-mimicking properties. For example, polyanhydride nanoparticles can be functionalized with carbohydrates that are
typically found on pathogens. Additionally, the morphometric properties and persistence of polyanhydride nanoparticles intracellularly is similar to the internalization and replication of microbial pathogens. Ultimately, a single intranasal dose of antigen-containing pathogen-mimicking nanoparticles resulted in 100% protection of mice against a live bacterial challenge 40 weeks post-vaccination. The success of polyanhydride nanoparticles in sustained release, protein stabilization, biocompatibility, and immune activation make them an excellent platform for subunit vaccine and drug delivery.

2.2. INTRANASAL VACCINATION

2.2.1. Pulmonary Immunology

The mucosal immune responses within the pulmonary system are unique, complex, and highly regulated processes. The lung is constantly exposed to airborne particulates in the external environment, and therefore must interact with foreign materials without producing inflammation that may damage the fragile air-capillary interfaces. Thus, the pulmonary system is composed of layered innate immune pathways to control and process antigen without stimulating adaptive immunity until real danger, such as bacteria or viruses, is present.

As with the rest of the body, the innate immunity in the lung begins with physical barriers. While most particulates become stuck within the mucociliary escalator and swallowed, there still remains a small percentage of foreign entities that reach the underlying epithelium (Figure 2.2). The epithelium plays a major role in regulating immune responses. For example, through expression of surface receptors and chemokines the epithelium cells control which leukocytes enter and respond to antigens within the lung. Epithelial cells also secrete antimicrobials, interferons, and complement components.

In addition to the epithelium, the lung contains many other innate cells that contribute to pulmonary immunity. For example, the epithelium contains specialized sampling cells called
nonciliated macro-fold cells. These cells pass antigens from the lumen of the lung to underlying APCs, such as dendritic cells (DCs) and alveolar macrophages (AMØs). The DCs of the lungs also extend dendrites into the lumen and sample antigens as well. Once activated, the DCs travel to the lymph node to stimulate and proliferate T cells. Perhaps more interesting are the alveolar macrophages, which traffic antigens to lymph nodes. Originally, it was generally believed that only DCs would migrate to lymph nodes, however, there is evidence now suggesting that within the pulmonary system AMØs may be, in fact, more important than DCs. AMØs have been shown to be steadily migrating from the lung to lymph nodes even in naïve mice, and once exposed to antigen, transport it to draining lymph nodes within two hours before DCs. AMØs also have immunosuppressive capabilities on T cells, contributing to the lung’s ability to tightly regulate inflammatory responses. Neutrophils also are key players in pulmonary immunity, phagocytizing antigen missed by AMØs. Although neutrophil infiltration into the lung tissues is highly restricted, they line the walls of pulmonary capillaries filtering materials from systemic circulation.

Adaptive immunity composed of T and B cell responses also have several distinct characteristics exclusive to the pulmonary system. T cells recruited to the lungs are thought to be hypo-responsive, again minimizing inflammatory damage to sensitive lung surfaces. B cells secrete IgA, a unique antibody to mucosal surfaces, neutralizing pathogens before entry into epithelial cells. While IgG can also be induced systemically, it is much more specific than IgA. Therefore, IgA can maintain cross reactivity against drifting pathogens, for example, the change in epitopes of seasonal influenza.

Pulmonary immunity is indeed a distinctive and highly regulated system. However, as many pathogens, such as influenza, commonly enter the body through the lungs, eliciting mucosal immunity is essential for protection. Since most routes of vaccination stimulate systemic but not mucosal immunity, intranasal vaccination provides a pathway of inducing appropriate responses for respiratory pathogens.
2.2.2. Advantages of Intranasal Vaccination

Vaccine formulations with “pathogen mimicking” characteristics are often the most successful at eliciting protective responses. With most pathogens entering the body at mucosal surfaces, intranasal vaccination has many advantages by inducing mucosal immunity.\textsuperscript{47} Most importantly, if mucosal immunity is desired then mucosal immunization must be used.\textsuperscript{48,49} Many other routes of immunization such as subcutaneous or intramuscular only induce systemic immunity that may not be protective against mucosal pathogens. Immunization at a mucosal site, however, has been found to induce both local and systemic immunity towards the antigen.\textsuperscript{48,49}

Antigen availability is an important part of intranasal immunization that leads to both pulmonary and systemic immunity. By administering the vaccine formulation to the lungs, antigens avoid degradation in the first pass metabolism, often caused by enzymatic activity and the extreme pH of the stomach.\textsuperscript{17,48-50} The increased availability of antigen works to the advantage of systemic immunity with the large permeable and adsorptive surface area of the lungs.\textsuperscript{17,48,49} However, local immunity is also induced by the many immune-reactive sites of the lungs which are more enriched in with T cells, B cells, and plasma cells than systemic circulation.\textsuperscript{50}

Delivery of free antigen intranasally is often not sufficient for protective immunity due to the mucosal barrier and clearance by the lungs.\textsuperscript{17} Therefore, carriers and/or adjuvants such as polymeric particles are often used to administer antigen successfully. As discussed previously, hydrophobic polymers enhance antigen uptake by APCs, but incorporation of hydrophilic groups can also assist penetration through the mucus layer.\textsuperscript{50} Manipulating the charge of particles, for example by modifying the surface for positive charge, allows the association and persistence of antigen at the negatively charged epithelial layer.\textsuperscript{50} Finally, immunomodulatory stimulants can be co-delivered with antigens to target the expansive innate immune pathways present in the lungs. Ligands for TLRs are often used to induce type I interferons, bridging the innate and
adaptive immune responses. By taking advantage of the unique innate pulmonary immune responses, efficacious vaccines can be designed for intranasal delivery.

2.3. H5N1 AVIAN INFLUENZA

2.3.1. Introduction

Influenza A virus is a single strand negative sense RNA virus of the Orthomyxoviridae family. The genome of influenza contains eight segmented RNA strands encoding for the glycoproteins hemagglutinin (HA) and neuraminidase (NA), matrix proteins (M), and ion channels (Figure 2.3). The segmented genome also allows for re-assortment of the segments to take place between different viruses infecting the same host cell, leading to genetic shift. Genetic shift is the basis for the sub-classification of the influenza virus based on the sixteen subtypes of hemagglutinin (HA) surface proteins and the nine subtypes of neuraminidase (NA) surface proteins, allowing for a possible 144 unique influenza strains. These surface proteins are the antigens towards which host antibody responses are directed, each of which maintains a unique function. The HA proteins are responsible for attaching the virus to epithelial surfaces, such as the lungs, by binding sialic acid receptors. Once attached, HA facilitates the fusion of the viral envelope with the cell membrane, allowing the virus entrance to the target cell. The NA protein is involved in preventing clumping during virus release, promoting the spread of infection to other cells. Both HA and NA perform their functions by binding and cleaving sialic acid on cell surfaces. In the H5N1 strain, both protein subunits have several cleavage sites instead of just one, increasing the rapid spread of infection from cell to cell, and therefore, pathogenicity.

A major concern with respect to H5N1 is the possibility of human-to-human transmission through antigenic shift and drift. During antigenic shift, two or more viruses reassort to form a new virus type, containing epitopes of both viruses. For example, it has been observed that
pigs co-infected with both avian and human flu types become a melting pot for the viral genome. The swine respiratory tract contains both α 2-3 (avian) and α 2-6 (human) sialic acid linkages, making the pig a potential mixing vessel for genetic re-assortment. Because of the ability of influenza to mutate very quickly, there are concerns that the mutations needed to change binding preferences of sialic acid linkages from the avian α 2-3 to the human α 2-6 could lead to a global pandemic, similar to the 2009 H1N1 pandemic. Recent work with serial passage of the wild type virus in ferrets, the preferred animal model for recapitulating human responses to influenza because of sialic acid receptor distribution and pathological similarities, has demonstrated that the virus will adapt to airborne transmission albeit with a loss in pathogenicity. These studies, while generating controversy within the scientific community, are essential to the understanding of the virus and the capabilities of the HPAI H5N1 to evolve and adapt to human-to-human transmission.

H5N1 is a highly pathogenic form of influenza affecting its host systemically as well as locally. The easy cleavage of HA and NA proteins, as well as the large gene re-assortment through shift and drift, allows H5N1 to infect a host very quickly resulting in symptoms such as fever, sore throat, coughing, and in severe cases pneumonia. Further insight into the mechanisms that manifest flu symptoms in both protective (full recovery by the patient) and pathogenic (poor) immune response will be discussed in later sections of this chapter.

2.3.2. H5N1 Pathogenesis

H5N1 is most commonly transmitted through aerosol droplets, infecting through sneezing or coughing. The most common symptoms of H5N1 influenza are fever, cough, throat congestion, and sore throat. Often times, pneumonia can develop as a result of inflammation causing the release and buildup of fluid in the lungs, or secondary bacterial infections. In such cases, hospitalization is needed for medical treatment (using anti-viral drugs) and careful monitoring is performed via chest exams and cell counts. Usually the duration of H5N1 flu
lasts seven to ten days in patients that recover and achieve protection. However, in pathological cases, H5N1 has been known to cause death within 1 to 3 days.\textsuperscript{62-64}

Recent studies have shown that the highly pathogenic H5N1 rapidly infects epithelial cells as well as important immune cells, including DCs, macrophages, and other APCs.\textsuperscript{65} In this case, cytokine production is inhibited due to the increasing apoptosis and disabling of virally infected APCs. Through this mechanism, both the innate and adaptive arms of the immune system lack the encouragement and signaling they need, leading to mortality.

More common, however, is the theory of a “cytokine storm,” or hypercytokinemia.\textsuperscript{66,67} In this case, the rapid spread of the virus elicits a strong, systemic response. A flood of immune cells, including DCs, macrophages, and NK cells, rush toward the infected areas, in this case the lungs, releasing large amounts of cytokines including TNF-\(\alpha\), IL-6, IL-1\(\beta\), IL-10, and IFN \(\alpha\) and \(\beta\) and the chemokines RANTES, MCP-1, MCP-3, MIP-1\(\alpha\), and MIP-1\(\beta\).\textsuperscript{68-70} \textit{In vitro} infection of bronchial alveolar epithelial cells showed an increase in IFN-\(\beta\), RANTES, and CXCL10 early in infection as compared to a seasonal H1N1 virus.\textsuperscript{71} Additionally, mutations in the HA viral sequence leading to multiple basic amino acids at the HA cleavage site of HPAI viruses skew the ability of the virus to replicate in multiple tissue sites as compared to other influenza viruses associated with the respiratory tract.\textsuperscript{72,73} The ubiquitous distribution of replicating HPAI virus contributes to an overabundant antiviral immune response. Normal immune responses regulate cytokine production, enabling cells to turn off production after some time. However, for reasons yet unknown, the production of cytokines is out of control and unregulated during H5N1 infection. This large overproduction of cytokines often causes more injury than help, for example, inflammatory responses lead to pneumonia and the accumulation of fluid in the lungs blocking of airways.\textsuperscript{66,67} In studies on hypercytokinemia, normal mice infected with H5N1 were compared to mice whose cytokine regulators had been knocked out. Both sets of mice reacted similarly to H5N1, with no survival after ten days.\textsuperscript{67} Researchers concluded that hypercytokinemia plays a major role in the pathology of H5N1 influenza, increasing damage to
The theory of the cytokine storm is important in understanding the fine balance of our immune system. Cytokines play an important role in activating immune cells, such as Th1 and CTLs, to combat infections. However, if not carefully controlled and regulated, the immune system can be more detrimental than helpful.

The cytokine storm is not the only process that mediates pathogenic responses to H5N1. Studies have shown that inhibition of cytokine production does not aid in protection. The role of cell receptors, such as TLRs, can also increase the lethality of H5N1. TLRs are pattern recognition receptors and found on a wide range of immune cells, binding non-specifically to an assortment of pathogens. The elusive H5N1 binds few TLRs during infection, limiting immune recognition and response. However, studies have shown that cross-linking of several combinations of TLRs promotes immune responses by increasing the avidity of T cell effector functions. TLRs are an increasing subject of H5N1 studies and many preventive methods, such as vaccines, depend upon the role of TLR binding during viral infection.

Macrophages commonly have C-type lectin receptors (CLRs) that bind to the hemagglutinin portion of viruses. Through this receptor, the virus gains entry into the cell and begins its process of replication. In influenza, the macrophage receptors have weak interactions with viral particles and limited entry is gained. Due to the limitation of infecting cells, and the cytokines released by macrophages, the virus is normally cleared. H5N1 virions are different in that they have high affinity for macrophage receptors. Strong interactions allow virions to gain easy access to macrophages, inducing large production of cytokines while inhibiting the regulators previously discussed in hypercytokinemia.

Another receptor, chemokine receptor 6 (CCR6), is found on DCs, B cells, and effector T cells. CCR6 is responsible for binding a pro-inflammatory chemokine and stimulating the immune system. H5N1 and other respiratory viruses are known to block CCR6 by an unknown pathway and disturb immune recruitment. By blocking CCR6 signaling, studies have found
that viral infection increases pathology of the lungs and diminishes important cell-mediated responses involving dendritic cells and CTLs.\textsuperscript{77}

2.3.3. Immune Response to Orthomyxoviruses

The innate immune response consists of protection we are born with. It is the first defense against pathogens, responding very quickly. Although innate immunity often is not enough to completely clear infection, it can hold it at bay until the slower, but stronger adaptive response arrives.

Important cytokines are released during viral infection via APCs such as macrophages. Inflammatory cytokines, such as IL-1, IL-6, and TNF-\(\alpha\), raise the body temperature to a fever and slow the replication of the virus.\textsuperscript{78,79} These cytokines are also responsible for encouraging complement pathways to bind released virus molecules in an immune complex that will be cleared by macrophages.\textsuperscript{78} Cytokines also provide a chemical gradient to recruit immune cells by a process called chemotaxis and allow cells to hone to the infected tissues.\textsuperscript{78,79} Finally, cytokines such as IL-2 and IL-4 encourage the adaptive immune response to help combat infection.

Virus-infected cells also produce the interferons, IFN-\(\alpha\) and IFN-\(\beta\). Both of these molecules inhibit viral replication, increase MHC class I expression (antigen presentation to immune cells), and activate natural killer (NK) cells.\textsuperscript{78,79} NK cells are large lymphocytes that circulate in the blood and migrate into tissue, carrying cytotoxic granules within their cytoplasm. When stimulated by IFN-\(\alpha\) and IFN-\(\beta\), NK cells release their granules to kill targeted cells. When stimulated by IL-12, NK cells produce cytokines, including IFN-\(\gamma\), which increases activity of NK cells as well as activated macrophages. Macrophages will in turn activate T cells of the adaptive immune system through cytokines of their own.\textsuperscript{78,79} As T cells activate, proliferate, and differentiate into CTLs, they take over the production of IFN-\(\gamma\) and turn off NK cells through the production of IL-10.\textsuperscript{79} Through this process, the innate immune system quickly responds and
activates the adaptive immune response while controlling infection, and then steps aside, leaving adaptive immunity to completely clear the virus.

During the adaptive immune response, T cells travel to the lymph nodes and check APCs for their specific antigen. Once T cells bind to its specific antigen, in this case a viral epitope, they activate, proliferate, and differentiate into effector T helper (CD4⁺) and cytotoxic T (CD8⁺) cells. During the adaptive immune response, T cells travel to the lymph nodes and check APCs for their specific antigen. Once T cells bind to its specific antigen, in this case a viral epitope, they activate, proliferate, and differentiate into effector T helper (CD4⁺) and cytotoxic T (CD8⁺) cells.⁷⁸,⁷⁹

Effector T helper cells are influenced by the presence of cytokines. For example, during viral infection NK cell production of IL-12 and IFN-γ mediate the production of Th1 cells. Th1 cells are responsible for the stimulation of both macrophages and cytotoxic T cells through the cytokines previously discussed, such as IFN-γ.⁷⁸,⁷⁹ Although Th2 cells can be sometimes involved in antiviral responses, they commonly stimulate B cells and the production of antibodies against extracellular pathogens.⁷⁹ In most cases of viral infection, pathways towards cell-mediated immunity are influenced by the cytokine signals of macrophages and NK cells, leading to a biased response of Th1 cells.

Effector CD8⁺ cells, or activated CTLs, take over for the innate NK cells by killing infected cells. CTLs target virally infected cells and bind to them, locally releasing cytotoxins such as perforin, granzymes, and granulysin, thus inducing cell death by apoptosis.⁷⁸,⁷⁹ Following the clearance of the virus and infected cells, some of these T cells develop into memory cells, scanning the body for future infection(s) of the virus.⁷⁸,⁷⁹ In this way, the adaptive immune system learns how to deal with the virus and can respond quickly to future infections.

2.3.4. Protective responses to H5N1

When 60% of all H5N1 cases result in mortality, it is puzzling to researchers as to why some patients survive and others, unfortunately, do not.⁶³ Currently, influenza pandemics affect mainly the young and healthy, as opposed to seasonal flu, where the elderly are most affected. Many researchers believe that today’s pandemics are variations of old flu strains, such as those
in the 1918 Spanish flu pandemic and the Asian flu pandemics of 1957 and 1968. New pandemic strains are usually the result of an antigenic shift, crossing a species barrier (i.e., bird to human) and presenting several new epitopes. Researchers believe that because the H5N1 strain is similar to earlier pandemics, previously exposed populations such as the elderly, may be protected against infection.

Other facets of protection are understood even less. In some cases H5N1 replicates faster or sometimes, a patient’s immune system responds faster. Highly pathogenic influenza strains, such as H5N1, have most commonly been found to have weak cytokine production in immune responses. However, when a patient experiences a robust cytokine response and is able to activate both the innate and adaptive branches of the immune system, protective immunity can be achieved.

Another important part of protective immunity is medical intervention, which will be discussed later in the chapter. Briefly, in cases when H5N1 can be identified early, anti-viral treatment, such as the popular oseltamivir (Tamiflu), can be administered in twice daily doses of 75 mg to reduce symptoms. Hospitalized patients can also be monitored for low white blood cell and platelet counts, a sign of severe infection, as well as pneumonia, which commonly presents itself with respiratory pathogenic H5N1.

Aside from treatment, prevention is beginning to be a focus in protective immunity. Current vaccinations for H5N1 are poorly immunogenic, but at least provide exposure to the H5N1 antigen. Some scientists believe that limited responses occur in all the new vaccines, such as the vaccines based on H5 HA protein, and large doses are needed to secure even a little protection, despite the lack of understanding of these mechanisms. However, H5N1 vaccination is a fast growing research area, working towards goals of increased immunogenicity and long term protection of patients.
2.3.5. Treatment of H5N1

The treatment of H5N1 is limited by the small selection of antiviral drugs available. In contrast to antibiotics, the development of antiviral medications has not been as successful. However, two treatments are capable in reducing the infection of H5N1 by inhibiting either the HA or NA proteins.

Oseltamivir, also known as Tamiflu, is an antiviral drug commonly used to treat influenza. When given within 48 hours of the first symptoms, oseltamivir can drastically reduce both the duration and severity of viral infection, and is safe and effective for patients over one year of age. The course of treatment is administration in 75 mg doses, with three doses the first day and twice daily for the following five days. In most cases, oseltamivir is paired with an antibiotic to protect against secondary bacterial infections that affect weakened patients, such as those with pneumonia. Oseltamivir is a neuraminidase inhibitor, preventing the release and spread of viral particles from one cell to another. First, the administered phosphate form is cleaved by esterase enzymes in the liver. Once converted into a carboxylate form, oseltamivir can bind and inhibit the NA protein of the viral envelope. With the NA protein inhibited, virus particles clump together and prevent their release to other cells.

Recent studies, however, show a rising disadvantage: new oseltamivir-resistant H5N1 strains. During treatment, an amino acid mutation in the NA portion of the virus allows the resistance to oseltamivir. In the study by de Jong et al., only 50% of H5N1 patients survived during treatment and had no H5N1 mutations. Initial treatments identified the current H5N1 strain in all patients, however, all the lethal cases showed mutation to the oseltamivir-resistant H5N1 strain.

Ion channel inhibitors such as amantadine and rimantidine are viable options for anti-viral treatment in pandemic HPAI situations. Amantadine and rimantidine form a barrier at the M2 influenza viral protein that functions to enable the entry of H+ ions. HA relies on an acidic environment for conformational changes and goes through to fuse the viral envelope with a cell
membrane. By modifying the pH, the virus cannot gain entry into the cell. Rimantadine has also experienced H5N1 resistance, but in this case, alterations are made to the HA instead of the NA protein. Oseltamivir still remains the most common treatment for influenza because it reduces symptoms and the spread of infection.

Corticosteroids have been used for decades in the treatment of influenza. The use of corticosteroids reduces inflammation by down-regulating the production of cytokines such as IL-2, TNF-α, and IFN-γ. Through this, corticosteroids control and reduce the effects of hypercytokinemia. It is important to note that corticosteroids do not clear the virus, but only help to control the immune response towards the virus. In the case of H5N1, it is necessary to use antiviral drugs, such as oseltamivir or rimantadine, to fully clear infection.

2.4. CURRENT VACCINE APPROACHES TO H5N1

The severe pathology of H5N1 infection in humans, the increased risk of secondary infections, as well as the high frequency of mutation of influenza which could lead to human to human transmission, have motivated research in the realm of preventive therapeutic approaches against HPAI. In 2007, the FDA approved the first H5N1 vaccine in the United States. The current H5N1 vaccine uses two 90 μg doses administered 28 days apart. However, the vaccine is generally poorly immunogenic. In fact, FDA studies show that only 45% of patients receiving the vaccine had antibody titers suitable for protection. Generally, many vaccines follow a Th2 route of immune response, creating antibodies against a pathogen. However, H5N1 is a virus, and hence an intracellular infection, and Th1 or cell-mediated responses may be important to clear the virus. Future research towards the development of a H5N1 vaccine (described below) needs to focus on inducing both cell-mediated responses and antibody production to promote immunogenicity and protection. In addition, new vaccine development strategies must take into account both the safety and potency of the formulation, which is often challenging when safer vaccines are typically less potent (Table 2.2). This
section will focus on the traditional seasonal influenza vaccines and the possibility of using those for pandemic preparedness to H5N1 as well as on emerging vaccine and anti-viral technologies which may enable pandemic preparedness (Figure 2.4).

2.4.1. Inactivated Viral Vaccines

The traditional seasonal influenza vaccine consists of formaldehyde or β-propiolactone inactivated virus propagated in embryonated hen eggs, and more recently, propagation of influenza virus in Vero cell cultures to replace hen egg propagation. The antigenic components of the vaccine are either administered as whole inactivated virion, purified subunit of surface glycoproteins, or chemically split virus vaccine. These aspects of the seasonal influenza vaccine may therefore be applied towards stockpiling H5N1-specific vaccines for pandemic preparedness. Seasonal influenza vaccine protection profiles are based on a close antigenic match and previous exposure to influenza. The previous exposure to the HPAI H5N1 will be absent due to the fact that the seasonal influenza A virus contains H1 and H3 (and not H5) HA. Inactivated influenza virus vaccines can result in rubor and tumor at injection sites with frequency of incidence dictated by age but no differences from placebo were observed. People with egg albumin allergies cannot be administered the traditional mass-produced inactivated vaccines because of hen egg virus propagation. The main conundrum of using traditional seasonal inactivated influenza virus techniques to produce pandemic vaccines is the time needed for the manufacture and production of the virus. Typical mass production timelines are estimated to be 6-9 months. However, a dominant pandemic strain would need to be identified prior to production delaying when the vaccine could make it to clinics.

The immune response induced by inactivated influenza virus vaccines is predominately humoral. Antibody responses are typically directed towards the surface glycoproteins HA and NA. Systemic antibody specific for HA and NA is predominately IgG1 with some IgM and IgA being identified with protective antibody responses elicited within two weeks post-vaccination.
IgG and IgA specific antibody secreting cells have been identified in peripheral blood following vaccination. The isotype switch associated with these humoral responses can be attributed to the prime/boost effect of the previous year’s vaccinations. Combinations of antiviral NA inhibitors with inactivated virus vaccines may also be an effective regimen during a pandemic due to little interference with the humoral response kinetics in clinical studies. Inactivated viral vaccines can also elicit stronger influenza antigen specific proliferative responses of T cells found in the palatine tonsils and peripheral blood, although this may also be attributed to the prime/boost response of either natural seasonal influenza infection or previous vaccinations.

Studies with inactivated HPAI H5N1 prepared and administered similarly to licensed seasonal vaccines still present the most viable option for pandemic formulations due to the vaccine not having to undergo licensure but rather be considered a new strain of influenza added to an existing licensed product to alleviate regulatory delays. Inactivated subvirion H5N1 virus vaccines were shown to provide virus neutralization titers in 54 percent of individuals receiving two doses of 90 μg of HA which is approximately six times the dose of seasonal influenza vaccines. A TLR7 dependent mechanism associated with increased immunogenicity of inactivated H5N1 vaccines because of residual viral RNA in the preparation has been identified. Aluminum salt adjuvanted inactivated split virion H5N1 vaccines were able to elicit 67% HA inhibition in patients after two doses of 30 μg HA. Post vaccination, immunological responses to split virion H5N1 were greater in infants and children. Additionally, whole virion inactivated H5N1 vaccines were able to produce 78% seropositivity when administered in a two dose regimen with aluminum salts at a dosage of 10 μg of HA. Vero cell derived inactivated H5N1 vaccines show similar moderate humoral immune responses with and without Al(OH)₃ adjuvant.
2.4.2. Cold adapted attenuated viral vaccines

The field of influenza vaccinology was expanded by the licensure of a cold adapted live seasonal influenza vaccine (Flumist®, MedImmune, Gaithersburg, MD) in 2003. Cold adapted live influenza viruses are based on the master strain A/Ann Arbor/6/60 (H2N2) being attenuated by passaging the virus through decreasing temperatures in embryonated hen eggs. The continuous passage through decreasing temperatures allows for mutational adaptations in the polymerase (PA), PB1, PB2, and M genes of influenza leading to the cold adapted phenotype in which replication is restricted to 33°C in the upper respiratory tract.\textsuperscript{108} The cold adapted virus expressing the HA and NA of interest, either the seasonal influenza strains or pandemic strains, is produced by genetic reassortment.\textsuperscript{109} The modified live cold adapted influenza virus allows for an intranasal vaccination that mimics the natural infection in both site and mode of infection, but under the virus replicative constraints of the upper respiratory tract. The upper respiratory replication of the virus allows for lower doses of influenza antigen.

The cold adapted influenza virus seasonal vaccines have shown cross-reactive immune responses to other antigenically drifted H1N1 and H3N2 strains.\textsuperscript{110-112} This cross reactivity could prove extremely beneficial in the stockpiling of pandemic influenza vaccines because prediction of the antigenically drifted strain of influenza will be difficult.\textsuperscript{113} The intranasal route also allows for ease of administration by individuals with minimal professional training during a pandemic.

Cold adapted H5N1 virus vaccines were able to provide incomplete protection after single dose vaccinations with homologous and heterologous viral challenge but two dose regimens provided heterologous cross protection in laboratory models.\textsuperscript{114} Safety and toxicological profiles of the cold adapted H5N1 vaccines were favorable in the ferret model.\textsuperscript{115} Clinical evaluations of a cold adapted influenza vaccine containing the H5 and N1 outer glycoproteins of two H5N1 isotypes A/Vietnam/1203/2004 and A/Hong Kong/213/2003 on the A/Ann Arbor/6/60 show limited viral replication leading to nominal neutralizing humoral immune response.\textsuperscript{113} Immune responses were elicited using a low pathogenicity avian influenza
A/Duck/Potsdam/1402-6/86 (H5N2) genetically reassorted to a cold adapted A/Leningrad/134/17/57(H2N2) that show cross reactivity to an H5N1 strain in a HA inhibition test.\textsuperscript{116} Interestingly, a cold adapted vaccine consisting of HA and NA of the 2009 pandemic H1N1 strain was able to protect mice from lethal H5N1 challenge.\textsuperscript{117,118}

2.4.3. Reverse Genetics Derived Virus

The traditional method of genetic re-assortment of influenza virus for vaccines in embryonated hen eggs can be inefficient and variable. Recent advances in molecular biological techniques have allowed for the formation of infectious influenza A virus completely from plasmid cDNA expression of the viral RNA segments transfected in human embryonic kidney cells (293T) or Madin-Darby canine kidney (MDCK) cells.\textsuperscript{119,120} The reverse genetics system of creation and propagation of influenza virus allows for molecular manipulation of the genetic elements to decrease pathogenicity, a more targeted approach for creation of genetically reassorted viruses, and alleviation of propagation validation issues associated with production of virus for vaccine purposes.

The initial reverse genetics systems demonstrated the rescue of seasonal influenza variants containing the HA and NA of A/New Caledonia/20/99 (H1N1), A/Panama/2007/99 (H3N2), A/teal/HK/W312 (H6N1), and A/quail/HK/G1/97 (H9N2) and the six internal genes of PR8, a high growth lab strain of influenza.\textsuperscript{121} Subsequently, reverse genetics derived strains containing the HA and NA of H5N1 were created\textsuperscript{122,123} and adapted for expression in Vero cells, which have been previously validated for the production of polio vaccine.\textsuperscript{124-126} The reverse genetics derived system for production of re-assorted viruses verified the decreased timeline needed for reverse genetics derivation compared to the traditional embryonated hen egg reassortment method. Vero cells present difficulties in transfection efficiency compared to non-production validated cell lines such as 293T cells. Adenovirus vectors that, when transduced in Vero cells, produce the viral RNA needed for creation of the viral ribonucleoprotein complex by
transferring the same cells with plasmids encoding the PB1, PB2, and PA subunits of the viral polymerase increased the viral production efficiency from the traditional eight plasmid method.\textsuperscript{127}

An inactivated reverse genetics virus was used for immunization and elicited cross neutralization to several H5N1 strains isolated from human infections in a guinea pig model and protection from challenge with three different H5N1 viruses in mice vaccinated with a two dose regimen.\textsuperscript{128} An H5N3 reverse genetics derived virus was used for an inactivated vaccination challenge model in ducks and chickens that provided protection in a two dose and a single dose regimen.\textsuperscript{129} Finally, a formalin inactivated reverse genetics derived H5N1 oil emulsion vaccine also showed protection 43 weeks post vaccination in chickens.\textsuperscript{130}

The reverse genetics platform allows for a more targeted approach towards generation of cold adapted influenza virus with the A/Ann Arbor/6/60 (H2N2) backbone and the H5N1 glycoproteins of HPAI H5N1. Reverse genetics derivation of cold adapted influenza virus for modified live vaccines have shown potential in two dose intranasal administrations in reducing wild type virus replication.\textsuperscript{131} Protection from homologous and heterologous virus challenge in mice, ferrets, and macaques has also been reported when boosted with a second immunization.\textsuperscript{114,132}

\subsection*{2.4.4. DNA Vaccines}

Viral infections are good candidates for DNA vaccination because infection of the host cells hijacks the cellular machinery for viral replication. DNA vaccination, in general, refers to the initiation of an immune response against an antigen introduced via purified DNA in supercoiled plasmids that encode for the antigen (poly)peptide sequence.\textsuperscript{133} The plasmid DNA incorporates into the host cells via direct gene transfer and the host cells produce the protein antigen. Mass production of plasmid DNA is less labor intensive than protein antigens and DNA
storage conditions are less intense when proper techniques are used, providing advantages in stockpiling pandemic vaccines.\textsuperscript{134}

The immune response elicited to DNA vaccines is primarily skewed to the Th1 type of immune response, in which cell-mediated immunity (CMI) to the DNA encoded antigen is more prevalent with IFN-γ and IL-12 being the predominant cytokines being reported in the vaccine response.\textsuperscript{133} Although the entirety of the plasmid DNA in the vaccine is non-immunogenic, the plasmid DNA can be a non-specific immunostimulatory component through pattern recognition receptors (PRRs) thus providing an innate adjuvant response.\textsuperscript{135,136} The proposed mechanism of DNA vaccines mimics the cellular pathogenesis of viruses. The post transcriptional and translational proteins are split into smaller peptides by intracellular proteosomes of either muscle cells, epithelial cells, or APCs.\textsuperscript{137,138} The peptides are presented by MHC class I molecules after trafficking through the endoplasmic reticulum of the host cell. The MHC class I molecule containing the peptide then elicits the appropriate CD8\textsuperscript{+} T cell responses.

The stability and ease of manipulation of plasmid DNA allows for encoding of additional immunostimulatory components to be transcribed and translated by the host cell. Some components that have been encoded to be co-expressed with the antigen of interest include the cytokines IFN-γ, IL-2, IL-12 and GM-CSF, co-stimulatory molecules such as B7 (CD80 and CD86) and CTLA4-Ig, as well as DNA sequences that are specifically immunostimulatory.\textsuperscript{139-144}

The molecular techniques developed in the last decades and the antigenic drift associated with influenza have provided resources to enable fast production of a pandemic H5N1 vaccine. Protection from lethal challenge of heterologous strains of H5N1 influenza with DNA vaccines encoding HA proteins as well as protection from homologous strains encoding for NA, NP, or M2 have been demonstrated.\textsuperscript{145-147} Dependency on the HA protein needing to be encoded in the formulation for humoral and cellular immunity is evident when combinations of all the antigenic targets are used.\textsuperscript{148-150} Addition of a virus induced signaling adaptor (VISA) molecule in the DNA antigenic preparation of an H5 HA peptide was able to convey protection
from lethal challenge in a mouse model. Heterotypic immunity against different clades of HPAI viruses has been reported making thus validating the approach. Clinical trials of H5N1 DNA vaccines have begun.

2.4.5. Virus Like Particles (VLPs)

Responding to the need for enhanced immunity and novel approaches for potential pandemics, VLPs present viral antigens in a more native and therefore immunogenic fashion. VLPs are self-assembled membranes (a virion shell) containing relevant viral proteins such as HA, NA, nucleoprotein (NP), and matrix protein 1 and 2 (M1, M2). The presented proteins are active and remain in their native structures similar to live virions.

VLPs are immunogenic due to their ability to mimic live virions at the cellular level. Due to the lack of the viral genome, VLPs are non-replicating and non-infectious and therefore, remain immunogenic and safe to those in high-risk groups such as the elderly, even after several administrations. Immunogenicity of VLPs can be further enhanced due to the particle’s adjuvant properties to stimulate both humoral and cellular immune responses, including cross-reactive antibodies that protect against the variability of influenza strains.

VLP proteins are commonly recombinant based, produced by the baculovirus system in yeast, insect, or mammalian cells. Independence from egg-derived vaccines is especially important for an avian influenza vaccine, due to the limited egg supply and the H5N1’s ability to kill egg embryos. Egg independence also eliminates the need for live viruses during production and manufacturing, and is therefore very favorable for production without the need for chemical inactivation or biosafety containment.

VLPs against H5N1 have been successful in eliciting cross-reactive responses between subtypes. H1N1 VLP intranasal vaccinations in mice and ferrets induced high levels of cross-reactive IgG and IgA and had two to three fold greater amounts of IL-2, IL-9, IL-10, and...
IL-17 than other vaccinations, while remaining protective against a heterologous H5N1 challenge. VLPs using HA and NA surface proteins, while internalizing the conserved epitopes of the M1 protein, stimulated the activation and proliferation of CD8+ T cells that increased production of IFN-γ, TNF-α, and IL-2 when compared to recombinant protein subunit vaccines. Although the HA protein is mainly responsible for inducing H5N1 specific antibodies, the M1 protein’s conserved epitopes are generally accountable for cellular immunity and cross-protection. This increase in cellular immunity was also apparent in the ratios of antibodies found with H5N1 VLP vaccine responses. Post-vaccination, IgG2a and IgG2b titers were largely increased resulting in a balanced IgG1:IgG2 ratio. H5N1 vaccines using VLP technology showed long lasting memory responses in challenge with single does as low as 0.4 μg and utilized needle-free technologies for inoculation. Protective efficacy of VLP vaccines against H5N1 have been shown in poultry and a computationally optimized broadly reactive antigen (COBRA) H5 VLP vaccine showed protection from challenge in a non-human primate model challenged with clade 2.2 A/Whooper Swan/Mongolia/244/05 (WS/05).

2.4.6. Subunit Vaccines

Recombinant subunit protein-based vaccines using a single protein antigen have also been examined due to the increased purity and safety profiles. Using this system, individual viral proteins, most commonly HA and NA, are recombinantly synthesized in mammalian cells or cells that support post-translational modifications such as glycosylation, allowing the production of correctly folded proteins.

While the baculovirus technology allows rapid production of recombinant proteins, there is some debate on using insect or mammalian cultures. During translation of recombinant H5 HA protein, mammalian cells commonly produced a high-molecular weight oligomer or trimer form of HA. Insect cells have been more likely to produce cleaved or monomer forms of the HA protein, although some studies have shown stable production of
trimer HA in insect cultures.\textsuperscript{167-169} In most cases, the immunogenicity of the H5 HA protein was highly dependent on its oligomer and trimer forms showing that in a weakly immunogenic protein it is optimal to present it in its natural trimeric state.\textsuperscript{167-169}

Besides the challenges associated with protein production, subunit antigens like H5 HA are a weak immunogens. Therefore, several large doses (up to 90 μg) are needed to achieve suitable protection. However, with the help of novel adjuvants and/or protein carriers, the immunogenicity of HA can be greatly improved to reduce both the number and size of vaccine doses.\textsuperscript{165,168,171,172} H5 HA has been shown to be the only protein inducing H5N1 specific neutralizing antibodies. While other proteins, such as NA, are also included in protein-based vaccinations, they have not been shown to be protective on their own. When used in tandem with HA protein, NA enhanced cross-reactive protection between H5N1 clades.\textsuperscript{167,166,170,171}

Like DNA vaccines and VLPs, recombinant proteins can be produced quickly in mass quantities without the dependence on a limited egg supply.\textsuperscript{167,169} Large scale production of recombinant proteins is feasible because no live viruses are required, eliminating the need for bio-containment facilities.\textsuperscript{167,169} Novel recombinant production techniques that use plant-based systems are being developed for influenza HA antigens.\textsuperscript{173} Likewise, recombinant proteins can be highly purified and don’t utilize chemical inactivators, reducing contamination and adverse effects on patients.\textsuperscript{167}

Oral and intranasal vaccinations of baculovirus produced H5 HA led to effective, long-lived production of IgA in mucosal tissues, as well as protection in mice.\textsuperscript{169,171} Likewise, H5N1 proteins such as M2, NP, and NA have also been used to induce cross-protection between clades of avian influenza.\textsuperscript{170,174,175} As mentioned previously, HA is the primary antigen that stimulates neutralizing antibody in H5N1.\textsuperscript{174} However, by using more conserved proteins, studies have shown an increase in HA-specific neutralizing antibodies, as well as IFN-γ producing CD8\textsuperscript{+} T cells, especially in the presence of an adjuvant.\textsuperscript{170,174-176} Recombinant production of HA proteins of H5 allows for manipulation of the antigen, specifically in the
creation of stabilized trimeric forms of the antigen that increase the immunogenicity of the protein.\textsuperscript{177-179} Licensure of a recombinant HA vaccine, FluBlok, grown in insect cells has been a recent advancement in this area.\textsuperscript{180}

\textbf{2.4.7. Immune Refocusing}

Immune refocusing, also termed as deceptive imprinting or epitope masking, is a new advance in influenza vaccine technology. Immune refocusing targets class II pathogens, such as influenza, or pathogens that have immune responses to strain specific epitopes, yet high rates of mutation within these epitopes.\textsuperscript{181} Tobin et al. theorize that pathogens, such as H5N1 avian influenza, have immunodominant epitopes, such as the globular head of the HA trimer, and subdominant epitopes, or more conserved proteins.\textsuperscript{181} These dominant epitopes are distractions to obstruct the immune system from recognizing and manufacturing antibodies against more protective or cross-reactive epitopes.\textsuperscript{182} This concept arises from the theory of original antigenic sin.\textsuperscript{183} For example, a secondary influenza infection containing epitopes previously recognized from a former strain as well as new epitopes will only produce antibodies against the previous epitopes, eliminating the infection quickly during a memory response.\textsuperscript{183} However, in the cases of HPAI, strain specific epitopes dominate the immune response and block reactions to epitopes that appear from strain to strain. In this case, the immune system must respond to each infection as a primary infection, with no memory response.\textsuperscript{186}

Recent evidence has identified wide cross reaction of the influenza HA immune responses by constructing a headless HA molecule that retains the correct conformation of the stalk structure.\textsuperscript{184} The HA protein has two subunits: the globular head and immunodominant HA1 and the more conserved and cross-reactive HA2.\textsuperscript{91} By removing a portion of the HA1 globular head, the most variable and strain specific subunit, the remaining portion of HA1 and conserved HA2 is retained and exposed to the immune response allowing cross-reactive antibodies to form.\textsuperscript{184} Immunizations with the headless HA allowed complete protection and
partial prevention of weight loss during a lethal homologous challenge and cross-reactive antibodies to different HA subtypes. This advancement could be exploited for a prospective pandemic platform for cross-reactive influenza vaccines.\textsuperscript{184}

2.4.8. Vectors

An increasing focus on utilizing vectors (i.e., bacteria or viruses) to deliver antigen to the appropriate cellular compartments has been identified with emphasis on production in poultry medicine.\textsuperscript{185,186} A commercially available fowlpox vectored avian influenza vaccine administered parenterally has demonstrated extensive efficacy in poultry against HPAI although administration requires handling of each individual bird.\textsuperscript{185,186} Subsequent studies focused on improving efficacy by overcoming immune suppressive effects of the fowlpox vector by including genes encoding for the cytokine IL-18.\textsuperscript{187} Modified Vaccinia virus Ankara (MVA) encoding H5 HA showed protective efficacy in mice.\textsuperscript{188} Vaccinia virus, smallpox and fowlpox viral vector vaccines encoding A/chicken/Indonesia/7/03 H5 hemagglutinin were examined in a swine model of vaccination and elicited protection from challenge with low pathogenic H5N1 virus.\textsuperscript{189} Replication defective human adenovirus vectors effectively delivered DNA encoding influenza antigens to host cells while being well tolerated in human trials.\textsuperscript{190} The adenovirus technology ensures a targeted approach towards delivery of the H5 HA DNA over the intramuscular injection of DNA discussed earlier and demonstrated protection in mouse and poultry models of HPAI challenge.\textsuperscript{191-193} A poultry specific attenuated live vaccine strain of Newcastle Disease Virus (NDV) was genetically modified to express HA of HPAI and showed protection from lethal challenge of both HPAI and NDV in poultry.\textsuperscript{194} Wang et al. demonstrated that a common poultry \textit{Lactobacillus} that colonizes the gut of poultry delivered H5 antigen to mucosal tissues and elicited specific IgA and cell mediated immune responses.\textsuperscript{195}
2.4.9. Adjuvants

HPAI vaccines present the same problems that hinder seasonal influenza vaccines in that the immunogenicity of the antigens is mediocre.\textsuperscript{196} Adjuvanting vaccines with immunostimulatory compounds has long been a proven technique for increasing the immune response towards the antigens of interest.\textsuperscript{196} However, adjuvants present safety concerns as vaccinologists and immunologists attempt to balance between sufficiently engaging the immune system to mount a response without crossing the line into a deleterious immune event. Adverse events associated with adjuvants have led to very few vaccines containing adjuvants being approved for use in humans.\textsuperscript{197,198} Seasonal as well as pandemic influenza vaccine research has led to significant advances in adjuvant development, leading to novel adjuvants like MF59, an oil-in-water emulsion adjuvant, being approved for human use in the European Union.\textsuperscript{199}

As mentioned in the earlier sections, many possible pandemic formulations for HPAI contain adjuvants. Here we highlight the advances in adjuvant research in the context of HPAI vaccines. Alum remains the principal adjuvant studied because of its approval for human use while maintaining favorable safety profiles.\textsuperscript{200-202} MF59 and AS03 (another oil-in-water emulsion) appear to increase efficacy as compared to Alum, although vaccines using these adjuvants are limited in their approval for use world-wide.\textsuperscript{203-207} Evidence has pointed to the spreading of the epitopes that the immune response recognizes in the context of individuals vaccinated with MF59 adjuvanted H5N1 vaccines versus un-adjuvanted vaccines providing insight into the mechanisms of efficacy.\textsuperscript{208}

The pandemic possibility of HPAI has pushed other avenues of adjuvant research. Inactivated whole virus adjuvanted with CoVaccine HT\textsuperscript{TM}, a mixture of squalene, polysorbate 80, sucrose fatty acid sulphate ester in water, demonstrated favorable HA inhibition titers in ferrets and macaques with a single dose regimen.\textsuperscript{209,210} A purified derivative of the saponin from \textit{Quillaja saponaria}, QS-21, has been identified as another potential adjuvant in pandemic HPAI vaccines.\textsuperscript{132} Addition of QS-21 with Alum adjuvanted vaccines greatly increased antibody
responses to the M2 protein of HPAI in rhesus monkeys. Immune stimulating complexes (ISCOMs) are 40 nm structures containing glycosides from *Quillaja saponaria* and lipids for which immunogens can attach via hydrophobic interactions. These compounds have shown promising protection data upon lethal challenge in avian models. Platycodin D, another saponin derived from *Platycodon grandiflorum*, has shown adjuvant potential with recombinantly produced H5.

Recent advances in molecular and structural biology have led to targeted approaches to stimulate innate immune cells through PRRs such as TLRs, RIG-1-like receptors, NOD-like receptors, and CLRs. Stimulating PRRs with ligands in conjunction with delivering antigens provides an adjuvanting mechanism. Stimulation of endosomal TLR3 and TLR9 with Poly ICLC or CpG, respectively, during HPAI infections led to protection and viral clearance. The adjuvant effect of TLR3 ligands was also demonstrated in intranasal HPAI vaccines in primates. Intranasal administration of Poly I:C with H5N1 HA as well as chitin microparticles delivered concurrently with Poly I:C led to protection upon lethal challenge. PIKA, a dsRNA analog that functions through ligation of TLR3, showed adjuvant effects to subunit H5N1 vaccines administered intranasally in mice as well as protection from heterologous challenge. Incorporation of monophosphoryl lipid A (MPLA), a non-toxic derivative of LPS that signals through TLR4, combined with Alum showed higher HAI responses and balanced IgG1 and IgG2a isotypes with a 3.8 μg HA dose of a split virion vaccine.

Cholera toxin (CT) has demonstrated mucosal adjuvant capabilities that appear to be STAT3 signaling dependent. Recombinant CT demonstrated more favorable safety profiles due to engineering the absence of the catalytic subunit. Recombinant CT adjuvanted inactivated H5N1 viral vaccines showed similar antibody titers to other adjuvanted inactivated H5N1 vaccines but with diminished protection. Another bacterial derivative adjuvant, a patch containing heat labile enterotoxin from *E. coli* (LT) administered over the injection site, proved to
be safe and provided enhanced mean HA inhibition titer in a randomized clinical trial in a prime
boost immunization regimen.\textsuperscript{226}

Combinations of adjuvants, as alluded to earlier with MPLA and Alum, are an exciting
avenue of HPAI vaccine research. A synthetic bioresorbable diblock tri-component copolymer
poly(ethylene glycol)-block-poly(lactide-co-ε-caprolactone) (PEG-b-PLACL) was used to create
a novel emulsion delivery system (PELC) that when combined with CpG, a TLR9 agonist,
induced higher antibody responses in single dose applications using inactivated H5N1 virus and
elicited cross reactive antibodies to heterologous virus.\textsuperscript{227,228} The increased immune response
with a particulate adjuvant as well as other studies using micelle-based carriers administered
orally\textsuperscript{229} indicate the importance of size and shape in designing adjuvants.\textsuperscript{230}

2.5. CONCLUSIONS

H5N1 influenza is a growing concern for public health officials all over the world. This
rapidly mutating virus has the potential to become the next influenza pandemic devastating the
global population with a 60% mortality rate. With this in mind, there is an urgent need for
research on the prevention of H5N1 through vaccination. Many vaccination strategies have
been studied, including DNA plasmids and VLPs, but perhaps the safest vaccine is a
formulation of highly purified protein or subunits. The purified proteins, which are non-infectious,
will minimize side effects from vaccination. However, these proteins are generally poorly
immunogenic and require the use of an adjuvant. Among the various classes of adjuvants that
have been studied as described in this chapter, polyanhydride nanoparticles have many
advantages: sustained protein release, preservation of protein antigenicity, activation of immune
cells, and immunomodulation. These nanoparticles also have pathogen mimicking properties
that take advantage of unique pulmonary immunity leading to the design of safe, efficacious
vaccines.
## Table 2.1 Biodegradable Polymers as Vaccine Adjuvants and Delivery Vehicles

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Table 2.2: Considerations for Pandemic Vaccines

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Figure 2.1. Bulk and surface erosion of biodegradable polymers. Poly(esters) exhibit bulk erosion mechanisms allowing water penetration into the bulk of the material (top). Surface eroding polymers, such as acid catalyzed polymers or polyanhydrides, only allow water penetration at the surface and erode layer by layer (bottom).
Figure 2.2. Features of the pulmonary immune system. The pulmonary immune system has several unique aspects that provide mucosal protection from foreign particles. The lung is lined with a mucosal escalator that traps particulates and moves them upward to be swallowed (upper right). Macro-fold cells sample antigens from the lumen and pass them to underlying APCs such as alveolar macrophages and dendritic cells (lower right). Finally, neutrophils line the pulmonary capillaries to catch antigen missed by other APCs (lower left).
Figure 2.3. The H5N1 virion capsid encloses an eight segmented, negative sense RNA genome. The virion also contains a lipid envelope with several major glycoproteins: hemagglutinin (HA), neuraminidase (NA), and ion channels.
Figure 2.4. A simplified overview of the immune responses generated from the different antigens used in the vaccines discussed in this chapter. Arrows are indicative of the route of administration being used for these vaccines.
2.8. REFERENCES


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CHAPTER 3: Research Objectives and Thesis Organization

The overall objective of this research is to design a synthetic nanoparticle-based intranasal vaccine against respiratory pathogens, specifically H5N1 influenza. In order to accomplish this objective, the deposition and internalization of intranasal nanoparticle vaccines were studied at early time points with an eye towards examining the role of the initial fate of these particles on the induction of long-lasting memory responses and protective immunity. These studies were followed by an examination of the long-term persistence of nanoparticle-based vaccines. Furthermore, the long-lasting memory responses of particle-based vaccines were examined by utilizing an adoptive transfer model of antigen-specific CD8$^+$ T cells and a model antigen (ovalbumin). Next, the stability and release kinetics of a nanoparticle-encapsulated H5 hemagglutinin trimer ($H5_3$) were investigated to rationally determine the optimal polyanhydride chemistries suitable for protein stabilization. The insights gained from all these studies were utilized to perform in vivo experiments in a rodent model to define the immune responses to $H5_3$-containing polyanhydride nanovaccine formulations and protection was evaluated against live viral challenge.

The specific goals (SGs) of this research are to:

- **SG1:** Characterize the deposition, internalization, and persistence characteristics of intranasally delivered polyanhydride nanoparticles;

- **SG2:** Assess the cell-mediated long-term memory responses induced by polyanhydride nanoparticle vaccine formulations using an adoptive transfer model of antigen-specific CD8$^+$ T cells;

- **SG3:** Evaluate the structural and functional stability and release kinetics of $H5_3$ hemagglutinin antigen encapsulated within polyanhydride nanoparticles; and
SG4: Investigate the efficacy of H5₃-containing polyanhydride nanoparticle formulations in a murine model.

The following four chapters in the thesis address these specific goals. The first specific goal is addressed in Chapters 4 and 5, which discuss the initial (i.e., within 48 h) deposition and cellular internalization of polyanhydride nanoparticles within the lungs as well as effects of long-term antigen persistence. Chapter 6 focuses on SG2, investigating the expansion, contraction, and phenotypes of CD8⁺ T cells after administration of polyanhydride nanoparticles. Chapter 7 is focused on rationally determining the optimal polyanhydride chemistries for encapsulating H5₃ through examination of protein structure and function upon release from nanoparticles. Finally, Chapter 8 addresses SG4 by investigating the humoral and cell-mediated responses to H5₃-loaded nanoparticles in vivo and the ability of those responses to protect against a live viral challenge.
CHAPTER 4:
Lung Deposition and Cellular Uptake Behavior of Pathogen-mimicking Nanovaccines in the First 48 Hours

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*These authors contributed equally to the design and performance of these studies.

Abstract

Pulmonary immunization poses the unique challenge of balancing vaccine efficacy with minimizing inflammation in the respiratory tract. While previous studies have shown that mice immunized intranasally with F1-V-loaded polyanhydride nanoparticles were protected from a lethal challenge with Yersinia pestis, little is known about the initial interaction between the nanoparticles and immune cells following intranasal administration. Herein, the deposition within the lung and internalization by phagocytic cells of polyanhydride nanovaccines encapsulating F1-V are compared to that of soluble F1-V alone or F1-V adjuvanted with monophosphoryl lipid A (MPLA). Encapsulation of F1-V into polyanhydride nanoparticles prolonged its presence while F1-V administered with MPLA was undetectable within 48 hours. The inflammation induced by the polyanhydride nanovaccine was mild compared to the marked inflammation induced by the MPLA-adjuvanted F1-V. Even though F1-V delivered with saline was detected in the lung 48 hours after administration, it is known that this regimen does not elicit a protective immune response. The prolonged F1-V presence in the lung in concert with the mild inflammatory response provided by the nanovaccine provides new insights into the development of protective

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immune responses with a single intranasal dose.

4.1. INTRODUCTION

Many pathogens enter the body through mucosal surfaces, especially those of the gastrointestinal and respiratory tracts. When designing vaccines against respiratory pathogens, mucosal immunization (e.g., oral, intranasal, pulmonary, intrarectal, conjunctival) will likely provide both systemic and mucosal immunity. Delivery to the lungs also increases the bioavailability of the antigen by avoiding first pass metabolism and by enabling high adsorption because of the large, permeable surface area. The lungs encounter many airborne particles and must differentiate between innocuous particles and pathogens, only initiating a response when true danger is present. Therefore, an ideal delivery system for a subunit vaccine must enhance antibody and/or T cell responses to often weakly immunogenic proteins without disrupting homeostasis within the respiratory tract.

Polyanhydride nanoparticles comprised of 1,6-bis(p-carboxyphenoxy)hexane (CPH), 1,8-bis(p-carboxyphenoxy)-3,6-dioxaoctane (CPTEG), and copolymers thereof are well suited for pulmonary administration because of their favorable safety profile, mild inflammatory response, and their ability to enhance the immune response. However, compared to an approved adjuvant such as alum, polyanhydride nanoparticles are markedly less phlogistic. Induction of a mild inflammatory response may be especially important when designing vaccine formulations to deliver antigens to the lungs, which are sensitive to inflammation. Additionally, polyanhydride nanovaccines provide pathogen-mimicking capabilities that enhance the activation of antigen presenting cells (APCs). These nanovaccines induce robust immune responses and provide protective immunity without the damaging effects of disease. A recent study utilizing F1-V, a recombinant antigen derived from Yersinia pestis, showed that a single dose of intranasally administered polyanhydride nanovaccine induced full protection against a subsequent lethal challenge.
While it has been demonstrated that intranasally administered polyanhydride nanovaccines provide long-lasting protective immunity, the role of deposition within the lung and internalization by phagocytic cells of nanoparticles on the induction of immune responses after delivery is largely unknown. The initial interactions between antigen and the immune system are often critical to the establishment of long-lasting protective immunity. For example, studies with nano-sized viral particles such as influenza virions have demonstrated pathogen deposition and infection in the lower respiratory tract. Likewise, vaccines that deposit within the same area of the respiratory tract would be ideal candidates for prevention of influenza. Such vaccines have demonstrated increased residence time and prolonged contact with APCs resulting in enhanced cell-mediated responses. It is known that dendritic cells (DCs) that reside within lymph nodes and injection-site DCs play important roles during the first 50 hours post-infection. This study by Jenkins and co-workers demonstrated that while many resident DCs acquired antigen in the draining lymph nodes and presented peptide-MHC II complexes within 30 minutes of injection of a soluble antigen, the interactions between these DCs and naïve CD4$^+$ T cells were relatively short-lived. Injection-site DCs that migrated to the lymph node, however, interacted with CD4$^+$ T cells for extended periods of time, inducing T cell proliferation despite arriving at the lymph nodes 18 hours post-injection. It was also observed that sustained exposure of antigen enriched these interactions and enhanced T cell activation. Based on these results, a reasonable hypothesis is that vaccine delivery vehicles that prolong the presence of antigen may enhance the immune response.

This work focuses on the initial (i.e., the first 48 hours) deposition within the lung and internalization by phagocytic cells of intranasally administered polyanhydride nanovaccines. Using fluorescently labeled F1-V antigen, the kinetics of soluble and encapsulated F1-V distribution within the lungs and its uptake by APCs were investigated. This work demonstrates key differences in antigen presence and inflammation when administered as part of a
polyanhydride nanovaccine compared to that associated with monophosphoryl lipid A (MPLA), providing new insights into the protective capabilities of pathogen-mimicking nanovaccines.

4.2. METHODS AND MATERIALS

4.2.1. Materials

Compounds for polymer synthesis included 1,6-dibromohexane, 1-methyl-2-pyrrolidinone, hydroxybenzoic acid, N,N-dimethylacetamide, sebacic acid, and tri-ethylene glycol (Sigma Aldrich, St. Louis, MO). Acetic acid, acetic anhydride, acetone, acetonitrile, chloroform, dimethyl formamide, ethyl ether, hexane, methylene chloride, pentane, petroleum ether, potassium carbonate, sodium hydroxide, sulfuric acid, and toluene were purchased from Fisher Scientific (Fairlawn, NJ). 4-p-fluorobenzonitrile was obtained from Apollo Scientific (Cheshire, UK). F1-V fusion protein was obtained from BEI Resources (Manassas, VA) and fluorescently labeled with Vivo Tag 680 according to manufacturer instructions (Vivo Tag 680, Perkin Elmer, Waltham, MA). The inflammatory probe ProSense® 750 FAST was also purchased from Perkin Elmer. Flow cytometric analysis utilized anti-mouse antibodies and their respective isotypes for PerCP-Cy5.5 labeled anti-CD11c, Biotinylated anti-CD324, Streptavidin-conjugated eFluor 710 (eBioscience, San Diego, CA), PE-CF594 labeled anti-CD11b (BD Bioscience, San Jose, CA), and PE-Cy7 labeled anti-F4/80 (BioLegend, San Diego, CA).

4.2.2. Polymer synthesis and characterization

The anhydride monomers, CPH and CPTEG, and the 50:50 CPTEG:CPH copolymer were synthesized as previously described.\textsuperscript{18,19} Copolymer composition, purity, and molecular weight ($M_w\sim5100$ Da, PDI = 1.5) were determined by $^1$H nuclear magnetic resonance spectroscopy (VXR 300 MHz, Varian, Palo Alto, CA) and found to be consistent with previously published results.\textsuperscript{19}
4.2.3. **Nanoparticle synthesis**

F1-V was conjugated to Vivo Tag 680 per the manufacturer's instructions (Perkin Elmer). 50:50 CPTEG:CPH nanoparticles loaded with 2% (w/w) Vivo Tag 680-conjugated F1-V were fabricated by nanoprecipitation as described previously.\(^7\) Briefly, 20 mg/mL polymer with 0.2 mg lyophilized F1-V was dissolved in methylene chloride and sonicated to ensure uniform protein distribution. The solution was then poured into chilled pentane (at a solvent:non-solvent ratio of 1:250) to precipitate the nanoparticles. Particles were collected using vacuum filtration and their morphology and size (mean diameter of 180 ± 57 nm) were characterized with scanning electron microscopy (FEI Quanta 250, FEI, Hillsboro, OR).

4.2.4. **Intranasal immunization**

Female C57BL/6 mice were purchased from Harlan (Haslett, MI). Mice were housed under specific pathogen-free conditions where all bedding, caging, water, and feed were sterilized prior to use. All studies were conducted with the approval of the Iowa State University Institutional Animal Care and Use Committee. There were five separate treatment groups of mice that were intranasally administered following administration of 100 µL of 20 mg/mL ketamine with 1 mg/mL xylazine anesthesia with either i) 10 µg of F1-V encapsulated within 500 µg of 50:50 CPTEG:CPH nanoparticles, ii) 50 µg of soluble F1-V and 10 µg encapsulated within 500 µg of 50:50 CPTEG:CPH nanoparticles, iii) 50 µg of soluble F1-V adjuvanted with 10 µg MPLA, iv) 50 µg of soluble F1-V alone, or v) saline (Figure 4.1). These groups were chosen based upon previous studies by Ulery et al.\(^7,10\) All formulations were administered in 50 µL sterile phosphate buffered saline (PBS). Six mice per group were used and two independent experiments were performed.
4.2.5. *Ex vivo* imaging of antigen distribution in lung tissue

Animals were euthanized 2 and 48 hours post-immunization. After perfusing the lungs with 5 mL sterile PBS, the lungs were excised and imaged to detect the distribution of the Vivo Tag 680-conjugated antigen within the lung using an *in vivo* imaging system (Carestream Multispectral FX, Rochester, NY). Images were captured using a white light image with a 2 second exposure followed by a fluorescent image with a 10 second exposure with an excitation wavelength of 670 nm and a 750 nm emission filter. All images were analyzed with ImageJ software (Version 1.46r, NIH, Bethesda, MD). To calculate MFI, regions of interest were selected around each fluorescent lung image to obtain a MFI value for each sample. To obtain *ex vivo* fluorescent lung images (Figure 4.1), the false-color look-up table “fire” was applied to the fluorescent image and an overlay was created with the white light and fluorescent images.

4.2.6. Inflammatory response of adjuvants

The pulmonary inflammation was measured using ProSense® 750 FAST (Perkin Elmer, Waltham, MA), a fluorescent probe activated by cathepsins. ProSense® 750 FAST was prepared according to manufacturer instructions and administered via tail vein injection six hours prior to imaging. Mice (*n* = 3) received intranasal administrations with 500 µg of blank 50:50 CPTEG:CPH nanoparticles or 10 µg MPLA in 50 µL, and euthanized at 6, 24, and 48 hours post-administration. Perfused lung tissue was excised from each mouse and imaged for the presence of activated ProSense® 750 FAST. Images were captured as described above using a white light image with a 2 second exposure followed by a fluorescent image with a 10 second exposure with an excitation wavelength of 750 nm and an 830 nm emission filter. Regions of interest were selected around each fluorescent lung image and the background was subtracted using a rolling ball radius of 40 to obtain a MFI value for each sample. To obtain *ex vivo* fluorescent lung images (Figure 4.1), the false-color look-up table “fire” was applied to the fluorescent image and an overlay was created using the white light and fluorescent images.
4.2.7. High-throughput multi-spectral imaging flow cytometry

Excised lungs were incubated in Hank’s balanced salt solution with 1 mg/mL collagenase D and 60 U/mL DNase II for 20 minutes at 37°C. Single cell suspensions were prepared using a gentleMACS™ dissociator (Miltenyi Biotec, Cambridge, MA). Debris was removed by passing the tissue homogenate through a 40 μm cell filter and red blood cells were lysed with ACK lysis buffer (150 mM ammonium chloride, 10 mM potassium bicarbonate, 0.1 mM EDTA). The cells (2.5 x 10^6 cells/mL) were washed with 2% heat inactivated fetal bovine serum and 0.1% sodium azide in PBS and re-suspended in 50 μL 1% paraformaldehyde in PBS. Cell samples were analyzed using multi-spectral imaging flow cytometry (ImageStreamX, Amnis, Seattle, WA) by exciting the samples with a 658 nm laser and collecting emission spectra using a 600-745 nm filter. Internalization of F1-V antigen was determined following the manufacturer’s instructions using IDEAS® software (Amnis).

4.2.8. Flow cytometry

Single cell suspensions were prepared as described above. Cell suspensions (2.5 x 10^6 cells/mL) were subsequently blocked for non-specific antibody binding using 0.1 mg/mL rat IgG and 10μg/mL mouse anti-CD16/32. Cells were surface-stained for CD11c, CD11b, CD324, and F4/80. All samples were fixed with stabilizing fixative (BD Biosciences) and analyzed using a FACS Canto flow cytometer (BD Biosciences, San Jose, CA) and data analysis was performed with FlowJo software (Treestar, Inc., Ashland, OR).

4.2.9. Statistics

The statistical tests were performed in MiniTab software (Minitab Inc, State College, PA). Significant differences between two indicated groups were evaluated by Student’s t-test. The level of significance was set at p < 0.05, unless otherwise stated.
4.3. RESULTS AND DISCUSSION

4.3.1. Encapsulation prolongs antigen presence

Mice received an intranasal administration of the vaccine formulations shown in Figure 4.1. After 2 and 48 hours post-administration, separate groups of animals were euthanized and their lungs were excised to visualize and quantify the distribution of fluorescently labeled F1-V antigen. The lungs of all mice showed a uniform spatial distribution of F1-V at 2 and 48 hours post-administration, regardless of the formulation (Figure 4.1). Ex vivo analysis of lung tissues showed that the mice administered the nanovaccine formulation showed prolonged presence of antigen. Figure 4.1 shows the percentage loss in fluorescence in the lung between 2 and 48 hours for each formulation administered. Statistically significant decreases in fluorescence intensity, i.e., statistically significant differences in mean fluorescence intensity (MFI) values at 2 and 48 hours post-immunization, were observed in the lungs of mice that received soluble F1-V or F1-V adjuvanted with MPLA (Figure 4.1) while that of mice that received only nanoparticle-encapsulated F1-V (i.e., no soluble antigen) displayed stable fluorescence intensity (i.e., no statistical significance between MFI values at 2 and 48 hours). The lungs of the mice that received the soluble F1-V + nanoparticle-encapsulated F1-V also showed a marked decrease in fluorescence between 2 and 48 hours. In this formulation, 80% of the antigen was delivered solubly and thus cleared quickly, accounting for the decreased fluorescence. However, the nanoparticles prolonged the presence of F1-V in the lungs, as noted by the stable fluorescence intensity between 2 and 48 hours in the lungs of the mice that received only the F1-V encapsulated nanoparticles (i.e., without any soluble antigen). In order to make comparisons with the formulations tested in previous work, the “encapsulated only” formulation contained 10 µg of antigen, while the soluble protein formulation contained 50 µg, and the “soluble + encapsulated” group received 60 µg of F1-V. Therefore, it is important to note that only differences between 2 and 48 hours within each group are being compared, because the actual MFI may vary between groups.
4.3.2. Reduced pulmonary inflammation after deposition of polyanhydride nanovaccine

Because inflammation may affect the disappearance of antigen from the site of deposition, the inflammatory response induced by MPLA and the nanovaccine was evaluated. It is known that MPLA induces a marked inflammatory response and recruits inflammatory cells to the site of antigen administration, which aids in the clearance of antigen. While subcutaneously administered polyanhydride nanoparticles have previously been shown to induce less inflammation in comparison to alum, the inflammation induced by intranasally delivered polyanhydride nanovaccines is unknown. Mice that were previously administered polyanhydride nanoparticles or MPLA intranasally were injected with ProSense®, a fluorescent imaging agent that is activated by cathepsins produced by inflammatory cells such as monocytes and macrophages. While both polyanhydride nanovaccines and MPLA displayed similar magnitudes of inflammation at 6 hours post-administration (Figure 4.2), by 48 hours the level of cathepsin-activated fluorescent probe detected in the lungs of mice treated with MPLA was double that of the mice that received nanoparticles. We hypothesize that the increased inflammation induced by MPLA led to a greater recruitment of macrophages that enabled the more rapid clearance of F1-V depicted in Figure 4.1.

Thus far, our data indicate that polyanhydride nanovaccines prolonged the presence of F1-V in the lungs (Figure 4.1) while inducing mild inflammation (Figure 4.2). The prolonged F1-V presence in the lung in concert with the mild inflammatory response provided by the nanovaccine suggests a novel paradigm related to the development of the long-lasting protective immune response following the administration of a single intranasal dose as previously observed. It is instructive to note that F1-V delivered with MPLA induced a relatively higher level of inflammation, and was cleared from the lung by 48 hours. F1-V delivered alone does remain in the lung for up to 48 hours, but failed to elicit a measurable immune response in previous studies. An important benefit of the F1-V nanovaccine formulation is to enhance the stimulation of the immune system through the induction of an inflammatory response (albeit mild
in comparison to MPLA), while simultaneously prolonging the presence of antigen within the lung, potentially leading to increased interactions with T cells and enhanced immune activation.\textsuperscript{13}

4.3.3. Cellular internalization of antigen is sustained by polyanhydride nanovaccine

It is known that continual exposure of antigen to APCs at the site of administration increases APC-T cell interactions and T cell proliferation in the draining lymph nodes.\textsuperscript{13} These enhanced cellular interactions suggest that vaccine delivery regimens which provide a sustained presence of antigen in the lungs would better enable the induction of long-lasting immunity. In this work, high-throughput multi-spectral imaging flow cytometry was used to detect APCs that had internalized F1-V. At 48 hours post-administration, cells recovered from the lungs of mice that received soluble and/or nanovaccine formulations were positive for internalized F1-V. In addition, there was a marked decrease (from 2 to 48 hours) in the percentage of cells containing F1-V from the mice that had been administered F1-V adjuvanted with MPLA (Figure 4.3). This observation is consistent with the results in Figure 4.1. While soluble F1-V is rapidly internalized by APCs, the nanovaccine formulations continue to release antigen that can be continually internalized by both resident as well as recruited APCs. In contrast, the mice treated with MPLA-adjuvanted F1-V demonstrated a significant decrease in both tissue fluorescence and cellular internalization within 48 hours after immunization.

It is important to recognize that the cellular population at the administration site is dynamic and involves both resident and recruited APCs. While the inflammatory responses induced by MPLA (Figure 4.2) recruits APCs that can internalize foreign antigen, the antigen may be rapidly exported to the lymph node and/or degraded, resulting in little antigen remaining in the lungs to interact with newly recruited cells. In contrast, more F1-V positive cells were present at 48 hours in the lungs of mice that received the polyanhydride nanovaccines. This
suggests that APCs recruited to the lung were able to internalize F1-V released from the nanovaccine.

Although soluble antigen is still present within the lung at 48 hours, it was previously demonstrated that soluble F1-V antigen alone did not elicit a protective immune response.\(^7\),\(^10\) Because soluble F1-V alone likely failed to induce an inflammatory response, it is conceivable that fewer APCs were recruited to the lungs that resulted in less clearance of the F1-V by 48 hours. This poses an additional consideration to the above argument. While presence (i.e., dose and time) of antigen is necessary for the induction of an immune response, soluble F1-V delivered alone was not sufficient to initiate an immune response.\(^7\) Therefore, an appropriate adjuvant is necessary to prolong the presence of the antigen within the lung for internalization by and activation of recruited APCs. As demonstrated in this work, polyanhydride nanovaccines are able to bridge this shortfall by prolonging the presence of antigen at the site of administration coupled with a mild inflammatory immunological stimulation that may explain the ability of the nanovaccines to induce long-lasting protective immunity.\(^7\)

### 4.3.4. Cellular uptake prolonged by polyanhydride nanovaccines

To ascertain the specific cell types interacting with antigen, flow cytometry was used to characterize cellular populations of F1-V\(^+\) cells. In these analyses, all F1-V\(^+\) cells were gated first before using the following cell surface marker combinations to characterize the cellular populations: DCs: CD11c\(^+\) CD11b\(^-\); alveolar macrophages (aMØs): CD11c\(^-\) CD11b\(^+\) F4/80\(^+\); and epithelial cells: CD11c\(^-\) CD11b\(^-\) CD324\(^+\) (Figure 4.4A). While the percentage of different cellular populations associated with antigen was independent of formulation (Figure 4.4B), the MFI or amount of F1-V within the cell populations varied among the different treatment groups (Figure 4.4C). Consistent with the data presented in Figures 4.1 and 4.3, the amount of antigen in the F1-V\(^+\) DCs and aMØs was reduced significantly to background by 48 hours for cells recovered from mice administered MPLA-adjuvanted F1-V (Figure 4.4C). This is likely associated with the
more rapid clearance of antigen (Figure 4.1) induced by inflammation (Figure 4.2). In contrast, mice administered nanovaccines or soluble antigen alone displayed stable or increased MFI of antigen associated with DCs or aMØs and was comparable to cells recovered from mice that received only the soluble antigen (Figure 4.4C). It is likely that the previously identified protective nanovaccine formulation, consisting of both soluble and nanoparticle-encapsulated antigen, demonstrated superior performance because of the initial priming of the immune response by soluble protein and the maturation of the serum antibody response (e.g., titer and avidity) facilitated by the prolonged presence of F1-V provided by the nanovaccines.

4.4. CONCLUSIONS

These studies provide confirmatory evidence relating to the ability of the pathogen mimicking 50:50 CPTEG:CPH nanovaccine to induce prolonged APC uptake of antigen in concert with a mild inflammatory response. Furthermore, the nanovaccine was able to overcome limitations associated with the MPLA and soluble dose formulations in its ability to bridge the gap between prolonged antigen presence and activation of APCs. These characteristics of polyanhydride nanovaccines suggest a novel paradigm related to the development of the long-lasting protective immune response following the administration of a single intranasal dose as previously observed. Together with their low phlogistic potential and their capacity to prolong antigen presence at the site of administration, polyanhydride nanovaccines are excellent candidates for pulmonary vaccination.

Acknowledgements

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Figure 4.1. *Ex vivo* detection of F1-V in the lungs of mice following intranasal administration. Images of excised lungs show presence of fluorescently labeled F1-V at 2 and 48 hours after administration. The table on the right depicts the different treatment groups with the respective dose of F1-V administered per mouse. The table also includes the percent decrease in mean fluorescence intensity (MFI) of the labeled-F1-V from the 2 to 48 hour time point for each group ± standard error of mean. * indicates statistical significance between the MFI values at 2 and 48 hours for that treatment at p < 0.05. Six mice per group were used from two independent experiments.
Figure 4.2. Comparison of the pulmonary inflammation induced following intranasal administration of 50:50 CPTEG:CPH nanoparticles compared to that induced by MPLA. ProSense® 750 FAST, a cathepsin activated fluorescent probe, was used to detect inflammation. Mean fluorescence intensity, represented on the y-axis, corresponds to the level of inflammation present at the indicated time points after administration. Error bars represent standard error of the mean (SEM) and n=3. * indicates statistical significance between 50:50 CPTEG:CPH and MPLA at the indicated time point (p < 0.05). Background levels of inflammation observed at 6 hours in the mice administered saline, n=2, had an average MFI of 63.8.
Figure 4.3. Assessment of internalized F1-V by lung cells using multispectral imaging flow cytometry. All error bars represent SEM with n=6. One thousand events per sample were acquired and analyzed as described in Materials and Methods. * indicates statistical significance between 2 and 48 hours for that treatment at p < 0.05. "Encap“ is an abbreviation for “Encapsulated“.
Figure 4.4. Use of flow cytometry to assess the pulmonary cellular populations of F1-V⁺ cells. (A) Representative contour plots demonstrate the gating used to determine dendritic (CD11c⁺ CD11b⁻), macrophage (CD11c⁻ CD11b⁺ F4/80⁺), and epithelial (CD11c⁻ CD11b⁻ CD324⁺) cell types. (B) Percent of each cell type within the F1-V⁺ population and (C) MFI of F1-V associated with dendritic cells (top), macrophages (middle), and epithelial cells (bottom). All error bars represent SEM and n=6. Ten thousand events per sample were collected. * indicates statistical significance between 2 and 48 hours for that treatment at p < 0.0001.
4.6. REFERENCES


CHAPTER 5:
Polyanhydride Nanoparticle Chemistry Affects In Vivo Antigen Persistence, Antigen Release Kinetics, and Immune Response

To be submitted to Biomaterials in December 2013

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Abstract

Acute respiratory infections represent a significant portion of global morbidity and mortality annually. There is a need for highly effective vaccines against respiratory pathogens. When designing a vaccine against respiratory diseases, pulmonary delivery is an attractive route as it mimics the route of natural infection and may confer both systemic and mucosal immunity. We have previously demonstrated that single dose, intranasal vaccines based on polyanhydride nanoparticles elicit a protective immune response up to 40 weeks after immunization. Herein we investigate the effect of nanoparticle chemistry on in vivo antigen persistence, antigen release kinetics, and the humoral immune response. We demonstrate that a tunable antigen persistence profile in vivo can be achieved based on nanoparticle chemistry, sustained antigen internalization by antigen presenting cells, and the development of avid antibodies towards protective epitopes over time. These studies shed new light on the role of prolonged antigen presence in vivo in inducing protective immunity and have important implications for the rational design of efficacious single dose vaccines against respiratory pathogens.

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5.1. INTRODUCTION

Acute respiratory infections represent a significant portion of global morbidity and mortality annually.\(^1\),\(^2\) With the increasing prevalence of multi-drug resistant pathogens and the threat of aerosolized bioterrorism agents, there is an urgent need for highly effective vaccines against respiratory pathogens.\(^3\),\(^4\) When designing vaccines against respiratory diseases, intranasal and pulmonary delivery are attractive routes because intranasal or inhaled vaccines can confer both systemic and mucosal immunity, deliver antigen to highly immunoreactive sites within the lung, and lead to increased protection.\(^5\),\(^6\) Recombinant protein technology has led to the development of protective antigens against many respiratory pathogens, often based on surface proteins of viruses and bacteria. It is often noted, however, that antigen delivered alone intranasally does not induce a protective immune response, and therefore, requires the use of adjuvants.\(^7\),\(^8\)

Polyanhydride nanoparticle-based vaccines (i.e., nanovaccines) have previously been demonstrated to be a safe and efficacious delivery platform for protein antigens against multiple pathogens.\(^3\),\(^9\),\(^10\) These biodegradable polymers demonstrate pathogen-mimicking properties that adjuvant poorly immunogenic subunit proteins to enhance the immune response.\(^11\) Additionally, particle chemistry may be tailored to stabilize fragile proteins as well as the protein release profile.\(^10\) A polyanhydride nanovaccine based on F1-V, a recombinant fusion protein that has been shown to be a protective antigen against pneumonic plague,\(^12\) has been demonstrated to provide protective immunity up to 40 weeks after vaccination.\(^13\)

Previously, we have described the deposition, distribution, and prolonged presence of antigen delivered with polyanhydride nanovaccines at early time points after administration (i.e., 48 hours), which enhances internalization by antigen presenting cells (APCs).\(^14\) While the initial interactions between antigen and APCs are important in laying a foundation for vaccine efficacy, the role of the nanovaccine formulation (i.e., the chemistry of the nanoparticle carrier) on the persistence of antigen \textit{in vivo} is critical for the continual recruitment of antigen-internalizing
APCs and the development of high antibody titers with increased avidity. In this work, we follow the persistence of antigen encapsulated into polyanhydride nanoparticles over nine weeks and provide an in-depth analysis of the effect of nanoparticle chemistry on pulmonary persistence of antigen, sustained internalization of antigen by immune cells, and the humoral immune response.

5.2. MATERIALS AND METHODS

5.2.1. Materials

The materials used for monomer synthesis include sodium hydroxide, hydrobenzoic acid, dibromohexane, 1-methyl-2-pyrrolidinone, triethylene glycol, and sebacic acid (Sigma Aldrich, St. Louis, MO). Acetone, sulfuric acid, potassium carbonate, dimethyl formamide, toluene, acetonitrile, N,N-dimethylacetamide, and acetic acid were purchased from Fisher Scientific (Fairlawn, NJ). 4-p-fluorobenzonitrile used in the synthesis of CPTEG monomer was purchased from Apollo Scientific (Cheshire, UK). Acetic anhydride, ethyl ether, petroleum ether, chloroform, methylene chloride, and hexane used in acetylation and polymerization were purchased from Fisher Scientific. Deuterated chloroform and dimethyl sulfoxide were used in 1H NMR analysis of the polymers and monomers (Cambridge Isotope Laboratories, Andover, MA). Pentane and methylene chloride used in nanoparticle fabrication were purchased from Fisher Scientific.

F1-V fusion protein (BEI Resources, Manassas, VA) was conjugated to the fluorescent label Vivo Tag 680 (Perkin Elmer, Waltham, MA). Flow cytometry utilized anti-mouse antibodies and their respective isotypes for PerCP-Cy5.5 CD11c, Biotin CD324, Streptavidin eFluor 710 (eBioscience, San Diego, CA), PE-CF594 CD11b (BD Bioscience, San Jose, CA), and PE-Cy7 F4/80 (BioLegend, San Diego, CA).
5.2.2. Polymer Synthesis

The CPH and CPTEG monomers were fabricated as described previously.\textsuperscript{15-17} Pre-polymers of CPH and SA were synthesized from monomers as described previously.\textsuperscript{16,17} Polymers and co-polymers of CPH, CPTEG and SA were synthesized using melt condensation as described by Kipper et al. and Torres et al.\textsuperscript{15,16} Polymer purity and molecular weight were determined using $^1$H NMR (Varian VXR300).

5.2.3. Nanoparticle Fabrication

The F1-V fusion protein was conjugated to VivoTag 680 fluorescent label according to manufacturer instructions (Perkin Elmer). Briefly, 10 µL VivoTag 680 was added per mg of F1-V and incubated at room temperature for 1 h. Excess unconjugated VivoTag 680 was then removed using a 5 kDa MWCO dialysis microcentrifuge tube. The protein was lyophilized overnight at -40˚C under vacuum. The F1-V loaded polyanhydride nanoparticles were formulated using an anti-solvent precipitation method as previously described.\textsuperscript{13} Polymer and 2% (w/w) F1-V were dissolved in methylene chloride at a concentration of 20 mg/mL. The solution was sonicated (Vibra-Cell\textsuperscript{TM}, Sonics & Materials, Newton, CT) at an output of 40 Hz to ensure a homogenized mixture. The resulting solution was rapidly poured into pentane at a ratio of 1:250 at room temperature for CPH:SA and poly(SA) formulations or at -40°C for the CPTEG:CPH polymers due to the lower glass transition temperature for these polymers.\textsuperscript{15} Nanoparticles were collected via vacuum filtration and characterized using scanning electron microscopy (FEI Quanta SEM, Hillsboro, OR). Particle size distribution was determined from resultant images using ImageJ (Version 1.46r, NIH, Bethesda, MD) software and found to be consistent with previous work.\textsuperscript{18,19}
5.2.4. Mice

C57BL/6 mice purchased at 5-6 weeks of age were used (Harlan, Haslett, MI). Mice were housed under specific pathogen-free conditions where all bedding, caging, water, and feed were sterilized prior to use. All studies were conducted with the approval of the Iowa State University Institutional Animal Care and Use Committee.

5.2.5. Immunization Protocol

Mice (between 7 and 9 weeks of age) were anesthetized with 100 µL of 20 mg/mL ketamine and 1 mg/mL xylazine and immunized intranasally. Experimental groups consisted of 10 µg of F1-V encapsulated into 500 µg of 50:50 CPTEG:CPH, 20:80 CPTEG:CPH, 20:80 CPH:SA, or poly(SA) nanoparticles with 40 µg soluble F1-V delivered concurrently in 50 µL PBS, 50 µg F1-V delivered with 10 µg MPLA derived from Salmonella enterica serotype Minnesota Re 595 (Sigma Aldrich), or 50 µg F1-V alone. Sterile PBS was used for control animals. Mice were euthanized at 14, 36, and 63 days post-immunization. Four mice were used per group per time point and the experiment was repeated for a total of 8 mice per group. The soluble protein alone and control groups were administered to half the number of animals as the treatment groups.

5.2.6. Ex vivo Lung Imaging

Mice were euthanized to quantify protein remaining in the lung at 14, 36, and 63 days after immunization. A lung perfusion with 5 mL of sterile PBS was performed to reduce background from autofluorescence of red blood cells and the lungs were excised. An in vivo imaging system (Carestream Multispectral FX, Rochester NY) was used to measure the fluorescence of antigen remaining in the lungs. A white light image (2 s exposure) followed by a fluorescent image (60 s exposure) with an excitation of 670 nm and a 750 nm emission filter was used.
Images were analyzed using ImageJ software. Mean fluorescence intensity (MFI) was calculated using the fluorescent lung images. A region of interest was drawn around the fluorescent image and the mean was recorded. Background was then subtracted from each sample and a MFI value was obtained. For the ex vivo lung images (Figure 5.1), background was subtracted from the fluorescent images with a rolling ball radius of 40, the images were smoothed, and the false-color look-up table “fire” was applied. White light images were adjusted to have the same minimum and maximum values and a z-projection of the two images was created.

5.2.7. Multi-spectral Imaging Flow Cytometry

After imaging, lung samples were incubated in Hank’s balanced salt solution (HBSS) with 1 mg/mL collagenase D and 60 U/mL DNase II for 20 min at 37°C. Tissue was then homogenized to a single cell suspension using a gentleMACS™ dissociator (Miltenyi Biotec, Cambridge, MA). The cell suspensions were centrifuged at 250 rcf for 30 seconds to remove large debris. The supernatants were then passed through a 40 µm cell filter and centrifuged at 250 rcf for 10 min at 4°C to collect the cell pellet. Remaining red blood cells in the lung samples were lysed with ACK lysis buffer (150 mM ammonium chloride, 10 mM potassium bicarbonate, 0.1 mM EDTA), and cells were centrifuged once more before enumeration using a Coulter counter (Beckman Coulter, Indianapolis, IN). Cells were washed once in buffer (2% heat inactivated fetal bovine serum and 0.1% sodium azide in PBS) and re-suspended in 60 µL 1% paraformaldehyde (PFA) in PBS. Samples were analyzed using an ImageStreamX (Amnis, Seattle, WA) with a 658 nm laser and 600-745 nm emission filter. Internalization of F1-V was determined using IDEAS® software following the manufacturer’s instructions (Amnis).
5.2.8. Flow Cytometry

Single cell suspensions were prepared as described above. Cell solutions were incubated with 0.1 mg/mL rat IgG and 10 μg/mL mouse anti-CD16/32 to prevent non-specific binding of fluorescent antibodies. Cell suspensions were surface-stained for CD11c, CD11b, CD324, and F4/80 for 30 min. After washing, the stained cell samples were re-suspended in 100 μL stabilizing fixative (BD Biosciences) and analyzed using a FACS Canto flow cytometer (BD Biosciences, San Jose, CA). Analysis of the flow cytometric data was performed with FlowJo software (Treestar, Inc., Ashland, OR).

5.2.9. Anti-F1-V Serum Antibody Titers and Avidity Assays

Antibody titers were determined using an enzyme-linked immunosorbent assay (ELISA) as described elsewhere. Briefly, high-binding microtiter plates were coated with 100 μL of F1-V (0.5 µg/mL) in PBS and incubated overnight at 4˚C. F1-V coated microtiter plates were incubated with blocking buffer (0.05 M PBS with 0.05% Tween 20 (PBS-T) supplemented with 2.5% powdered skim milk) for 2 h at room temperature before washing three times with PBS-T. Serum from immunized mice was added to the first well at a 1:200 dilution in PBS containing 1% goat serum and serially diluted at a three-fold ratio per well. After incubating at 4˚C overnight, plates were washed three times with PBS-T. Alkaline phosphatase-conjugated goat anti-mouse IgG (heavy and light chain) (Jackson ImmunoResearch, West Grove, PA) was added at a concentration of 1 μg/mL and incubated for 2 h at room temperature. Plates were washed again and 1 mg/mL alkaline phosphatase substrate (Fisher Scientific, Pittsburgh, PA) dissolved in 50 mM sodium carbonate, 2 mM magnesium chloride buffer (pH 9.3) was added for colorimetric development. The optical density (OD) was recorded after 30 min at 405 nm. All the samples were tested in technical replicates of two. Herein, we define titer as the serum dilution value that produced an OD greater than twice that of the saline group.
Avidity assays were performed as described above for ELISA. After overnight incubation with 100 µL per well serum at a 1:200 dilution, a 5 M solution of sodium thiocyanate (NaSCN) in a 0.1 M sodium phosphate buffer was added to the first well and serially diluted two-fold five times. Six control wells were used per sample and received sodium phosphate buffer alone. Solution was incubated for 15 min before washing thoroughly. The remainder of the assay follows the steps described above for ELISA. Avidity indices were calculated by determining the concentration of sodium thiocyanate at which the OD is 50% of that of the control wells.

5.2.10. Peptide Array Assay

In order to measure the immune response to specific F1-V epitopes an overlapping peptide array (BEI Resources) assay was performed. High-binding microtiter plates were coated with individual F1-V peptides at a concentration of 5 µg/mL in PBS. The ELISA protocol described above was followed with a single serum dilution of 1:200 used for each sample. The optical density (OD) was recorded at 405 nm after incubating for 2 h at room temperature.

5.2.11. Statistics

Statistical significance using the Graph Pad Prism software (Version 6.01, Graph Pad Software, Inc., La Jolla, CA) was determined by one-way analysis of variance (ANOVA) followed by Tukey's post-test. P-values less than or equal to 0.05 were considered significant.

5.3. RESULTS

5.3.1. Immunization with polyanhydride nanovaccines induced prolonged presence of antigen within the lung up to 63 days after administration

In order to compare the pulmonary persistence and immune response across different nanoparticle chemistries, a 50 µg dose of the protein F1-V was used, with 40 µg delivered as soluble protein and 10 µg encapsulated into 500 µg of polyanhydride nanoparticles. This
formulation has been demonstrated previously to protect against lethal challenge with *Yersinia pestis* with 50:50 CPTEG:CPH nanoparticles.\textsuperscript{11, 13} The F1-V antigen was tagged with the fluorescent label Vivo Tag 680 in order to track the persistence of encapsulated protein. Anesthetized C57B/6 mice were administered a single dose vaccine formulation intranasally and were euthanized at 14, 36, and 63 days in order to quantify the remaining fluorescence in the lungs and characterize antigen uptake by lung cells.

Figure 5.1 demonstrates the persistence of polyanhydride nanoparticles within the lung and the effect that nanoparticle chemistry has on the persistence of encapsulated antigen. Consistent with our previous work,\textsuperscript{14} protein adjuvanted with MPLA was cleared rapidly from the lung, with less than 0.4\% of the original fluorescence (which was observed 2 h after immunization) remaining 14 days after administration. In contrast, the nanovaccine formulations continued to persist in the lung and released antigen as a function of the nanoparticle chemistry. The nanoparticles based on the CPH-rich chemistries, i.e., 20:80 CPTEG:CPH and 50:50 CPTEG:CPH, maintained visible and measurable fluorescence for at least 63 days after immunization, indicating the prolonged presence of antigen within the lung. In contrast, nanoparticles based on the SA-rich chemistries, poly(SA) and 20:80 CPH:SA, showed a marked decrease in fluorescence within the lung 36 days after immunization and returned to baseline levels by 63 days.

### 5.3.2. Nanoparticle chemistry influences antigen internalization and association

The quantification of antigen internalization within the lung demonstrates the combined effects of nanoparticle chemistry and persistence. SA-rich chemistries have been demonstrated previously to be the most readily internalized by APCs.\textsuperscript{18, 20, 21} Consistent with this observation, Figure 5.2 shows that 14 days after administration, antigen internalization is highest in mice immunized with the 20:80 CPH:SA nanovaccine. However, 36 days post-immunization, the percent of cells internalizing the 20:80 CPH:SA nanovaccine formulation decreased
dramatically. This decrease can be attributed to the fast erosion of 20:80 CPH:SA, which therefore, reduces antigen availability. In contrast, the hydrophobic 20:80 CPTEG:CPH nanovaccine shows comparatively modest internalization at day 14, but continues to be internalized above background for at least 63 days. This observation can be attributed to the slow erosion profile of CPH-rich chemistries, which enabled sustained release of antigen.

Additionally, flow cytometric analysis (Figure 5.3) showed increased cellular association of F1-V with dendritic cells (CD11c+) in mice that were administered the poly(SA) nanovaccine compared to that in saline-administered animals and the animals that received F1-V alone 14 days after administration. Although macrophage association (CD11b+) did show some increase over the saline background, the differences between the various nanovaccine formulations were less pronounced. After 14 days, the fluorescence was no longer detectable.

5.3.3. Polyanhydride nanovaccines elicit sustained high titer antibody responses

The data in Figures 5.1-3 provide evidence regarding the persistence and sustained release of antigen within the lung facilitated by intranasal immunization with polyanhydride nanovaccines. These data also suggest that antigen is continuously being internalized by lung cells. Next, new insights are presented on the combined effects of polymer chemistry and nanoparticle persistence on the immune response to the antigen. Figure 5.4 shows that antibody titer is a strong function of adjuvant chemistry, while antigen persistence has less of an impact on the antibody titer over time. The mice administered the highly persistent 20:80 CPTEG:CPH nanovaccine demonstrated the highest serum antibody titers over the course of the nine week study. However, the titers appear to be trending downward by day 63. In contrast, the mice that received the 50:50 CPTEG:CPH nanovaccine showed increasing serum antibody levels through nine weeks. This is consistent with previously published work demonstrating that the 50:50 CPTEG:CPH nanovaccine contains pathogen-mimicking abilities, which may have led to a sustained immune response similar to that induced by the microbially-derived MPLA. It
is interesting to note that although the soluble protein alone persisted in the lung and continued being internalized by cells for 14 days, the animals that received this treatment did not show demonstrable antibody titers.

5.3.4. Antigen persistence and release kinetics affects antibody avidity

The release kinetics of antigen, which is controlled by the chemistry of the biodegradable nano-carrier, affects the quality of antibody response, as measured by antibody avidity (Figure 5.5). Nanovaccines based on 20:80 CPTEG:CPH, 20:80 CPH:SA, and poly(SA) nanoparticles induced highly avid antibodies which are sustained for nine weeks. In contrast, the serum of mice that received the amphiphilic 50:50 CPTEG:CPH nanovaccine showed an increasingly avid IgG antibody with time. These differences in avidity may be attributed in part to the differences in the protein release kinetics from these polymers.\textsuperscript{10,15,22} The poly(SA) and 20:80 CPH:SA nanoparticles release antigen too rapidly to stimulate a sustained immune response which is important for high maturation affinity (i.e., highly avid) antibody. The most hydrophobic 20:80 CPTEG:CPH nanoparticles release antigen so slowly that there may not be sufficient stimuli to elicit a sustained response. In contrast, the 50:50 CPTEG:CPH nanoparticles exhibit the appropriate release rate of antigen to induce a sustained immune response that favors the production of highly avid antibody. This finding is consistent with the antibody titer data in Figure 5.4.

5.3.5. Polyanhydride nanovaccines result in a focused epitope response

A peptide map of the F1-V antigen was used to determine how the nanovaccine formulation, delivery method, and protein release kinetics affected the immune response to specific regions of the protein. As shown in Figure 5.6A, F1-V delivered alone (i.e., without any adjuvant) generated antibodies to a broad spectrum of epitopes, but at relatively weak optical densities. In contrast, F1-V delivered either in the context of polyanhydride nanoparticles or
MPLA elicited a more specific antibody response towards a few dominant epitopes. Consistent with the antibody and avidity responses discussed in Figures 5.4 and 5.5, the responses to specific epitopes were highly dependent upon the nanoparticle chemistry and the kinetics of antigen release. The 20:80 CPTEG:CPH and 20:80 CPH:SA nanovaccines responded by day 14 to the V1 and V2 epitopes, which have been shown to be important for protection. However, this response waned over the nine-week duration of the study, likely due to the clearance of soluble protein, and the too slow (20:80 CPTEG:CPH) or too fast (20:80 CPH:SA) release of the encapsulated antigen. In contrast, the 50:50 CPTEG:CPH nanovaccine enhanced its response to this region over 63 days. Similar responses were observed for the F18 epitope, noting that the addition of responses to the F1 antigen enhances the protection provided by the V antigen. The data in Figure 5.6B compares the immune response to the dominant epitope F18, V1, and V2 in response to each of the formulations. The immune response to these dominant epitopes induced by the 50:50 CPTEG:CPH nanovaccine exhibits a time dependent evolution to the V1 and V2 epitopes. In this case, the optical density increases steadily over the course of the study as the immune response narrows to these epitopes. This type of kinetics was not observed with any of the other nanovaccine formulations of with MPLA.

5.4. DISCUSSION

In this work, polyanhydride nanoparticle formulations representing a range of hydrophobicity, amphiphilicity, and degradation kinetics were examined for their effect in vivo on antigen persistence, antigen release kinetics, and the resultant immune response phenotype. The most hydrophobic of the anhydride chemistries studied is CPH, which has the slowest rate of degradation (of the order of several months). Therefore, CPH-rich chemistries maintain a sustained release of payload over long periods of time and persist longer in vivo. In contrast, CPTEG, which has ethylene glycol moieties incorporated into the polymer backbone, has been shown to be pathogen-mimicking, based on its backbone oxygen moieties, presence of hydroxyl
end groups, etc.\textsuperscript{11, 15, 20} CPTEG-based polymers degrade the fastest among the formulations studied and exhibit bulk erosion.\textsuperscript{15} The degradation rate of poly(SA) is in between that of CPTEG and CPH.\textsuperscript{25}

While all the formulations tested produced similar levels of antibody, the immune response to F1-V is altered when delivered in the context of different polymer nanovaccine formulations, presumably because the antigen release kinetics are strongly affected by the nano-carrier chemistry. Previous work has demonstrated that poly(SA) and 20:80 CPH:SA nanoparticles provide the fastest antigen release kinetics \textit{in vitro} of the four nanoparticle formulations tested, with a majority of the antigen released in one month.\textsuperscript{26, 27} Consistent with these studies, the amount of antigen \textit{in vivo} is almost completely cleared from the lung 36 days post-immunization (Figure 5.1). In contrast, the two CPH-rich chemistries (i.e., 20:80 CPTEG:CPH and 50:50 CPTEG:CPH), which have slower antigen release kinetics,\textsuperscript{15} prolonged the presence of antigen in the lung for up to 63 days as shown in Figure 5.1. Together, these data raise an important question on the effect of persistent antigen and nanoparticle presence on the generation and sustenance of immune responses over time.

To answer this question, the antigen release kinetics from 50:50 CPTEG:CPH nanoparticles needs to be carefully considered. The antigen internalization data presented in Figure 5.2 displays a unique balance between antigen release and persistence of the 50:50 CPTEG:CPH nanovaccine. Consistent with previous work, SA-rich chemistries are rapidly internalized by dendritic cells and macrophages\textsuperscript{18, 20, 21} (Figures 5.2 and 5.3). However, most of the antigen is cleared by 14 days post-immunization, leading to the decline in antibody responses discussed below. Despite the fact that CPH-rich chemistries are less readily internalized by cells, the 50:50 CPTEG:CPH formulation enables antigen release kinetics that provides sustained antigen presence for internalization, and thus, maturation of the immune response.
Figure 5.4 demonstrates that the antibody titers induced in mice that received 50:50 CPTEG:CPH nanovaccine steadily increased over time. The evidence that the levels of circulating antibody increased between 36 and 63 days after immunization for the mice that received the 50:50 CPTEG:CPH nanovaccine formulation suggests that the immune response is being driven by the continuous presence of the right amounts of antigen released from this formulation. The F1-V antigen release kinetics from this formulation is “just right”, neither too slow nor too fast, enabling both increased antibody titers as well as highly avid antibody over time as shown in Figure 5.5. In contrast, the remaining nanovaccine formulations and MPLA show similar profiles for the development of anti-F1-V antibody titers that peak or plateau between day 36 and 63. The release kinetics of antigen from these formulations is either too fast (i.e., MPLA, poly(SA), 20:80 CPH:SA) or too slow (i.e., 20:80 CPTEG:CPH) and resulted in titers that peaked between 36 and 63 days and induced antibody that is not highly avid. Together, these data suggest that the presence of antigen alone is not sufficient to stimulate the immune response. The chemistry of the nano-adjuvant must be selected carefully to interact with immune cells and enable sustained antigen release at an appropriate rate to generate long-lived antibody titers.

It may be possible that differences in antibody titer profiles are a result of plasma cell development.\textsuperscript{28} Perhaps nanovaccine formulations that sustain antigen presence (and hence re-expose B cells to antigen over time) lead to the development of long-lived plasma cells, accounting for the increase in antibody titers between 36 and 63 days. In contrast, formulations that induce the production of short-lived plasma cells, which have a half-life of only seven days,\textsuperscript{28} may account for the plateau or decreasing antibody titers at day 63. In addition, repeated antigen exposure (and therefore generation of long-lived plasma cells) typically leads to the maturation of antibody responses over time. In this work, the avidity of antibodies in response to 50:50 CPTEG:CPH nanovaccine immunization increased over time, while the other formulations did not (Figure 5.5). There are a number of factors to consider when analyzing this
observation. CPTEG-rich polymers have lower glass transition temperatures compared to the other polyanhydrides studied herein.\textsuperscript{15} This may induce more agglomeration of these particles within the lung, leading to a reduced surface area to volume ratio and result in slower release of antigen, thereby prolonging antigen release kinetics optimally.\textsuperscript{15, 16} Additionally, the pattern provided by 50:50 CPTEG:CPH formulations may imitate a replicating pathogen, or a replication-competent vaccine, as suggested previously based on demonstration of protective immunity 40 weeks after a single intranasal immunization,\textsuperscript{11, 13} leading to memory development.

Furthermore, the selection of protective epitopes by nanovaccine formulations provides additional evidence of antibody maturation, and therefore, the presence of long-lived plasma cells (Figure 5.6). Consistent with the increasing antibody titers and avidity, the 50:50 CPTEG:CPH nanovaccine formulation led to a more focused epitope spread over nine weeks as shown in the peptide heat map in Figure 5.6A. The gradual development of an immune response to the V1 and V2 epitopes (Figure 5.6B) over time indicates that the release rate of antigen is sufficient to maintain B cell maturation, leading to the production of antibodies towards specialized epitopes. It is well known that the V1 and V2 epitopes are important in Y. pestis pathogenesis and antibody to these epitopes is critical for protective immunity against infection.\textsuperscript{23, 24} In contrast, the other nanovaccine formulations (which may release antigen too fast or too slow) demonstrated early recognition of protective epitopes that waned over the course of the study. F1-V antigen delivered alone without adjuvant elicited a broad, unfocused response to many F1-V epitopes, indicating that while the antigen is recognized, plasma cell maturation and antibody refinement did not occur and this formulation failed to protect animals upon live challenge.\textsuperscript{13}

While the prolonged presence of antigen \textit{in vivo} may play a large role in the development and maturation of antibody responses, it is instructive to note that the mice administered the slowest eroding (and therefore, most persistent) 20:80 CPTEG:CPH nanovaccine formulation showed decreased antibody titers over time. Therefore, the
persistence of antigen alone is not enough to drive an efficacious immune response. The kinetics of antigen release must be appropriate and play a key role in the rational design of efficacious vaccines.

The persistence of antigen has a large impact on the development of short-lived and long-lived plasma cells, as well as the development of T cell memory.\textsuperscript{29-30} Short-lived plasma cells are quick to respond and proliferate, but often at the expense of antibody maturation.\textsuperscript{29} In instances where antigen is released and cleared quickly, these short-lived responses are often ineffective in developing protection. However, sustained antigen presence may enhance the development of germinal centers and long-lived plasma cells producing high avidity, high affinity antibodies as well as memory B cells.\textsuperscript{28, 29} Likewise, the kinetics of antigen release from nanovaccine formulations has a large role in the development of T cell responses. The initial activation and expansion of T cells is largely antigen-dependent, requiring optimal concentrations to successfully prime the immune response.\textsuperscript{30} Too much antigen, however, may lead to accelerated activation-induced cell death and short-lived immune responses.\textsuperscript{30} Similar to B cells, optimal concentrations of persisting antigen may lead to the development of T cell memory.\textsuperscript{30} The development of optimal vaccine formulations, such as 50:50 CPTEG:CPH-based nanovaccines, which release the right amount of antigen at the right time, bring balance to the adaptive immune response, effectively bridging the gap between priming early expansion and sustaining long-lived memory through persistent antigen.

5.5. CONCLUSIONS

We have herein demonstrated the importance of polymer chemistry in the design of nanovaccines. There are several important factors that influence the design of an efficacious intranasal vaccine, including nanoparticle interactions with immune cells and the kinetics of antigen release. The performance of the 50:50 CPTEG:CPH nanovaccine formulation clearly demonstrates the impact of sustained release of antigen on the immune response to the F1-V
antigen over time. The formulation delivered with this nano-carrier chemistry showed an increase in antibody titer throughout the duration of the experiment, indicating a continual addition of long-lived plasma cells to the pool of antibody-generating cells. In contrast, the other nanovaccine formulations as well as MPLA demonstrated a stable or decreased antibody titer at longer time points, suggesting a higher percentage of short-lived plasma cells. Finally, the maturation of antibody avidity and focus towards protective epitopes demonstrates the importance of persistent antigen in developing an efficacious immune response.
Figure 5.1. Polyanhydride nanovaccines persist in the lung up to 63 days. Images represent fluorescence of remaining labeled F1-V antigen in excised lung tissue at the indicated time points. Mean fluorescence intensity (MFI) is represented graphically as the average of eight mice per group from a total of two independent experiments (Days 14 and 36) and four
mice per group from one experiment (Day 63). Error bars represent the standard error of the mean. Letters represent statistical differences comparing experimental groups across one time point. Asterisks represent statistical differences across time points for one experimental treatment group. $p \leq 0.05$.

Figure 5.2. Persistence of particles leads to sustained internalization of antigen.
Multispectral imaging flow cytometry was used to determine the internalization of F1-V by lung cells. Error bars represent the standard error of the mean with $n = 8$ from two independent experiments (Day 36) and $n = 4$ from one experiment (Days 14 and 63). One thousand events per sample were acquired and analyzed as described. Letters represent statistical differences between experimental groups at one time point. Asterisks represent statistical differences across time points for one experimental treatment group. $p \leq 0.05$. 
Figure 5.3. Flow cytometric analysis indicates increased cellular association at 14 days after immunization for dendritic cells (CD11c⁺) and macrophages (CD11b⁺). Flow cytometry was used to assess the mean fluorescent intensity (MFI) of F1-V associated with dendritic cells and macrophages 14 day post-immunization. Ten thousand events per sample were collected. Error bars represent the standard error of the mean (n = 8) from two independent experiments. Letters represent statistical differences between experimental groups. p ≤ 0.05.
Figure 5.4. Polyanhydride nanovaccines induced high titer anti-F1-V antibody responses.

Error bars represent the standard error of the mean (n = 8) from two independent experiments (Days 14 and 36) and n = 4 from one experiment. Letters represent statistical differences between experimental groups at one time point. Asterisks represent statistical differences across time points for one experimental treatment group. p ≤ 0.05.
Figure 5.5. Polyanhydride nanovaccine formulations induced highly avid antibodies to F1-V. Error bars represent the standard error of the mean (n = 8) from two independent experiments (Days 14 and 36) and n = 4 from one experiment. Letters represent statistical differences between experimental groups at one time point. Asterisks represent statistical differences across time points for one experimental treatment group. p ≤ 0.05.
Figure 5.6. Epitope recognition of antibodies induced by nanovaccine formulations. A heat map of the responses to eighty F1-V peptides was evaluated by ELISA, beginning at the top with the amino-terminal peptide and then
moving down sequentially through the F1-V protein (A). The optical density of each peptide is indicated by a range of color from blue (no response) to red (maximum response). Data presented is the average of eight individual mice for days 14 and 36, and four individual mice for the day 63 time point. Graphical representation of the evolution of the immune response to the F18, V1, and V2 peptides as fold change over saline (B). Data was collected from groups of animals for days 14 and 36 from two individual experiments and from four animals for day 63 from one experiment. Epitope recognition is considered to be 1.5-fold or higher fold change over saline. Dashed line represents a 1.5-fold change from saline controls for that peptide.
5.7. REFERENCES


CHAPTER 6:
Polyanhydride Nanovaccine Platform Enhances Antigen-specific Cytotoxic T Cell Responses

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Abstract

Vaccination remains the most cost effective preventive intervention against disease. Polyanhydride nanovaccines stabilize protein antigens, provide sustained antigen release leading to prolonged antigen presence, enhance activation of antigen presenting cells, and elicit protective immunity against respiratory infections upon challenge. However, evaluation of cell-mediated immune activation when mice are immunized with polyanhydride nanoparticles has not been carried out. Using a transgenic ovalbumin-specific T cell adoptive transfer model, we report the enhanced expansion of antigen-specific cytotoxic CD8⁺ T cells expressing an effector memory phenotype at early time points post-immunization with nanovaccine formulations. Furthermore, mice immunized with polyanhydride nanoparticles also showed superior ability to respond to tumor challenge, indicative of memory T cell generation post-immunization.

6.1. INNOVATION

Polyanhydride nanoparticles represent a unique biodegradable platform for the delivery of vaccine antigens. Polyanhydride copolymers contain hydrophobic properties, which exclude water from the bulk of the material. This limitation of water exposure maintains the stability of

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fragile protein antigens, as well as allows for sustained release kinetics. Previously, polyanhydride nanoparticles successfully induced long-lived humoral responses (i.e., high avidity, high titer antibody) that provided protection against live bacterial challenge. For many infectious pathogens, humoral immunity (antibody) alone will not provide complete protection and cell-mediated immunity is required to provide host protection. Many subunit vaccines fail to produce or produce weak cellular immune responses towards single protein antigens. Herein, we demonstrate that polyanhydride nanovaccines elicited cell-mediated immunity via expansion of antigen-specific T cells of memory phenotypes and enabled tumor regression. The ability of polyanhydride nanovaccines to induce memory CD8+ cytotoxic T cells provides proof of concept for this platform to be used in the development of efficacious vaccines for cancer, influenza, and intracellular pathogens.

6.2. INTRODUCTION

The use of preventive vaccines remains the most effective public health intervention strategy to decrease the morbidity and mortality associated with infectious diseases. Challenges associated with design of next generation vaccines include eliciting effective cell-mediated immune responses from highly purified antigens and achieving long-lived immunological memory against the purified antigen. To date, the live attenuated yellow fever vaccine remains the most successful human vaccine.1-3 This efficacy is primarily due to its ability to elicit cell-mediated (i.e., CD4+ and CD8+ T cell) responses within the first two weeks of immunization and subsequently a long-lived protective antibody response that can last for 45 years.1-3

The effectiveness of the yellow fever vaccine can be attributed to two important factors. First is the engagement of the innate immune system resulting in enhanced humoral and cell-mediated immunity. Second is the ability of the attenuated virus to establish a mild sub-clinical infection and a low level of replication, which promotes the development of long-lived CD8+ T cells.1-3 Unfortunately, not all organisms can be attenuated, and adverse side effects must be
given careful consideration in vaccine design. Subunit (i.e., purified or recombinant proteins) vaccines are a safe alternative to live, attenuated pathogens; however, these proteins are often poorly immunogenic requiring adjuvants to enhance immune responses and induce long-lived T cell memory.

Pola**nhydride nanoparticles based on 1,6-bis(p-carboxyphenoxy)hexane (CPH) and 1,8-bis(p-carboxyphenoxy)-3,6-dioxaoctane (CPTEG) have been shown to sustain the release of and enhance the immunogenicity of encapsulated subunit proteins, which promotes robust B cell responses with sustained antibody titers with high avidity.** Furthermore, immunization with polyanhydride nanovaccines has induced protective responses upon live bacterial challenge. However, no direct evaluation of the cell-mediated immune responses elicited by the polyanhydride nanovaccine platform has been reported. In this work, we sought to examine the CD8⁺ T cell responses elicited by the 20:80 CPTEG:CPH nanovaccine utilizing an adoptive transfer model of ovalbumin (OVA)-specific transgenic OTI CD8⁺ T cells to recipient mice receiving a vaccine regimen of soluble and encapsulated OVA, as previously described.

This study demonstrates that encapsulation of antigen in 20:80 CPTEG:CPH nanoparticles enhanced the expansion of OTI CD8⁺ T cells after immunization. The subsequent expansion led to higher frequencies of memory precursor effector cells and central memory CD8⁺ T cells. Mice immunized with 20:80 CPTEG:CPH nanovaccine significantly expanded antigen-specific CD8⁺ memory T cells in response to challenge with OVA-secreting EG7 tumor cells, delaying tumor progression. Altogether, our data demonstrates that immunization with polyanhydride nanoparticles leads to the development of memory T cells capable of being recalled in response to challenge.
6.3. METHODS

6.3.1. Synthesis and characterization of copolymers

Polyanhydrides were synthesized using the chemicals listed: 4-p-hydroxybenzoic acid, 1,6-dibromohexane, 1-methyl-2-pyrrolidinone, N,N-dimethylacetamide, and triethylene glycol. All these chemicals and sebacic acid (99%) were purchased from Sigma Aldrich (St. Louis, MO); 4-p-fluorobenzonitrile was obtained from Apollo Scientific (Cheshire, UK); potassium carbonate, dimethyl formamide, toluene, sulfuric acid, acetic acid, acetonitrile, acetone, acetic anhydride, methylene chloride, chloroform, sodium hydroxide, hexane, ethyl ether, and petroleum ether were purchased from Fisher Scientific (Fairlawn, NJ). Synthesis of CPH and CPTEG diacids was performed as previously described.\textsuperscript{8, 9} The 20:80 CPTEG:CPH copolymer was synthesized using a melt polycondensation process as described previously.\textsuperscript{8, 10} The molecular weight and polymer purity were determined using \textsuperscript{1}H nuclear magnetic resonance (NMR) spectroscopy (Varian VXR-300 MHz, Palo Alto, CA).

6.3.2. Fabrication and characterization of particles

Nanoparticles encapsulating ovalbumin (OVA) (Sigma-Aldrich) (5.0% w/w) were fabricated using the anti-solvent nanoencapsulation method outlined previously.\textsuperscript{5} Briefly, the copolymer was dissolved in methylene chloride at a concentration of 20 mg/mL at 4°C. Lyophilized OVA was added to the dissolved copolymer and the solution was sonicated for uniform dispersal of the protein. The OVA-loaded copolymer (OVA-loaded) or copolymer (blank) solution was poured into chilled pentane (-20°C) at a solvent to non-solvent ratio of 1:250 and vacuum filtration was used to recover the nanoparticles. The shape and size of the resulting nanoparticles were characterized using scanning electron microscopy (FEI Quanta 250, FEI, Hillsboro, OR).
6.3.3. Animals

Recipient female C57BL/6 Thy 1.1\textsuperscript{+}, recipient albino C57BL/6, OTI mice were purchased from Jackson Laboratories (Bar Harbor, ME). C57BL/6 mice were obtained from Harlan Laboratories (Indianapolis, IN). T-lux mice were a kind gift from Dr. Casey Weaver of the University of Alabama. A T-lux breeding colony was established at Iowa State University. Heterozygous breeding between T-lux and OTI mice was established to produce T-lux/OTI offspring. Phenotyping of offspring for T cell receptor rearrangement (V\textalpha{}2 Clone B20.1 V\textbeta{}5 Clone MR9-4 (eBioscience, San Diego, CA) and luciferase-positive status was examined to establish which mice were the desired T cell donors (e.g., OT I\textsuperscript{+}:T-Lux\textsuperscript{+}). All mice were housed under specific pathogen-free conditions where all bedding, caging, water, and feed were sterilized prior to use. Animal procedures were conducted with the approval of the Iowa State University Institutional Animal Care and Use Committee.

6.3.4. Adoptive transfer of antigen-specific T cells

For the in vivo imaging studies, 5 \times 10^5 T-lux OTI CD8\textsuperscript{+} T cells were transferred into albino C57BL/6 recipients performed on day -1.\textsuperscript{11} For the low frequency adoptive transfer studies, 3 \times 10^3 OTI Thy 1.2\textsuperscript{+} 5-(and-6)-carboxyfluorescein diacetate, succinimidyl ester (CFSE) (Molecular Probes/Life Technologies, Grand Island, NY) labeled cells were adoptively transferred to each recipient mouse.

6.3.5. Immunization Regimens

On day 0, mice were immunized with 1.75 mg of soluble OVA and 5 mg of 5% OVA-loaded 20:80 CPTEG:CPH polyanhydride nanoparticles, 2.0 mg of soluble OVA (sOVA), or 2.0 mg of OVA adjuvanted 1:1 with Imject\textsuperscript{TM} Alum (ThermoFisher, Rockford, IL).
6.3.6. *Ex vivo* T cell phenotype and activity assays

At multiple time points post-immunization, mice were euthanized and draining lymph nodes were excised and single cell suspensions were created using a glass homogenizer. Cell numbers and phenotype were quantified using flow cytometry (BD FACS Aria III, BD Biosciences, San Jose, CA). For the low frequency adoptive transfer experiments, positive selection for donor T cells was accomplished using biotinylated anti-CD90.2 (Thy 1.2 Clone 53-2.1) (eBioscience) and streptavidin magnetic microbeads (Miltenyi Biotech, Auburn, CA). Positive magnetic bead selection was performed via methods outlined by Moon *et al.*

(AutoMACS Pro, Milteny Biotech). Cell suspensions were blocked for non-specific antibody binding using 0.1 mg/mL rat IgG (Sigma Aldrich) and 10 µg/mL mouse anti-CD16/32 (eBioscience). For T cell assays, fluorescently conjugated antibodies specific for CD8β (APC, Clone eBioH35-17.2, eBioscience), CD62L (PE, Clone 2G8, eBioscience), Thy 1.2 (APC-eFluor 780, Clone 53-2.1, eBioscience), Thy 1.1 (PerCP-Cy 5.5, Clone HIS51, eBioscience), CD197 (eFluor 450, Clone 4B12, eBioscience), KLRG1 (PE-Texas Red, Clone 145-2C11, eBioscience), or CD44 (v500, clone IM7, BD, Franklin Lakes, NJ) were diluted in FACS buffer and used to label the cells in order to quantify donor antigen-specific cell populations. Antibodies were used with appropriate combinations of fluorochromes.

6.3.7. *In vivo* imaging of T-lux cells

Five minutes prior to imaging for the presence of T-lux positive T cells, mice were administered D-luciferin (215 µg/g body weight) via intraperitoneal injection. *In vivo* images (Carestream Multispectral FX, Rochester, NY) were captured while mice were anesthetized as described above. Bioluminescent images were captured using 10-minute exposures with high amounts of binning for increasing sensitivity. All image analyses were performed using Image J software version 1.46. Raw fluorescence images were inverted and background subtracted via a rolling ball radius of 150 pixels. The mean luminescence intensity (MLI) of the region of
interest (ROI) of 0.39 mm x 0.32 mm were quantified via ImageJ and presented thereto. Composite images of luminescent channel, fluorescence channel, and white light image were created using Image J software.

6.3.8. Tumor challenge

EG7 ovalbumin expressing tumor cells (2.5 x 10^6) were administered subcutaneously in the left rear flank on day 35 post-immunization. Tumor measurements were recorded using a digital caliper and tumor volume was calculated using the ellipsoid volume equation, where volume = (4/3) * π * length * width * height. Per criteria outlined in consultation with the attending veterinarian, animals were removed from the study when tumor volume reached 1000 mm^3. ProSense® 750 was administered (2 nmol) via tail vein injection seven days post-tumor challenge to visualize the inflammation associated with the tumor and to quantify changes in tumor size. ProSense® 750 is an activatable fluorescent reagent that is optically visible when the dye is cleaved by degradative enzymes, including cathepsin B, L, S, or plasminogen, that are common at sites of tumor growth.\textsuperscript{13} After 24 h, \textit{in vivo} images were captured while mice were kept under anesthesia using 2 % isoflurane in 100% O\textsubscript{2} at 2.5 L/min. Images were captured using 30 second exposures with an excitation filter of 730 nm and an emission filter of 790 nm.

6.3.9. Statistical analysis

Differences in mean responses among treatment groups were tested with either unpaired T test with Welch’s correction or one-way ANOVA F-test followed by a post-hoc Tukey’s t-test using GraphPad Prism version 5 (GraphPad Software, Inc., La Jolla, CA). Statistical tests with p values ≤ 0.05 were regarded as significant. Data was log transformed when variances were not equal.
6.4. RESULTS

6.4.1. Polyanhydride nanovaccine enhances early expansion of OTI CD8⁺ T cells

Induction of antigen-specific cell-mediated responses to cognate antigen occurs early following antigen encounter. Upon antigen encounter, antigen-specific T cells undergo expansion and acquire effector function. Once antigen is cleared, about 90% of antigen-specific T cells will undergo apoptosis while the remaining 10% will become long-lived memory T cells. In order to determine the ability of OVA-loaded 20:80 CPTEG:CPH polyanhydride nanovaccines to expand memory CD8⁺ T cells, we adoptively transferred 5 x 10⁵ transgenic luciferase-expressing OTI CD8⁺ T cells into naïve mice prior to immunization with OVA-loaded 20:80 CPTEG:CPH nanovaccine, OVA adjuvanted with alum (Alum), soluble OVA (sOVA) or sham vaccine (saline). Cohorts from each treatment group were imaged at 1, 3, 5, 7, and 10 days post-immunization to determine the level of OTI CD8⁺ T cell expansion in each of the treatments. Based on mean fluorescence intensity (MFI) in all groups, peak T cell expansion occurred at 5 days post-immunization (DPI) and contraction was achieved by 10 DPI (Figure 6.1). Mice immunized with the OVA-loaded 20:80 CPTEG:CPH nanovaccine formulation and alum had the highest levels of MFI, while sOVA-treated groups also induced expansion of OTI CD8⁺ T cells, albeit at a lower magnitude (Figure 6.1).

6.4.2. 20:80 CPTEG:CPH nanovaccine expands memory precursor T cells

To better assess the expansion and phenotype of antigen-specific T cells following a 20:80 CPTEG:CPH nanovaccine regimen, we again utilized an adoptive transfer model, as outlined by Moon et al. In this study, 3 x 10³ OTI CD8⁺ Thy 1.2⁺ cells were transferred into naïve recipients and immunized as described before. Seven days post-immunization, draining lymph nodes were harvested and the antigen-specific T cell population was analyzed for expansion and surface phenotype. Immunization with 20:80 CPTEG:CPH nanovaccines resulted in a statistically significant increase not only in the frequency, but also the total
numbers of OTI CD8+ T cells in comparison with all the other treatment groups (both panels of Figure 6.2A).

Surface phenotype analysis indicates that following all immunizations, the antigen-specific CD8+ T cells acquired both central memory (T_CM) (CD44_{high} CD62L_{high}) and effector memory (T_EM) (CD44_{high} CD62L_{low}) phenotypes (Figure 6.2B, flow cytometry panels) with a higher frequency of T_CM generation as compared to T_EM. Furthermore, 20:80 CPTEG:CPH nanovaccine-immunized mice showed significantly higher total numbers of T_CM phenotype in comparison with all the other treatment groups (Figure 6.2B, graph). Further surface phenotype analysis demonstrated that the 20:80 CPTEG:CPH nanovaccine immunization strategy resulted in higher numbers of antigen-specific CD8+ T cells with a memory precursor effector cell (MPEC) phenotype (CD44_{high} CD62L_{high} KLRG1_{low} CD127_{high}) in comparison with all the other immunization regimens (both panels of Figure 6.2C).

6.4.3. Cytotoxic CD8+ T cells respond to tumor challenge following immunization with 20:80 CPTEG:CPH nanovaccine

To determine if the 20:80 CPTEG:CPH nanovaccine was able to elicit cytotoxic CD8+ T cells capable of responding to challenge, albino C57BL/6 recipient mice receiving adoptive transfer of T-lux OTI CD8+ T cells and immunized with the regimens outlined previously were challenged with 2.5 \times 10^6 OVA-expressing EG7 lymphoma cells into the right rear flank at 35 DPI. **In vivo** imaging of bioluminescent OTI CD8+ T cells at seven days post-tumor challenge showed measurable T cell infiltrate into the tumor site in 20:80 CPTEG:CPH nanovaccine-immunized mice compared to mice immunized with Alum or sOVA (Figure 6.3A). Quantitative luminescent analysis of the OTI CD8+ T cell response at the site of tumor implantation (Figure 3B) showed more robust OTI CD8+ T cell responses in mice treated with the 20:80 CPTEG:CPH nanovaccine formulation in contrast to all the other treatments (Figure 6.3B).
To determine if the observed CD8⁺ T cell infiltration had a functional effect on tumor progression, a protease cleavable probe, Prosense® 750, was administered to tumor-challenged mice. Mice immunized with the 20:80 CPTEG:CPH nanovaccine formulation showed inhibited tumor growth as evidenced by the decreased tumor fluorescence intensity (Figure 6.4). This tumor inhibition by the 20:80 CPTEG:CPH nanovaccine may be attributed to the enhanced T cell response observed in Figure 6.3A-B. Altogether, these data suggest that polyanhydride nanovaccines promote the generation of antigen-specific OTI CD8⁺ T cells with a long-lived MPEC phenotype, capable of recall effector function.

6.5. Discussion

A major obstacle in an increasingly stringent regulatory environment is the development of safe and efficacious vaccines with purified antigens and reduced adverse side effects. Another major challenge is the generation of cell-mediated immunity against vaccine antigens. The ability to expand cytotoxic T cells and generate memory T cells using immunization is largely biased towards replicating viral vectors or DNA vaccines expressing targeted antigens, which provide both innate viral stimulatory mechanisms as well as extended presence of antigen.¹⁵,¹⁶ Targeting the small number of antigen-specific naïve T cells that would be available for a given T cell epitope, expanding that population of T cells, and creating stable central memory populations capable of responding to a subsequent infection is difficult to achieve with subunit immunizations.¹⁴,¹⁷⁻¹⁹ Polyanhydride nanoparticles can bridge the gap between safe subunit vaccines and efficacy with a broad-based repertoire of immune responses because of their pathogen mimicking attributes such as particle size, chemical structure, and perhaps most importantly to the development of memory T cells, sustained release of antigen.⁴,⁷,²⁰

The timing of antigen exposure to naïve T cells entering the draining lymph nodes is limited by antigen availability, making it critical in programming memory T cells.²¹ Short-lived effector cells are generated initially, while naïve T cells arrive later or during reduced antigen
load, and are subject to differential programming resulting in the development of T cells with a central memory phenotype. Continued antigen presence is a key component in driving memory populations since naïve T cells activated as the antigen load is waning acquire more central memory characteristics. While the initial expansion of T cells was similar between Alum- and 20:80 CPTEG:CPH nanovaccine-immunized mice (Figure 6.1), the persistent release of antigen from 20:80 CPTEG:CPH nanoparticles would likely increase the duration of antigen availability for late-arriving naïve CD8+ T cells. Polyanhydride nanoparticles have been shown to persist up to 12 weeks in vivo and sustain antigen release.

The expansion of OTI CD8+ T cells following 20:80 CPTEG:CPH nanovaccine administration resulted in higher total numbers of T cells expressing a precursor memory (MPEC) phenotype at 7 DPI, characterized by increased expression of CD127 (IL-7Rα and decreased expression of KLRG1 (Figure 6.2C). KLRG1low CD127high T cells demonstrate greater ability to effectively convert into central memory T cells. These data indicate that the polyanhydride nanovaccine formulation elicits not only an effector immune response, but also promotes the development of long-lived memory CD8+ T cells.

Likewise, the kinetics of T cell infiltration in response to the tumor challenge (Figure 6.3) and the amount of OTI CD8+ T cells in the periphery (data not shown) indicated higher levels of memory T cells developed from polyanhydride nanovaccine immunization. The high frequency adoptive transfer model demonstrated the ability of 20:80 CPTEG:CPH nanovaccine immunization to activate OTI CD8+ T cells and showed that an effector memory CD8+ T cell response can be enhanced by the inclusion of polyanhydride nanovaccines.

Adaptive immunity is contingent on innate immune response to “foreign” antigens. Previous work examining the immunomodulatory potential of the polyanhydride nanovaccine platform indicated that CPTEG:CPH chemistries appear “pathogen-like” in their ability to induce innate immune functions. These “pathogen-like” properties most likely are a synergistic combination of: the nanoparticles mimicking pathogen size; sustained antigen release
mimicking the kinetics of replicating pathogens; and interaction of conserved innate immune receptors responding to the patterns of repeated polymeric units.\textsuperscript{20} The data herein demonstrate that the polyanhydride nanovaccine platform can be harnessed to induce cellular immunity by expanding antigen-specific T cells that acquire a long-lived and functional memory phenotype.

Particulate-adjuvanted subunit antigen is regarded as better able to access intracellular endosomal compartments crucial for antigen presentation via major histocompatibility complex (MHC) I.\textsuperscript{16} Cross-presentation or endosomal escape into the cytosol leads to MHC I antigen presentation critical for CD8\textsuperscript{+} T cell activation.\textsuperscript{16} This work suggests that MHC I presentation may be enhanced by polyanhydride nanovaccines, thus requiring future examination of antigen presentation pathways in APCs using these formulations. Further studies are needed to examine if the nanovaccine platform is capable of inducing cellular immunity to other subunit antigens. Memory CD8\textsuperscript{+} T cell expansion and generation against antigens typically thought of as humoral dominant antigens can lead to increased vaccine efficacy and cell-mediated memory.\textsuperscript{16} The data reported herein demonstrates the polyanhydride nanovaccine platform performs as both an effective particulate vaccine delivery system as well as a potent immunomodulator, leading to enhanced CD8\textsuperscript{+} T cell immunity.

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Figure 6.1. 20:80 CPTEG:CPH nanovaccine immunized groups expanded OTI CD8+ T cells at the immunization site. (A) Representative images of albino C57BL/6 mice receiving transfer of T-lux OTI CD8+ T cells from immunized groups (20:80 CPTEG:CPH nanovaccine, Alum, sOVA, or PBS) at 1, 3, 5, 7, or 10 days post-immunization (DPI) examining bioluminescent CD8+ T cell activity. (B) Regions of interest of equal area were drawn at the immunization site to measure intensity of T cell luminescence and quantified over time. Error bars represent standard error of mean.
Figure 6.2. 20:80 CPTEG:CPH nanovaccine immunization expanded OTI CD8$^+$ T cells early after immunization and promotes memory development. (A) Representative flow cytometric histograms of donor OTI Thy 1.2$^+$ (y-axis) CD8$^+$ (x-axis) T cell expansion from draining lymph nodes (LNs) seven days post-immunization (DPI) and total CD8$^+$ Thy 1.2$^+$ T cells recovered from the axial and brachial LNs at seven DPI. (B) Representative flow cytometric histograms of OTI Thy 1.2$^+$ CD8$^+$ T cells analyzed for expression of CD44 (y-axis) and CD62L (x-axis) and total numbers of CD8$^+$ Thy 1.2$^+$ CD44$^{high}$ CD62L$^{high}$ T cells recovered from the axial and brachial LNs at seven DPI. (C) Representative flow cytometric histograms of gated OTI Thy 1.2$^+$ CD8$^+$ T cells for surface markers CD127 (y-axis) and KLRG1 (x-axis) and total numbers of CD8$^+$ Thy 1.2$^+$ CD127$^{low}$ KLRG1$^{high}$ (MPEC) T cells recovered from the axial and brachial LNs. * indicates statistical difference at a p-value < 0.0001 value obtained via one-way ANOVA with Tukey’s post-test. Data is from a single experiment.
Figure 6.3. 20:80 CPTEG:CPH nanovaccine immunized groups demonstrated cellular recall responses. (A) Representative images of albino C57BL/6 mice receiving transfer of T-lux OTI CD8+ T cells from immunized groups (20:80 CPTEG:CPH nanovaccine, Alum, sOVA, or PBS) challenged with EG7 tumor cells visualizing OTI CD8+ T cells. (B) Regions of interest of equal area were drawn at the tumor challenge site of representative mouse cohort and luminescent intensity of T cell luminescence was quantified post-tumor challenge (n = 4).
Figure 6.4. 20:80 CPTEG:CPH nanovaccine immunized groups demonstrated cellular recall responses capable of controlling tumor growth. Representative images of albino C57BL/6 mice receiving transfer of T-lux OTI CD8$^+$ T cells from immunized groups (20:80 CPTEG:CPH nanovaccine, Alum, sOVA, or PBS) challenged with EG7 tumor cells visualizing inflammation (ProSense® 750, Red) at the site of tumor implantation.
6.7. REFERENCES


Abstract

While H5N1 avian influenza has not yet acquired the capacity to readily infect humans, should it do so, this viral pathogen would present an increasing threat to the immunologically naïve human population. Subunit vaccines based on the viral glycoprotein hemagglutinin (HA) can provide protective immunity against influenza. Polyanhydride nanoparticles have been shown to enhance efficacy of subunit vaccines, providing the dual advantages of adjuvanticity and sustained delivery resulting in enhanced protein stability and immunogenicity. In this work, a recombinant trimer of H5 (H5₃) was encapsulated and released from polyanhydride nanoparticles. Release kinetics of the encapsulated H5₃ were found to be dependent on polymer chemistry (i.e., hydrophobicity and molecular weight). Polyanhydride nanoparticles composed of sebacic anhydride and 1,6-bis(p-carboxyphenoxy)hexane (CPH) (that degrade into more acidic monomers) released structurally stable hemagglutinin H5₃, while H5₃ released from formulations composed of CPH and 1,8-bis(p-carboxyphenoxy)-3,6-dioxaoctane (CPTEG) (that are amphiphilic and whose degradation products are less acidic) displayed unfolding of tertiary structure. However, the antigenicity of the H5₃ based on binding of a H5-specific monoclonal antibody was preserved upon release from all the formulations studied.

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demonstrating the value of polyanhydride nanoparticles as a viable platform for hemagglutinin-based influenza vaccines.

7.1. INTRODUCTION

Seasonal influenza affects people of all ages and induces characteristic symptoms that include cough, fever, and body aches; patients typically recover in a short period of time. However, other strains of influenza such as H5N1 avian influenza can be much more devastating due to the immunological naiveté of the population and ease of spread worldwide. H5N1 is an influenza A virus whose host range has been primarily restricted to waterfowl because of the binding of the HA to sialic acid with an α2,3 linkage to galactose; however, cases of H5N1 influenza viral infections have occurred in humans over the last several years.\(^1,2\) These cases, while isolated, have shown that up to 60% of human H5N1 infections are fatal within one to three days.\(^3^-7\) While mutations are common in RNA viruses, as observed by the change(s) in seasonal influenza each year, the mutations in H5N1 influenza virus strains are of a greater concern.\(^3,4\) If the appropriate mutations occur in the HA protein, the virus would be efficiently transmissible among a global population of immunologically naïve individuals. With its high fatality rate and resistance to antiviral treatments, it is predicted that without implementing appropriate public health measures, an H5N1 influenza virus pandemic would cause an estimated 50 million deaths in the U.S. alone, indicating an urgent need for research to develop efficacious vaccines against this virus.\(^3,4\)

Current manufacturing processes of viral vaccines require strictly regulated biocontainment facilities because of the need to generate large quantities of live virus for vaccine production.\(^8^-10\) In contrast, subunit vaccines do not require biocontainment and have reduced patient side effects (i.e., pain, swelling, allergic reactions) because the proteins are highly purified.\(^8^-10\) By using a protein-based subunit vaccine, the risks to both manufacturers and
patients can be largely reduced. Recently, progress has been made in the development of a subunit vaccine against H5N1 influenza virus.\textsuperscript{8}

The hemagglutinin (HA) protein has been identified as a protective antigen against infections induced by the homologous influenza virus.\textsuperscript{8-10} HA is a trimeric membrane glycoprotein present on the surface of influenza virions and is responsible for binding to and entry of the virus into host cells.\textsuperscript{11} During infection, HA binds to host cell sialic acid receptors and the virion is internalized by receptor-mediated endocytosis. Once inside an acidified endosome a conformational change in the trimeric structure occurs, exposing a fusion peptide and allowing endosomal escape of the viral RNA. Due to the involvement of HA in initiating viral infection, HA proteins are good candidates for inducing neutralizing antibodies.

As with most subunit vaccines, HA protein administered alone generally results in a weak immune response requiring multiple doses for efficacy.\textsuperscript{8-10} Therefore, adjuvants are needed to reduce the dosage and cost while enhancing immune stimulation and vaccine potency.\textsuperscript{9,12,13} Unlike traditional adjuvants (e.g., alum), polyanhydride nanoparticles can be used to perform the dual functions of providing sustained delivery of structurally stable and functional proteins, as well as enhancing the capacity to induce a robust immune response.\textsuperscript{14, 15, 16}

Polyanhydrides degrade into non-toxic, non-mutagenic carboxylic acids.\textsuperscript{17,18} The anhydride monomers most commonly studied include sebacic acid (SA), 1,6-bis(\(\rho\)-carboxyphenoxy) hexane (CPH), and 1,8-bis(\(\rho\)-carboxyphenoxy)-3,6-dioxaoctane (CPTEG). Polyanhydrides exhibit surface erosion, resulting in a sustained release of antigen.\textsuperscript{17,19} The composition of polyanhydride copolymers can be varied to modulate the release kinetics of the encapsulated protein, which can be tailored from days to weeks and even months.\textsuperscript{18-20} The sustained release kinetics of F1-V antigen from vaccine formulations based on amphiphilic 50:50 CPTEG:CPH nanoparticles proved beneficial, by inducing high titer, high avidity antibody responses that correlated with protection against a lethal \textit{Yersinia pestis} challenge.\textsuperscript{14}

Amphiphilic polyanhydride nanoparticles have also been shown to stabilize recombinant
proteins, which is an important consideration in designing subunit vaccines because many recombinant proteins, including HA, are functionally and immunogenically labile. In contrast to the acidic microenvironment created by the bulk erosion of poly(esters), which may be detrimental to protein function, polyanhydride degradation products are less water soluble and consequently create less acidic and more protein-friendly microenvironments. This study examines the structural and antigenic stability of a recombinant H5 HA trimer (H5₃) upon release from several polyanhydride nanoparticle formulations with an eye towards rationally determining the optimal chemistries for development of efficacious H5-based nanovaccines.

7.2. MATERIALS AND METHODS

7.2.1. Materials

For monomer and polymer synthesis, 1,6-dibromohexane, 1-methyl-2-pyrrolidinone, hydroxybenzoic acid, N,N-dimethylacetamide, sebacic acid, and tri-ethylene glycol were purchased from Sigma Aldrich (St. Louis, MO). Acetic acid, acetic anhydride, acetone, acetonitrile, chloroform, dimethyl formamide, ethyl ether, hexane, methylene chloride, pentane, petroleum ether, potassium carbonate, sodium hydroxide, sulfuric acid, and toluene were purchased from Fisher Scientific (Fairlawn, NJ); 4-p-fluorobenzonitrile was obtained from Apollo Scientific (Cheshire, UK). Protein analysis was performed with a microBCA kit (Pierce, Rockford, IL), alkaline phosphatase conjugated goat anti-mouse IgG (H+L) (Jackson ImmunoResearch, West Grove, PA), Flamingo fluorescent gel stain, and Mini-protean TGX gels from Bio-Rad (Hercules, CA). Monoclonal Anti-Influenza Virus H5 Hemagglutinin (HA) Protein (VN04-8), A/Vietnam/1203/2004 (H5N1), (ascites, Mouse), NR-2731 was obtained through the NIH Biodefense and Emerging Infections Research Resources Repository, NIAID, NIH.
7.2.2. Protein production and purification

The HA0 ectodomain of the H5N1 influenza virus (A/Whooper Swan/244/Mongolia/05) HA gene was cloned and expressed using the Bac-to-Bac baculovirus expression system (Invitrogen, Grand Island, NY). Purification was facilitated by the addition of a HIS-tag to the C-terminus of the HA0 ectodomain, and the trimeric structure was stabilized with a GCN4pII trimerization module. Briefly, recombinant H53 baculoviruses were produced by transfecting insect SF9 cells with bacmid DNA. After three rounds of amplification, the recombinant baculovirus was titered and used to subsequently infect SF9 cells. Supernatant containing the expressed H53 was collected after 96 hours and affinity-purified utilizing nickel-agarose beads.

7.2.3. Polymer synthesis and characterization

CPH and CPTEG diacids were synthesized as previously described. Pre-polymers of CPH and SA were then synthesized followed by melt polycondensation to synthesize 20:80 CPH:SA, 20:80 CPTEG:CPH, and 50:50 CPTEG:CPH copolymers. To determine the final composition, purity, and molecular weight, the polymers were dissolved in deuterated chloroform and characterized using $^1$H nuclear magnetic resonance spectroscopy (VXR 300 MHz, Varian, Palo Alto, CA). Polymer molecular weight was also measured using gel permeation chromatography (Waters HPLC, Milford, MA). The number average molecular weight was approximately 5,100 Da for both 20:80 and 50:50 CPTEG:CPH (PDI = 1.3 and 1.5, respectively) and 17,400 Da for 20:80 CPH:SA (PDI = 1.4), and found to be consistent with previous work.

7.2.4. Nanoparticle fabrication

H53-loaded nanoparticles were synthesized by concentrating 10.5 mg H53 into approximately 100 μL of nanopure water utilizing 5000 Da MWCO Vivaspin centrifugal concentrators (Viva Products, Sartorius, Goettingen, Germany). The protein solution was added
to 20 mg/mL of polymer in methylene chloride and homogenized for 90 s. Following homogenization, the solution was poured into chilled pentane at a solvent: non-solvent ratio of 1:250 and filtered to collect nanoparticles. Blank nanoparticles (i.e., no protein) were synthesized similarly without the protein/water emulsion step. Scanning electron microscopy (FEI Quanta 250, FEI, Hillsboro, OR) was used to determine the size and morphology of the nanoparticles. Image J (Version 1.46r, NIH, Bethesda, MD) analysis determined the nanoparticles were approximately 200 nm in diameter (Table 7.1), similar to that observed in previous studies.  

### 7.2.5. H53 release from polyanhydride nanoparticles

Approximately 10-20 mg of 7% H53-loaded nanoparticles were incubated at 37°C in 350 μL of PBS buffer containing 0.01% sodium azide. Periodically, the nanoparticles were centrifuged to the bottom of the tube and PBS buffer containing released protein was collected using a pipet and an equivalent amount of fresh buffer was added back to the tubes. To determine the release kinetics, the protein in buffer was quantified at each time point using a micro-bicinchoninic acid (BCA) assay. After one month of protein release, the PBS buffer was replaced with a 25 mM sodium hydroxide solution to rapidly degrade the particles and extract the remaining protein. The total amount of H53 released in the first 30 days point plus the amount of H53 extracted was used to calculate the encapsulation efficiency as described previously.  

### 7.2.6. Structural analysis of H53 released from polyanhydride nanoparticles

Soluble H53 proteins were released from the nanoparticles for 1 h in PBS and analyzed by SDS-PAGE. Briefly, 0.19 μg of released H53 was loaded into each well of Mini-Protean TGX gels and electrophoreosed at 150 V for 60 min at 4°C. Gels were incubated in fixative (40% ethanol, 10% acetic acid) for three hours and stained overnight with Flamingo fluorescent gel
stain (BioRad). Subsequently, images of each gel were collected using a Typhoon 9400 flatbed scanner (GE Healthcare, Pittsburgh, PA).

A blue native gel and western immunoblot was used to assess the oligomeric form of H5₃ following release. Briefly, protein samples were prepared with a sample loading buffer of 2 mM EDTA, 20 mM NaCl, 20 mM Bis-Tris, 8% glycerol, and 0.08% CBG-250. A control of non-encapsulated H5₃ to show the monomer, dimer, and trimer forms of the protein was prepared in sample loading buffer containing 0.5% SDS. Next, 200 ng of protein was loaded into each well of the blue native gel and subjected to a voltage of 100 V for 3 h. After electrophoresis, the separated proteins were transferred to a PVDF membrane at 100 V for 80 min. The membrane was blocked with 5% non-fat milk in Tris buffered saline (pH 7.2) containing 0.1% Tween 20, and incubated with a rabbit polyclonal anti-H5 Vietnam 03/04 antibody (Dr. Carol Weiss, FDA) followed by HRP-conjugated donkey anti-rabbit antibody (Jackson ImmunoResearch). The immunoblot was developed with ECL substrate (Thermo Scientific, Waltham, MA) and detected with a FOTODYNE imaging system (FOTODYNE Incorporated, Hartland, WI).

To determine changes in tertiary structure, H5₃ solutions (15 μg/mL) were excited at a wavelength of 280 nm. The emission spectrum was analyzed over a range of 295-405 nm using a SpectraMax M3 fluorescent spectrometer (Molecular Devices, Sunnyvale, CA). Changes in the tertiary structure were qualitatively assessed by the relative peak fluorescence compared to a non-encapsulated H5₃ protein control.

7.2.7. H5₃ antigenicity upon release from polyanhydride nanoparticles

The antigenicity of released H5₃ was analyzed by ELISA. High binding 96-well plates were coated with 0.5 μg/mL protein in 100 μL PBS. After incubating overnight at 4°C, each well was blocked for two hours at room temperature with 300 μL of 1% bovine serum albumin in PBS supplemented with 0.05% Tween 20 (PBS-T). The plates were washed three times with PBS-T, and incubated with 100 μL/well of 1:1000 anti-H5 monoclonal antibody (NR2731, BEI
Resources) overnight at 4°C. After washing three times with PBS-T, 100 μL/well of 1:1000 alkaline phosphatase-conjugated goat anti-mouse was added for two hours at room temperature. Finally, the colorimetric reaction was developed after washing the microtiter plates three times with PBS-T followed by the addition of 100 μL of 1 mg/mL phosphatase substrate in buffer to each well. Optical density of each well was determined by measuring the absorbance at 405 nm after 45 min. The relative antigenicity was determined by dividing the optical density of the experimental sample by the optical density obtained using control H5₃ to coat the microtiter wells.

In addition, the presence of neutralizing epitopes on the released H5₃ protein was evaluated in a neutralization inhibition assay using an H5-pseudotyped lentivirus vector. Influenza lentiviral pseudotyped viruses were produced in HEK293T cells by co-transfecting 5 μg of pEV-53B which encodes a lentiviral core, 5.5 μg of the luciferase reporter plasmid pG SIN6.1Luc, and 1 μg of plasmid DNA expressing H5₃ from H5N1 influenza strain A/Whooper Swan/244/Mongolia/05 using the ProFection mammalian transfection system (Promega, Madison, WI). Pig anti-H5 antiserum was diluted 1:5,000 and incubated with 10-fold serial dilutions of H5₃ protein at 37°C for 30 min. The mixtures were then incubated with 50,000 RLU of the H5-WhooperLuc pseudovirus at 37°C for 1h, and added into 96-well plates that contained 293T cells grown for 24 h at 2 x 10⁴ cells per well. After 48 h, the levels of the luciferase in the transduced cells were evaluated using ONE-Glo luciferase assay system (Promega). Neutralization inhibition was calculated as the concentration of H5₃ that inhibited 50% pseudovirus neutralization (IC₅₀) compared to non-treated pig serum.

7.2.8. Statistical Analysis

Statistical significance using the Graph Pad Prism software (Version 6.01, Graph Pad Software, Inc., La Jolla, CA) was determined by a one sample t-test (Figure 7.4) or one-way analysis of variance (ANOVA) followed by Dunnett's post-test (Figure 7.3 & 7.5). Each sample
was compared to a protein only control and p-values less than or equal to 0.05 were considered significant.

7.3. RESULTS

7.3.1. Polyanhydride nanoparticles provide sustained release of H5$_3$

The H5N1 hemagglutinin trimer (H5$_3$) was encapsulated into three polyanhydride nanoparticle formulations: 20:80 CPH:SA, 20:80 CPTEG:CPH, and 50:50 CPTEG:CPH (Figure 7.1A-C). In this work, the water/oil/oil (w/o/o) double emulsion method was used to fabricate the protein-loaded nanoparticles. It is known that the w/o/o method of nanoparticle synthesis affects the encapsulation efficiency due to the propensity of proteins to migrate to water/organic solvent interfaces. The encapsulation efficiency was found to be chemistry-dependent with encapsulation efficiencies of approximately 17% for 20:80 CPH:SA, 13% for 20:80 CPTEG:CPH, and 29% for 50:50 CPTEG:CPH nanoparticles (Table 7.1).

The degradation products of polyanhydrides range from mildly acidic pH (4-5 for 20:80 CPH:SA), which is a pH that has been shown to stabilize hemagglutinin, to neutral pH (6.5-7 for 20:80 CPTEG:CPH and 50:50 CPTEG:CPH). These polymers also have different hydrophobicities and molecular weights both of which directly affect their erosion rates (and consequently, payload release kinetics). Protein release kinetics from these three formulations was studied for one month. As shown in Figure 7.1D, all three polyanhydride nanoparticle formulations demonstrated near-zero order release kinetics of H5$_3$ after an initial burst. The initial burst of protein release (i.e., protein release within the first hour) was relatively small, ranging from ~1% for protein released from 20:80 CPH:SA nanoparticles up to ~20% for protein released from 50:50 CPTEG:CPH nanoparticles. The H5$_3$ release rate was observed to decrease with increasing hydrophobicity and molecular weight of the polymer. The amphiphilic 50:50 CPTEG:CPH nanoparticles, which have the lowest hydrophobicity of the three
formulations tested, released H53 at the highest rate compared to the other formulations studied.32 In contrast, both 20:80 CPTEG:CPH and 20:80 CPH:SA provided slower release kinetics due to the increased hydrophobicity of the polymer. In addition, the 20:80 CPH:SA formulation, which had a molecular weight approximately three times higher than the other two polymers, displayed the slowest protein release rate with only ~4% of the protein released in one month.

7.3.2. Polyanhydride nanoparticles release structurally stable H53

The stability of structure and folding of the H53 protein released from the three polyanhydride nanoparticle formulations were examined. SDS-PAGE analysis of H53 protein released from all three nanoparticle formulations identified a dominant band at 66 kDa, the molecular size of H5 monomer, similar to the dominant band of the control indicating little degradation of the released protein (Figure 7.2A). While no smeared bands or low molecular weight proteins were observed in the gel, the decreased intensity of the bands in comparison to the control may indicate the presence of small amounts of degraded protein.

To determine the effect of encapsulation and release on the oligomeric structure of H53, released protein was electrophoresed in a blue native gel and analyzed by immunoblot. Unlike SDS-PAGE, the blue native gel allows for the observation of H53 in its trimeric, dimeric, and monomeric states. The western blot showed that the H53 trimeric form was the predominant oligomeric form of H53 following release from all the polyanhydride nanoparticle formulations (Figure 7.2B).

The stability of the tertiary structure of the protein was qualitatively determined using fluorescence spectroscopy. Emission spectra of protein solutions excited at 280 nm is dependent upon the presence of tryptophan and tyrosine residues.33, 34 Typically, these hydrophobic amino acids are buried within the protein’s tertiary structure. If the protein undergoes unfolding, these residues are exposed to a hydrophilic environment leading to a
lower fluorescence emission. In this study, the relative fluorescence intensity was determined by normalizing the relative fluorescent units (RFUs) of each sample emitted at 335 nm, which is a signature for emission by tryptophan and tyrosine residues,\textsuperscript{33,34} with the non-encapsulated protein control.

The tertiary structure of H5\textsubscript{3} was significantly perturbed upon release from both CPTEG:CPH nanoparticle formulations as indicated by the data shown in Figure 7.3. This decreased fluorescent intensity observed for the protein released from the CPTEG:CPH formulations indicates unfolding of the tertiary structure, while the tertiary structure of H5\textsubscript{3} released from the 20:80 CPH:SA nanoparticles was preserved.

7.3.3. Polyanhydride nanoparticles preserve H5\textsubscript{3} antigenicity and function

An anti-H5 monoclonal antibody (NR-2731, BEI Resources, Manassas, VA) was used to evaluate the antigenicity of the H5\textsubscript{3} released from the polyanhydride nanoparticles. As shown in Figure 7.4, H5\textsubscript{3} protein released from all three nanoparticle formulations was recognized by the monoclonal antibody and not statistically significant from a protein only control (i.e., non-encapsulated protein), indicating preservation of antigenic epitopes.

The antigenicity of the released protein was further evaluated by investigating the preservation of neutralizing epitopes in the released H5\textsubscript{3} using a neutralizing inhibition assay. When the released H5\textsubscript{3} is pre-incubated with antiserum capable of neutralizing a H5 pseudovirus, neutralizing epitopes expressed on the protein will be bound by neutralizing antibodies, resulting in decreased neutralization of the pseudovirus. The H5\textsubscript{3} released from all the nanoparticle formulations was able to inhibit the neutralizing capacity of the anti-H5 pig antiserum (Figure 7.5). These results indicate that the protein released from the polyanhydride nanoparticle formulations retained the epitopes capable of neutralizing pseudovirus.
7.4. DISCUSSION

The development of vaccines for H5N1 influenza is critical due to the ability of the H5N1 virus to resist anti-viral treatments and its potential to become a pandemic infectious agent. Subunit vaccines have been increasingly utilized due to their favorable safety profile. Subunit proteins, however, are typically weak immunogens. This is often problematic as the generation of recombinant protein is expensive to produce and requires adjuvants to increase the titer of protective (i.e. neutralizing) serum antibodies. This work is focused on testing the stability of HA protein following release from the multi-functional adjuvant platform of polyanhydride nanoparticles (with varying hydrophobicities, molecular weight, and pH of degradation products) to help evaluate their use for vaccine delivery.

The data presented herein demonstrate the ability of polyanhydride nanoparticles to encapsulate and release structurally and antigenically stable H5. Polymer chemistry was found to influence the encapsulation efficiency of H5 as shown in Table 7.1. The 50:50 CPTEG:CPH formulation had the highest encapsulation efficiency (29%) suggesting that the glycosylated H5 may be more compatible with amphiphilic polymers. In contrast, the 20:80 CPTEG:CPH formulation had the lowest encapsulation efficiency, likely due to its increased hydrophobicity. The hydrophobicity of the polymer, and, therefore, its rate of erosion, plays a major role in the release kinetics of H5 (Figure 7.1). For example, encapsulating H5 within amphiphilic 50:50 CPTEG:CPH nanoparticles resulted in the most rapid H5 release rate among the three formulations tested. While hydrophobicity is an important factor to consider in tailoring the release kinetics of H5, polymer molecular weight also influences the release kinetics. The release kinetics of H5 from 20:80 CPH:SA nanoparticles was the slowest, likely because of the higher molecular weight of this polymer (~three times greater than that of the other two polymers studied). The initial burst (i.e., within the first hour) of released protein was found to be relatively small (ranging between 1 - 20%) for all three formulations. This is likely due to the low...
encapsulation efficiency of H5 into the nanoparticles (Table 7.1), and is indicative of a uniform distribution of protein within the polymer. However, the differences between the initial burst release among the various chemistries can be attributed to variations in actual loading of H5 as well as the stability of H5 within each chemistry (discussed below).

The primary structure of H5 was largely preserved when released as observed by SDS-PAGE, although differences in band intensity may indicate slight degradation (Figure 7.2A). Likewise, as shown in Figure 7.2B, the trimeric state of H5 was also preserved upon encapsulation and release. This preserved structure is likely to enhance the induction of neutralizing antibodies following immunization with the H5, as studies have suggested that H5 monomer is not as immunogenic as the native trimeric structure.

Fluorescence spectroscopy demonstrated unfolding of H5 tertiary structure when released from CPTEG:CPH formulations (Figure 7.3). The decreased fluorescent intensity can be attributed to a combination of completely unfolded protein and/or partially unfolded protein molecules. It is known that the degradation products of CPH:SA formulations are slightly more acidic (pH ~4-5) when compared to their CPTEG:CPH counterparts (pH ~6.5). It is possible that the pH microenvironment may have contributed to the observed changes in the conformational structure of H5.

Although there was no statistical significance of the relative antigenicity of the protein released from the different nanoparticle formulations (Figure 7.4), the small decrease in antigenicity of H5 released from 20:80 CPTEG:CPH nanoparticles may be attributed to the unfolding of tertiary structure observed in Figure 7.3. The anti-H5 antibody (NR-2731) used to determine antigenicity is specific for H5 as determined by hemagglutinin inhibition, suggesting that the antibody binds to conformational epitopes, and maintenance of conformational epitopes of H5, or folding, may be important. Similarly, released H5 from all three nanoparticle formulations was found to inhibit neutralizing serum as well as the control (Figure 7.5). However, it is important to note that while the H5 preparations recovered from the three
nanoparticle formulations were each capable of blocking the neutralizing activity of anti-H5 antiserum, it required approximately four to five times more H5\textsubscript{3} released from the CPTEG:CPH formulations to achieve the same level of inhibition as the H5\textsubscript{3} released from 20:80 CPH:SA nanoparticles. As the conformational epitopes presented by H5 are known to be most important for neutralization,\textsuperscript{31, 36} the loss of tertiary structure exhibited by the H5\textsubscript{3} released from the CPTEG:CPH formulations (Figure 7.3) is consistent with the lesser ability to block the neutralizing antiserum.

The ability of polyanhydride nanoparticles to release stable and antigenic antigen has many advantageous implications for vaccine design. The sustained release of antigen from polyanhydride nanoparticles has been shown to prolong the presence of antigen leading to long-lived high titer, high avidity antibody responses.\textsuperscript{14} In addition, maintaining the structural stability, and therefore a broad spectrum of protective epitopes, may also be beneficial in influenza where antigenic drift is common.\textsuperscript{1} It is also important to note that dry powder formulations with nanoparticles can be easy to administer with needle-free technologies (e.g., intranasal administration), allowing for rapid deployment in pandemic scenarios.\textsuperscript{37} Furthermore, encapsulating antigen into dry powder polyanhydride formulations has been shown to maintain antigen stability and activity even when stored for long periods of time at room temperature.\textsuperscript{38} The ability to maintain stockpiles of easy-to-use, broadly protective vaccines would be highly valuable in preparation for possible influenza pandemics.

7.5. CONCLUSIONS

The studies described herein demonstrated that polyanhydride nanoparticles provided sustained release of stable H5\textsubscript{3}. Protein released from CPH:SA nanoparticles maintained both structural and antigenic stability. Despite changes in the tertiary structure of H5\textsubscript{3} released from CPTEG:CPH nanoparticles, the antigenicity of protein released from all the nanoparticle
formulations was preserved. Together, these studies demonstrated that there was release of stable H5\textsubscript{3} from polyanhydride nanoparticles and that this platform is a potentially useful delivery vehicle for efficacious HA subunit nanovaccines against influenza viruses.

**ACKNOWLEDGMENTS**

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# 7.6. TABLES

## Table 7.1. Properties of Polyanhydride Nanoparticle Formulations

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Molecular Weight (g * mol(^{-1}))</th>
<th>Particle Diameter (nm)</th>
<th>Encapsulation Efficiency</th>
<th>Actual H5(_3) Loading</th>
</tr>
</thead>
<tbody>
<tr>
<td>20:80 CPH:SA</td>
<td>17,472</td>
<td>220 ± 60</td>
<td>17.1 ± 0.9%</td>
<td>1.2 ± 0.06%</td>
</tr>
<tr>
<td>20:80 CPTEG:CPH</td>
<td>5,117</td>
<td>195 ± 59</td>
<td>13.1 ± 0.3%</td>
<td>0.9 ± 0.02%</td>
</tr>
<tr>
<td>50:50 CPTEG:CPH</td>
<td>5,153</td>
<td>192 ± 54</td>
<td>28.7 ± 8.1%</td>
<td>2.0 ± 0.57%</td>
</tr>
</tbody>
</table>
Figure 7.1. Encapsulation and release of H5₃ from polyanhydride nanoparticles. Scanning electron photomicrographs showing H5₃-loaded 20:80 CPH:SA (A), 20:80 CPTEG:CPH (B), and 50:50 CPTEG:CPH (C) nanoparticles. Scale bar represents 1 μm. Release kinetics of H5₃ from the three polyanhydride nanoparticle formulations over 28 days (D). Error bars represent standard error of mean. Three replicates of each formulation were analyzed.
Figure 7.2. Integrity of the primary structure and oligomeric forms of H53 released from polyanhydride nanoparticles. SDS-PAGE analysis of H53 released from nanoparticles (A). Lanes represent molecular weight ladder (1), H53 control (2), H53 released from 20:80 CPH:SA (3), 20:80 CPTEG:CPH (4), and 50:50 CPTEG:CPH nanoparticles (5). Western blot of released H53 separated by blue native gel electrophoresis (B). Lanes represent non-encapsulated H53 control in 0.5% SDS (1), H53 released from 20:80 CPH:SA (2), 20:80 CPTEG:CPH (3), and 50:50 CPTEG:CPH nanoparticles (4).
Figure 7.3. Integrity of the tertiary structure of H5₃ released from polyanhydride nanoparticles. Tertiary structure of H5₃ released from various polyanhydride nanoparticle formulations was examined by fluorescent spectroscopy. The fluorescent intensity of the spectral peak (335 nm) was normalized to a H5₃ control. Three replicates of each formulation were analyzed. Error bars represent standard error of mean. * indicates statistical significance from the protein only control with p ≤ 0.0167.
Figure 7.4. Relative antigenicity of H5$_3$ released from polyanhydride nanoparticles. An ELISA was used to determine the antigenicity of H5$_3$ released from various polyanhydride nanoparticle formulations. Relative antigenicity was calculated by dividing the optical density (OD) of samples by the OD obtained using a control H5$_3$ as the antigen. Three replicates of released H5$_3$ were evaluated for each formulation. Error bars represent standard error of mean. No statistical significance was found in comparing each treatment group to the protein only control ($p \leq 0.4656$).
Figure 7.5. Neutralizing inhibition of H5₃ released from polyanhydride nanoparticles. A neutralization inhibition assay was used to evaluate expression of neutralizing epitopes on H5₃ released from various polyanhydride nanoparticle formulations. The IC₅₀ was calculated as the concentration of H5₃ that inhibited 50% of neutralizing activity of convalescent pig sera. Three replicates of each formulation were analyzed. Error bars represent standard error of mean. No statistical significance was found comparing each treatment group to the protein only control (p ≤ 0.4907).
7.8. REFERENCES


CHAPTER 8:  
Polyanhydride-based H5 Hemagglutinin Influenza Nanovaccines Elicit  
Protective Virus Neutralizing Titers and Cell-mediated Immunity

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Abstract

H5N1 avian influenza is a significant concern throughout the globe, having the potential to become the next pandemic threat. Recombinant subunit vaccines are an attractive alternative for pandemic vaccines compared to traditional technologies. Additionally, polyanhydride nanoparticles encapsulating subunit proteins have been shown to enhance humoral and cell-mediated immunity. In this work, a H5 hemagglutinin trimer (H5₃) was expressed and encapsulated into polyanhydride nanovaccines. The studies performed indicate that the expressed H5₃ antigen is a robust immunogen. Encapsulation into polyanhydride nanoparticles also induced high neutralizing antibody titers as well as enhanced CD4⁺ T cell memory responses. Finally, the H5₃-based nanovaccines were protective against a low pathogenic, H5N1 viral challenge.

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8.1. INTRODUCTION

Influenza virus is a major cause of serious respiratory illness and has been responsible for significant morbidity and mortality in humans worldwide. The major strains of influenza A virus circulating in human populations are H3N2 and H1N1, which are associated with seasonal flu epidemics. In addition, avian strains H5N1 and H7N9 have been found to infect humans,\(^1\) however, they do not appear capable of sustained human-to-human spread. Should these highly pathogenic avian viruses develop such capability, the disease could rapidly spread resulting in a global influenza pandemic. Vaccination represents a critical control measure against yearly seasonal influenza viruses and is an essential component of pandemic preparedness plans.\(^2\) Nearly all of the current influenza vaccine technologies are based on the use of embryonated eggs and require a relatively long production cycle and limited manufacturing capacity.\(^2\) The response to the 2009 H1N1 pandemic clearly demonstrated the limitations of these methods of vaccine production with respect to rapid deployment in response to an emerging influenza pandemic, which can lead to vaccine shortages. Consequently, there is great interest in developing new technologies for rapid, large scale, and safe and efficacious influenza vaccine production.

Recombinant hemagglutinin (rHA)-based vaccines produced in mammalian or insect cell culture systems are considered attractive alternatives to egg-based vaccine technologies.\(^3\) Production and purification of rHA protein can effectively limit the production time, and mammalian and insect cell culture systems will glycosylate proteins important for neutralizing epitope generation.\(^4\) Recent studies demonstrated that rHA engineered to form a stable trimeric configuration elicited a protective immune response in vaccinated animals.\(^5\)\(^-\)\(^10\) Neutralizing antibody levels and protection from disease were enhanced in mice vaccinated with soluble rHA trimers as compared to animals vaccinated with rHA monomers,\(^5\)\(^,\)\(^8\) indicating the importance of immunogens that mimic a natural infection. Along those lines, the composition and/or number of N-linked glycans on sHA trimers has been shown to modify the level of the protective antibody
These data suggest that recombinant soluble HA trimers could be an important component of a subunit vaccine strategy against influenza.

HA-specific serum antibody titers correlate with protection from disease; however, recombinant proteins are often weak immunogens and often require multiple immunizations with high doses to achieve protection. Polyanhydride nanoparticles have been shown to be a versatile vaccine adjuvant/delivery platform capable of enhancing the immune response to recombinant proteins. Polyanhydrides are a class of surface erodible, biodegradable materials that provide sustained release kinetics of encapsulated antigen, resulting in long lived, high avidity antibody titers even with suboptimal doses of antigen. Aside from amplifying humoral immunity, polyanhydride nanovaccines have also been known to be immunomodulatory and are capable of promoting cell-mediated immunity. Previous work with polyanhydride nanovaccines has observed higher frequencies of both antigen-specific CD8$^+$ and CD4$^+$ T cells following nanovaccine immunization, resulting in greater memory T cell populations that responded to challenge (CD8$^+$ T cells) and increased germinal center B cells (CD4$^+$ T cells). This broad repertoire of immune responses produced by polyanhydride nanovaccines may provide a large advantage in influenza vaccines as robust cell-mediated responses are often associated with broader protective immunity and directed at conserved epitopes.

In this study, soluble H5 HA trimers (sH5$_3$) from H5N1 influenza virus A/Whooper Swan/244/Mongolia/05 were produced using a baculovirus insect cell expression system. After characterizing the oligomeric structure of the protein and examining its immunogenicity in vivo, the protein was subsequently encapsulated into polyanhydride nanoparticles. The virus-neutralizing antibody titer in response to nanoparticle immunization was observed for approximately two months and concluded with an analysis of the memory T cell responses generated. Finally, the efficacy of lead nanoparticle formulations was examined using a low-pathogenic, live viral challenge. The data demonstrate that polyanhydride nanoparticles
encapsulating an immunogenic hemagglutinin trimer represent a potentially viable platform for pandemic influenza vaccines.

8.2. MATERIALS AND METHODS

8.2.1. Plasmids and antibody

Plasmid pHW500 (Genbank DQ659326), obtained from Dr. Bruce Janke of Iowa State University, contains the full length HA gene from HPAI H5N1 influenza virus A/whooper swan/244/Mongolia/05 (H5N1). The pHW500 HA gene was modified by replacement of the cognate polybasic cleavage site with that from a low pathogenic H6N1 avian influenza virus. The FDA-VN plasmid, obtained from Dr. Carol Weiss of U.S. FDA, contains the full length HA from A/Vietnam/1203/2004 (H5N1) (Genbank EF541403) in pCMV/R. The pWS-HA was constructed by replacing the H5 Vietnam HA gene in FDA-VN with the HA gene from pHW500. The low pathogenic cleavage site in pWS-HA was replaced with the polybasic cleavage site from A/whooper swan/3/Mongolia/05 (H5N1) (Genbank AB233320.1). The equine infectious anemia lentivirus vector plasmids pEV53B and pSIN6.1ClucW were obtained from John Olsen. Hyperimmune swine sera containing high-titers of H5 neutralizing antibody was obtained from Dr. Bruce Janke. H5-specific polyclonal rabbit antisera was obtained from Dr. Carol Weiss.

8.2.2. Cloning and expression of soluble H5 trimer (sH5₃)

The HA ectodomain (nucleotides 1-1723) was amplified from pHW500 and modified at the 3’ end by addition of linker sequences, a GCN4pII trimerization domain and His-tag sequences at the 3’ end (Figure 8.1A). The modified gene was cloned into pFastBac I, transformed into DH10Bac cells, and recombinant baculoviruses were generated using Bac-to Bac Baculovirus Expression System (Invitrogen, Grand Island, NY). Sf9 cells (Invitrogen) were infected with recombinant baculovirus and supernatants collected 96 h after infection were
clarified by centrifugation, dialyzed against 10 mM Tris buffer (10 mM Tris, 50 mM NaCl pH 8.0), and incubated with Ni-NTA beads (Thermo Scientific, Waltham, MA) overnight at 4°C. The beads were washed with 10 mM Tris buffer containing 10 mM imidazole and recombinant sH5 was eluted in 10 mM Tris buffer containing 250 mM imidazole. The eluted proteins were dialyzed against 10 mM Tris buffer to remove imidazole, concentrated and stored at 4°C for up to a month or at -80°C for long term storage.

8.2.3. HA-pseudotyped reporter virus

HA pseudotyped viruses were produced in HEK293T cells as previously described.23,24 Briefly, HEK293T cells were co-transfected with 5 μg of pEV-53B encoding the lentiviral core proteins of equine infectious anemia virus, 5.5 μg of the luciferase reporter plasmid plgSIN6.1Luc and 1 μg of pFDA-VN or pWS-HA plasmid DNA expressing HA from A/Vietnam/1203/2004 (clade1) or A/Whooper Swan/244/Mongolia/05 (clade 2.2), respectively. At 18 h post-transfection, cells were incubated with fresh medium containing 7 mU/mL of Vibrio cholera Type II neuraminidase (Sigma) and 1M NaB to induce the release of H5-pseudovirions from the surface of the producer cells. Supernatants were collected 48 h post-transfection, clarified by centrifugation, and stored at −80°C.

Reporter virus pseudotyped with HA from A/Vietnam/1203/2004 (H5-VN-Luc) or A/Whooper Swan/244/Mongolia/05 (H5-WS-Luc) were titered on HEK293T cells. Cells were seeded in 96-well plates at 2 x 10^4 cells/well in Dulbecco’s modified Eagle medium supplemented with antibiotics and heat-inactivated fetal bovine serum. The following day, cells were inoculated in triplicate with 10-fold serial dilutions of pseudovirus stock in the presence of 8 μg/mL polybrene. At 48 h post-transduction, cells were lysed and assayed for luciferase activity using the ONE-Glo Luciferase assay system (Promega). Luciferase activity was quantified using a Centro XS3 LB960 illuminometer (Berthold Technologies) and results reported as relative light units (RLU)/mL supernatant.
8.2.4. Electrophoresis and Immunoblot

For protein analyses in denaturing conditions, 1 μg of purified sH5₃ protein was boiled for 5 min in SDS loading buffer in (50mM Tris, 1% β-mecaptoethanol, 2% SDS, 0.005% bromophenol blue, and 10% glycerol) and electrophoresed in 10% SDS-PAGE. For analyses in non-denaturing gels, 1 μg of purified sH5₃ protein was mixed with Blue native loading buffer (2 mM EDTA, 20 mM NaCl, 20 mM Bis-Tris, 10% glycerol, 0.08% coomassie blue G-250) and separated on 10% Blue native PAGE gel containing Bis-Tris, glycerol and acrylamide in Bis-Tris buffer in the outer chamber and Tricine, Bis-Tris with Coomassie blue G250 in the inner chamber. Following electrophoresis, gels were stained with coomassie blue and imaged with a GelDoc XR+ imaging system (BioRad).

For immunoblot, 200 ng of protein was electrophoresed in SDS-PAGE and Blue native gels as described above. Proteins were transferred to PVDF membrane (GE Healthcare), blocked overnight in blocking buffer (5% Nonfat dried milk in Tris-buffered saline with 0.1% Tween 20) and incubated with 1:2000 of anti-H5 Vietnam 03/04. Blots were washed 3X, and incubated with 1:5000 dilution of HRP-conjugated donkey anti-rabbit antibody (Jackson ImmunoResearch). Blots were developed with ECL substrate (Thermo Scientific) and bands were visualized by FOTODYNE imaging system (Fotodyne Inc.).

8.2.5. Polymer and nanoparticle synthesis

Diacids based on 1,8-bis(p-carboxyphenoxy)-3,6-dioxaoctane (CPTEG) and 1,6-bis(p-carboxyphenoxy)hexane (CPH) diacids were synthesized as previously described²⁵,²⁶ using: 1,6-dibromohexane, 1-methyl-2-pyrrolidinone, hydroxybenzoic acid, N,N-dimethylacetamide, and tri-ethylene glycol (Sigma, Aldrich, St. Louis, MO); acetic acid, acetone, acetonitrile, dimethyl formamide, potassium carbonate, sulfuric acid, and toluene (Fisher Scientific, Fairlawn, NJ); 4-p-fluorobenzonitrile (Apollo Scientific, Cheshire, UK). Following diacid synthesis, 20:80 CPTEG:CPH copolymer was synthesized by melt polycondensation.²⁶ The final composition of
the polymer (23:77 CPTEG:CPH), molecular weight (6055 Da), and purity were determined with
$^1$H nuclear magnetic resonance spectroscopy (VXR 300 MHz, Varian, Palo Alto, CA). H5$_3$-
loaded nanoparticles were synthesized using a water-oil-oil (w/o/o) double emulsion process$^{27}$. First, 4.5 mg of sH5$_3$ was concentrated into 100 μL of nanopure water. The solution of sH5$_3$ was
then added to 45 mg of 20:80 CPTEG:CPH dissolved in 2.25 mL of methylene chloride and
homogenized for 90 s. The H5$_3$-loaded nanoparticles were precipitated by pouring into 562.5
mL of chilled pentane and collected via vacuum filtration. Blank nanoparticles, 1% poly I:C
(InvivoGen, San Diego, CA)-loaded nanoparticles, and 1% poly dA:dT (InvivoGen)-loaded
nanoparticles were synthesized similarly without water. Scanning electron microscopy (FEI
Quanta 250, FEI, Hillsboro, OR) was used to characterize the size and morphology of the
nanoparticles, which were found to be consistent with previous work.$^{13}$ The encapsulation
efficiency of protein was determined by degrading 10 mg of nanoparticles in 250 μL of 40 mM
sodium hydroxide and quantifying the total protein released using a microBCA protein kit
(Pierce, Rockford, IL).$^{13}$

8.2.6. Mice

Female BALB/c mice were purchased from Harlan Laboratories (Indianapolis, IN). All
mice were housed under specific pathogen-free conditions where all bedding, caging, water,
and feed were sterilized prior to use. Animal procedures were conducted with the approval of
the Iowa State University and University of Nebraska Medical Center Institutional Animal Care
and Use Committees.

8.2.7. Immunizations

Mice were administered 10 μg of sH5$_3$ alone or in conjunction with 10 μg of
monophosphoryl lipid A (MPLA) from Salmonella enterica (Sigma) as outlined in Table 8.1. In
studies testing the efficacy of sH5$_3$-loaded nanoparticles, blank nanoparticles were used to bring
the total amount of nanoparticles to 300 μg in all the groups tested (Table 8.1). All nanoparticle formulations were suspended in 250 μL (subcutaneous immunization) or 50 μL (intranasal immunization) sterile saline. Subcutaneous (SC) immunizations were administered at the nape of the neck; intranasal (IN) immunizations were carried out using droplet admission via pipettor after ketamine/xylazine chemical anesthetic. Boost immunizations were prepared and administered the same way as primary immunizations. Serum samples were obtained at the time points indicated via saphenous vein bleeding.

8.2.8. Neutralization Assay

Neutralizing antibody assays were carried out using HA-pseudotyped reporter virus as described previously.23,24 HEK293T cells were seeded in 96-well plate at 2 x 10^4 cells/well and grown for 24 h. Sera samples were serially diluted threefold in culture medium containing 8 μg/mL of polybrene (Sigma), and mixed with an equal volume of diluted pseudoviruses containing 5 x 10^4 RLU/mL. After incubation at 37°C for 1 h, virus and serum mixtures were added to the cells. Infectivity was evaluated 48 h after transduction using One-glo Luciferase assay system (Promega). The percent neutralization was calculated as (1-[virus+sera RLU/virus only RLU]) x100. The percent neutralizations for each sera dilution were plotted and neutralizing titers reported as ID₅₀, calculated as the reciprocal of the serum dilution that neutralized 50% of the virus.

A neutralization inhibition assay was used to evaluate the immunogenic properties of sH5₃ protein. Ten-fold serial dilutions of sH5 trimer or monomer were incubated 37°C for 30 min with a 1:5,000 dilution of pig H5-antiserum, which neutralizes 90% of 50,000 RLU of H5-WS-Luc (Dr. Bruce Janke, Iowa State University). Following pre-incubation with H5 protein, 50,000 RLU of H5-WS-Luc were added and the samples incubated an additional 1 h at 37°C and inoculated onto HEK293T cells in 96 well plates. At 48 h after transduction, the levels of the luciferase in the transfected cells were evaluated using One-glo Luciferase assay system (Promega) and
percent neutralization was calculated as described above. The percent neutralization at each protein concentration was plotted and the IC$_{50}$ was calculated as the concentration of soluble H5 protein that inhibited 50% of the neutralizing activity of the diluted pig sera.

8.2.9. **Flow cytometry for T cell memory populations**

Brachial and axillary draining lymph nodes were harvested 63 days post-immunization and homogenized into single cell suspensions in complete tissue culture medium. Single cell populations were labeled with 2.5 µM 5-(and-6)-carboxyfluorescein diacetate, succinimidyl ester (5(6)-CFDA, SE) (CFSE) (Life Technologies, Grand Island, NY). Cells ($2.5 \times 10^5$) were incubated in 96 well U-bottom plates with 0.5 µg of sH5$_3$ antigen for 96 h. Cells were aspirated and quantified using flow cytometry (BD FACScanto). Cell suspensions were blocked for non-specific antibody binding using 0.1 mg/mL Rat IgG (Sigma Aldrich, St. Louis, MO) and mouse anti-CD16/32 (eBioscience, San Diego, CA) 10 µg/mL. Fluorescently conjugated antibodies for CD4 and CD8 (eBioscience) were used to stain in FACS buffer, gate, and quantify specific cell populations.

8.2.10. **Low-pathogenic viral challenge and clinical evaluation**

The efficacy of sH5$_3$-based nanoparticle vaccines were evaluated in mice challenged at 63 days post-immunization with the low pathogenicity influenza virus A/H5N1 VNH5N1-PR8CDC-RG, obtained from the Centers for Disease Control and Prevention (Atlanta, GA). This is a PR-8-based reassortant virus that contains the HA and NA genes of H5N1 A/VietNam/1203/04, a clade 1 virus. Mice were anesthetized with 20 mg/ml of xylazine and 100 mg/ml of ketamine (1:4 ratio) and inoculated intranasally with $2.7 \times 10^3$ TCID$_{50}$ virus in 30 µL PBS. Three days after challenge, half of the mice were euthanized with 600 µL of 20 mg/mL xylazine and 100 mg/mL ketamine delivered via intraperitoneal injection. Bronchoalveolar lavage (BAL) fluid was collected as described previously$^{28}$ and lung tissue was collected for
inflammatory cytokine and viral load quantitation (described below). The remaining mice were monitored for weight loss for two weeks post-challenge before being removed from study.

8.2.11. Virus load quantitation

Following the procedure of Alsharifi and co-workers, lung tissue was preserved in 3 mL of RNA\textit{later} Stabilizing Reagent (Qiagen).\textsuperscript{29} Tissue was held submerged in the RNA\textit{later} for three days at 4°C. Tissues were then removed from the RNA\textit{later}, weighed and cut into 30 mg pieces and individually frozen at -80°C in 1.5 mL microcentrifuge tubes. For the extraction process (total RNA), individual 30 mg tissue pieces were homogenized in 200 μL Buffer RLT (Qiagen RNEasy Mini Kit) using a disposable pellet pestle (Fisher Scientific) in conjunction with a cordless motor (Fisher Scientific). An additional 400 μL of RLT buffer was added to each tube after homogenization was completed. Tissue was extracted into 60 μL final volume in sterile, RNAse-free H₂O (Qiagen) and frozen at -80°C until PCR was performed. Samples containing the extracted RNA were thawed, mixed well, and the total RNA concentration was determined, in duplicate measurements, using the Nanodrop method for RNA content. Total RNA concentration for each sample was adjusted to 40 μg/μL and 5 μL was used as the template for the PCR reaction. PCR was performed on an Applied Biosystems 7500 Fast Real-Time PCR System, on the standard mode, using the AgPath-ID One-Step RT-PCR Reagents (Life Technologies, Grand Island, NY) in conjunction with the Fast-Track Diagnostics FTD-21-96/12 Kit (Junglinster, Luxembourg) which contains Brome Mosaic Virus (BMV) internal PCR extraction control, positive control and primer/probes for universal influenza A antigen. For the standard curve, normal, non-influenza challenged mice lungs (naive controls) were homogenized using the procedure outlined above. RNA from stock influenza A H5 virus was extracted using the Qiagen QiAmp Viral RNA Kit Mini Kit (Qiagen). Extracted RNA was quantified using the Nanodrop procedure. For the standard curve, ten-fold dilutions of the H5
extracted viral RNA were mixed with extracted RNA from the normal mouse lungs that had been standardized to 40 ng/μL. Standard curves were obtained with each set of PCR reactions.

8.2.12. Cytokine quantitation

BAL fluid samples collected three days post-viral challenge were analyzed for the inflammatory cytokines IL-6, IP-10, MIG, G-CSF, IFN-γ, MCP-1, KC, and MIP-2 using a MILLIPLEX® MAP assay kit (EMD Millipore Corporation, Billerica, MA). The assay was performed according to manufacturer instructions. Briefly, 25 μL of BAL fluid, 25 μL assay buffer, and 25 μL MILLIPLEX® MAP beads were added to each well of a 96-well plate. After shaking overnight at 4°C, the plate was washed and incubated with 25μL/well detection antibody for 1 h at room temperature. Following, 25μL/well streptavidin-phycoerythrin was added for an additional 30 min. The plate was washed once more before measuring fluorescence intensity on a Bio-Plex 200 system (Bio-Rad).

8.2.13. Statistics

Statistical significance among formulations (p ≤ 0.05) was determined by a one-way analysis of variance followed by Tukey’s post test using Graph Pad Prism (Version 6.01, Graph Pad Software, Inc., La Jolla, CA).

8.3. RESULTS

8.3.1. Characterization of soluble H5 trimer protein (sH5₃)

A recombinant baculovirus containing a GCN-modified ectodomain of HA from A/Whooper Swan/244/Mongolia/05 (Figure 8.1A) was expressed in Sf21 insect cells and the secreted soluble H5 trimer (sH5₃) protein was purified from culture supernatant by affinity chromatography using nickel-agarose beads. Analyses of the purified protein by electrophoresis in SDS-PAGE blue native gels indicated the predominant form of sH5₃ protein was trimeric
A neutralization inhibition assay was used to determine whether the sH5\textsubscript{3} retained neutralizing epitopes of native virus (Figure 8.1C). In this assay, convalescent pig sera containing high titers of H5-neutralizing antibody was incubated with serial dilutions of sH5\textsubscript{3} or sH5 monomer and tested for neutralizing activity against H5-WS-Luc pseudotyped reporter virus. Both the monomeric and trimeric forms of sH5 inhibited neutralizing antibody at fmol concentrations; however, the inhibitory activity of the sH5\textsubscript{3} trimer was about 2.5 fold higher than the monomer.

8.3.2. Immunogenicity of soluble recombinant H5 HA trimer

BALB/c mice (6-8 weeks) were immunized either subcutaneously or intranasally with 10 µg of soluble protein at 0, 21 and 42 days and sera collected at 21, 42, and 63 days post-immunization were tested for neutralizing antibody against the homologous H5-WS-Luc pseudovirus. No neutralizing antibody was detected at day 21 following primary immunization (Figure 8.2A). By day 42, the majority of mice in both immunization groups had detectable neutralizing antibody to the homologous challenge virus. However, the response was highly variable among individual mice, with ID\textsubscript{50} titers ranging from undetectable to greater than 1,000. Neutralization titers significantly increased following a second boost, with mean neutralization antibody titers of greater than 10,000. In addition, there was less variability in in the neutralizing antibody titers among individual mice, especially those immunized subcutaneously.

As highly pathogenic H5N1 has continued to spread in avian and mammalian hosts, different lineages have emerged that are now classified into distinct, but phylogenetically related clades.\textsuperscript{1} To examine the cross-clade breadth of the neutralizing antibody response elicited with A/Whooper Swan/244/Mongolia/2005-based sH5\textsubscript{3} (Clade 2.2), day 63 sera was tested for neutralizing activity against H5-VN-Luc, a luciferase reporter virus pseudotyped with HA from the A/Vietnam/1203/2004 HA, an H5 clade 1 strain. Although the cross-clade neutralization titers were 2-10 fold lower than titers to homologous virus, all mice were able to neutralize
Vietnam/1203 pseudotyped virus, with mean neutralizing antibody titers of $10^3$ (Figure 8.2B). Together, these data indicate that non-adjuvanted sH5₃ antigen is able to elicit high titers of H5 cross-clade neutralizing antibody when administered using a prime-boost-boost regimen. However, consistent with previous reports,⁶,⁷,¹² multiple immunizations of sH5₃ recombinant protein were required to generate consistently high neutralizing antibody titers.

### 8.3.3. Neutralizing antibody response in mice vaccinated with H5₃-based nanovaccines

Our previous studies indicate that polyanhydride nanovaccines can enhance the immune response to recombinant proteins.¹³⁻¹⁵ Therefore, we examined the immune response of sH5₃ delivered with and/or encapsulated into polyanhydride nanoparticles (Table 8.1). Mice were immunized subcutaneously with either a single dose regimen or a prime/boost regimen of three immunizations 21 days apart. Serum was collected via saphenous vein at 42 and 63 days post-immunization and tested for neutralizing antibody titers against H5-WS-Luc. As we observed above, sH5₃ alone elicited low neutralizing titers in the absence of booster immunizations with most mice having ID₅₀ titers of less than 100 through 63 days post-immunization (Figure 8.3). Higher titers were observed using a single dose immunization regimen with sH5₃ in the presence of adjuvant, with MPLA eliciting higher neutralizing antibody titers than any of the nanovaccine formulations or sH5₃ alone. In the absence of booster immunizations, the neutralizing antibody response in groups vaccinated with nanoparticle formulations was lower than the MPLA adjuvant group at 42 days with the exception of the formulation including poly I:C; however, there were a greater number of nanoparticle vaccinated mice with detectable neutralizing antibodies than in mice vaccinated with sH5₃ alone.

The prime/boost vaccination regimen resulted in enhanced neutralizing antibody titers in all vaccine formulations, with most groups showing a 10-20 fold increase in mean neutralizing antibody titer over groups receiving a single booster immunization (Figure 8.3). In almost all
groups, the highest and most consistent titers were observed after receiving three immunizations, with little difference observed among the different formulations.

8.3.4. Cell-mediated immune response

Cell-mediated immune responses are often associated with broader protective immunity and can play an important role in protection against antigenically diverse strains of influenza.\textsuperscript{19,20} Therefore, we examined the proliferative T cell populations 63 days post-immunization in mice receiving the prime/boost/boost regimen (Figure 8.4). Draining lymph nodes were removed and homogenized to single cell suspensions and labeled with CFSE to observe proliferating cell populations. After 96 h of \textit{ex vivo} stimulation with sH5\textsubscript{3}, CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells were quantified via flow cytometry. A significant expansion of CD4\textsuperscript{+} T cells, but not CD8\textsuperscript{+} T cells, was observed in all groups of vaccinated mice as compared to mice receiving saline alone as shown in Figure 8.4. In addition, the inclusion of poly I:C within the nanovaccine formulation resulted in significantly higher numbers of both CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells as compared to other vaccinated groups. These data suggest that inclusion of nanoparticles encapsulating poly I:C to induce an appropriate innate immune response may enhance antigen-specific adaptive immune responses.

8.3.5. H5\textsubscript{3} vaccination protects against live viral challenge

To examine the efficacy of sH5\textsubscript{3}-loaded nanoparticles in protection from clinical disease, mice were vaccinated with various nanovaccine formulations (Table 8.1) using a prime/boost regimen and challenged at 63 days post-immunization with the low pathogenic VNHN5N1-PR8/CDC-RG, a reverse genetics-derived influenza virus containing the HA and neuraminidase (NA) genes of A/Vietnam/1203/04 (H5N1) virus in the genetic background of the high-growth master strain A/Puerto Rico/8/34 (H1N1). Pre-challenge antibody response to the various vaccine regimens was evaluated in serum collected one week before challenge. All vaccinated
groups had high levels of total anti-H$_5$ IgG, with no significant difference among the groups (data not shown). Similar to our earlier studies, all vaccinated mice had detectable neutralizing antibody titers of greater than 100 to the clade 2.2 H5-WS-Luc pseudotyped reporter virus, with mean titers among the group ranging between 3000-4000 (Figure 8.5).

The body weight of each mouse was observed over the course of 14 days post-challenge. All of the vaccine formulations protected mice from challenge with body weight increasing post-challenge similar to naïve mice that were not challenged (Figure 8.6A). In contrast, mice receiving saline immunizations began to lose a significant portion of body weight approximately five days post-challenge. Saline-administered mice continued to lose approximately 20% of their total by eight days post-challenge before recovering. Based on clinical response, as measured by weight loss following virus challenge, all vaccine regimens resulted in a protective immune response and there was no significant difference among the various vaccine regimens.

At three days post-challenge, half of the mice in each group were sacrificed and lung homogenates were assayed for virus load using quantitative RT-PCR. All vaccinated mice showed significant reduction in virus load upon challenge as compared to the virus load in the saline-administered mice (Figure 8.6B). There was wide mouse-to-mouse variability in virus load observed in all vaccine regimens, with no significant differences among the groups in mean virus loads.

Finally, a multiplex assay was used to quantitate the concentrations of inflammatory cytokines (IL-6, IP-10, G-CSF, IFN-γ, MCP-1, KC, and MIP-2) present in BAL fluid. Consistent with virus load, vaccinated mice produced very little inflammatory cytokines similar to the naïve control (Figure 8.6C). Mice receiving saline had significantly greater concentrations of inflammatory cytokines with the exception of IFN-γ (data not shown).
8.4. DISCUSSION

Subunit vaccines comprised of recombinant hemagglutinin are a viable alternative to traditional vaccine technologies against influenza. However, recombinant proteins are typically weak immunogens and require large doses as well as adjuvants to induce an immune response.\textsuperscript{6,7,12} In this work, the cloned and expressed recombinant protein was found to be highly immunogenic. It is known that hemagglutinin presented in its native trimeric state is often more immunogenic than the monomeric form\textsuperscript{5,8} and this was confirmed with a neutralization inhibition assay as shown in Figure 8.1. The trimeric oligomer of the H5 protein required significantly less protein in comparison with monomeric forms to inhibit neutralizing antibodies. Additionally, subcutaneous and intranasal immunizations with 10 μg soluble H5 trimer (sH5\textsubscript{3}) elicited neutralizing antibody titers greater than protective titers reported in the literature (Figure 8.2).\textsuperscript{7,8}

While sH5\textsubscript{3} may be immunogenic it requires refrigeration and/or freezing at low concentrations to maintain stability, and therefore, function. Previous work has demonstrated that the encapsulation of H5\textsubscript{3} into polyanhydride nanoparticles preserved the stability of the protein while simultaneously providing sustained release.\textsuperscript{31} Polyanhydride nanoparticles have also been shown to provide adjuvant properties, enhancing both cellular and humoral immunity.\textsuperscript{13,15,16} Other excipients known to enhance the immune response against viral pathogens include poly I:C, a synthetic analog of dsRNA interacting with TLR3, and poly dA:dT, a synthetic analog of dsDNA interacting with RIG-I.\textsuperscript{32,33,34} To understand the immune response to polyanhydride nanovaccines encapsulating H5\textsubscript{3}, mice were immunized subcutaneously with nanovaccine formulations (Table 8.1) with and without excipients (i.e., poly I:C and poly dA:dT) and compared to sH5\textsubscript{3} alone or MPLA (positive control).

The neutralizing antibody titers were examined at 42 and 63 days after the initial immunization. In mice receiving the single dose immunization regimen, the formulation including poly I:C enhanced neutralizing antibody titers at 42 days similar to the MPLA positive control.
(Figure 8.3). It is known that poly I:C confers protection in influenza vaccines through the activation of dendritic cells early resulting in marked antibody responses.\textsuperscript{32} The remaining formulations, while resulting in low titers at 42 days, were able to exhibit equivalent antibody titers at 63 days with the exception of the poly dA:dT formulation. The polyanhydride nanovaccine formulations release antigen slowly, which may explain the delayed antibody kinetics of nanovaccines delivered without excipients.\textsuperscript{15}

The mice administered a prime/boost immunization regimen (three immunizations, 21 days apart) generally displayed increased neutralizing titers in comparison with the single dose regimen at 42 days (i.e., after the first boost) (Figure 8.3). All formulations using a prime/boost regimen were found to have antibody titers similar to the positive control. While the mean antibody titer for each prime/boost formulation was similar at both 42 and 63 days, the addition of a second booster immunization enhanced almost all the treatments in comparison to the single dose regimen with less mouse-to-mouse variability at 63 days.

Although humoral immunity is often enough to provide protection against homologous strains of influenza, cell-mediated immunity is necessary when confronting heterologous variants.\textsuperscript{19} CD8$^+$ T cells secrete antiviral cytokines and often have a broader epitope spectrum than that of the humoral response.\textsuperscript{19,20} While not fully understood, CD4$^+$ T cells also play a role in protection against heterologous influenza infection by producing cytokines to restrict viral replication and even providing some lytic activity as well.\textsuperscript{19,20,35} Likewise, vaccines that can stimulate both humoral and cell-mediated immunity would likely have an advantage in cross-clade protection. As shown in Figure 8.4, polyanhydride nanovaccines enhanced T cell proliferation upon ex vivo stimulation with antigen. While all the formulations studied increased CD4$^+$ T cell proliferation, only the nanovaccine formulation containing poly I:C induced the greatest proliferation of both CD4$^+$ and CD8$^+$ T cells. It is known that poly I:C signaling through TLR3 induces the production of several Th1 and Th2 cytokines as well as type I interferons,\textsuperscript{32} which may explain the T cell expansion observed in Figure 8.4.
To examine whether the immune responses to H5\textsubscript{3} vaccination led to protection, mice administered a prime/boost immunization regimen were challenged intranasally with a low pathogenic virus. As expected, mice receiving saline injections were not protected from viral challenge. Saline-administered mice began to lose weight 4-5 days post-challenge and continued to lose up to 20\% of their body weight by eight days post-challenge, confirming that the viral challenge was successful (Figure 8.6A). In contrast, all the mice receiving H5\textsubscript{3} immunizations maintained or gained body weight post-challenge similar to naïve, non-infected mice.

At three days post-challenge, half of the mice were euthanized to further characterize the viral load and inflammatory cytokines present in the lungs. Saline-administered mice displayed significantly increased viral load compared to immunized mice (Figure 8.6B). All mice receiving H5\textsubscript{3} immunizations displayed at least a 10-fold reduction in viral load with some individual mice completely clearing the virus. Upon examining the presence of inflammatory cytokines within BAL fluid, saline mice had significantly increased levels of all cytokines examined (Figure 8.6C), which correlated with the increased viral load observed in Figure 8.6B. Consistent with the reduction of viral load, and therefore a reduction in antigen and inflammation, all vaccinated mice showed little to no inflammatory cytokines present in the lungs.

While no significant advantages of polyanhydride nanovaccines were found in terms of neutralizing antibody responses or reduction of clinical signs, the inclusion of nanoparticles in the vaccine formulations did not adversely affect the immune response. Noting that the H5\textsubscript{3} immunogenicity was relatively high, it is likely that the dose of antigen was greater than optimal. Polyanhydride nanovaccines have been shown to induce equivalent antibody titers with suboptimal (i.e., 64 times less) doses than that induced by soluble antigen.\textsuperscript{15} Likewise, previously observed enhancements of T cell memory after immunization with polyanhydride
nanovaccines\textsuperscript{17,18} will play an important role in future studies with high pathogenic and/or cross-clade influenza viral challenges.

8.5. CONCLUSIONS

The studies presented herein demonstrated the strong immunogenic properties of the H5\textsubscript{3} antigen as well as the immune responses to H5\textsubscript{3} containing polyanhydride nanovaccines. All formulations were able to achieve detectable neutralizing antibody titers 42 days post-vaccination even with a single dose regimen. The formulation including poly I:C also conferred the additional advantages of enhanced antibody titers at earlier time points and proliferative T cell responses. All vaccine formulations were able to induce protection against a low pathogenic, live viral challenge. The current studies lay a platform to further exploit the advantages of encapsulation into polyanhydride nanoparticles in future studies.

Acknowledgements

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## 8.6. TABLES

### Table 8.1. Immunization Formulations

<table>
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<th>Group</th>
<th>Encapsulated H5&lt;sub&gt;3&lt;/sub&gt;</th>
<th>Soluble H5&lt;sub&gt;3&lt;/sub&gt;</th>
<th>Total 20:80 CPTEG:CPH Nanoparticles</th>
<th>Additional Excipients</th>
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<tr>
<td>H5&lt;sub&gt;3&lt;/sub&gt; NP</td>
<td>2 µg</td>
<td>8 µg</td>
<td>300 µg</td>
<td>-</td>
</tr>
<tr>
<td>H5&lt;sub&gt;3&lt;/sub&gt; &amp; Poly I:C NP</td>
<td>2 µg</td>
<td>8 µg</td>
<td>300 µg</td>
<td>2.5 µg Poly I:C</td>
</tr>
<tr>
<td>H5&lt;sub&gt;3&lt;/sub&gt; &amp; Poly dA:dT NP</td>
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<td>8 µg</td>
<td>300 µg</td>
<td>2.5 µg Poly dA:dT</td>
</tr>
<tr>
<td>Blank NP</td>
<td>-</td>
<td>10 µg</td>
<td>300 µg</td>
<td>-</td>
</tr>
<tr>
<td>MPLA</td>
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<td>10 µg</td>
<td>-</td>
<td>10 µg MPLA</td>
</tr>
<tr>
<td>sH5&lt;sub&gt;3&lt;/sub&gt;</td>
<td>-</td>
<td>10 µg</td>
<td>-</td>
<td>-</td>
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<tr>
<td>Saline</td>
<td>-</td>
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</table>
Figure 8.1. Expression and characterization of H5. The HA ectodomain (nucleotides 1-1723) was amplified from pHW500 and modified at the 3’ end by addition of linker sequences, a GCN4pII trimerization domain and His-tag sequences at the 3’ end (A). SDS-PAGE (Lane 1), blue native gel electrophoresis (Lane 3), and their subsequent immunoblots (Lane 2 and 4, respectively) confirmed the expression of the H5 antigen as well as its trimeric form (B). A neutralization assay demonstrated that the immunogenicity of trimeric H5 was significantly greater than that of monomeric forms, requiring a lower concentration of protein to inhibit 50% of the neutralizing sera (IC$_{50}$). (C) Error bars represent the standard error of mean. $p \leq 0.0243$. 

- HA1
- HA2
- A/Whooper Swan/244/Mongolia/05 Ectodomain
- Linker
- GCN4pII
- His-Tag
- 66 kDa
- 198 kDa
Figure 8.2. Immunogenicity of soluble H5₃ antigen. Mice were immunized subcutaneously or intranasally with 10 μg soluble H5₃ on day 0, 21, and 42. Neutralizing antibody titers were not detected until after the second immunization, however, titer generated by days 42 and 63 were robust (A). The neutralizing antibodies demonstrated cross-clade protection at 63 days with both immunization routes (B). ID₅₀ = dose that inhibits 50% pseudovirus neutralization. Error bars represent standard error of mean.
Figure 8.3. Prime/boost polyanhydride nanovaccines induced robust neutralizing antibodies 42 days post-immunization. The neutralizing antibody responses to H5<sub>3</sub>-polyanhydride nanovaccines were examined 42 and 63 days post-primary immunization. Single dose vaccination regimens utilizing poly I:C encapsulated with H5<sub>3</sub> demonstrated neutralization titers similar to a MPLA-adjuvanted control at day 42, with most of the remaining formulations reaching equivalency at day 63. The use of a prime/boost immunization regimen enhanced the production of neutralizing antibodies and reduced mouse-to-mouse variability. ID<sub>50</sub> = dose that inhibits 50% pseudovirus neutralization. Error bars represent standard error of mean. Different
letters indicate statistical significant among treatments of the same regimen (i.e., single dose or prime/boost) at each time point. * indicates statistical significance between the different regimens for each treatment. No statistical significance was found comparing among prime/boost formulations. p ≤ 0.0434.
Figure 8.4. Enhanced CD4$^+$ T cell memory with poly I:C polyanhydride nanovaccine.

Draining lymph nodes were harvested for prime/boost immunized mice 63 days after the primary immunization. Ex vivo antigen stimulation and CFSE-labeling demonstrated enhanced CD4$^+$ T cell proliferation of mice immunized with poly I:C nanovaccines. Error bars represent standard error of mean. $p \leq 0.0147$. 
Mice immunized with the lead nanovaccine formulations were challenged with a low pathogenic influenza virus 63 days after initial vaccination. One week before challenge, all vaccine formulations induced high neutralizing antibody titers consistent with previous work and suggested by literature to be protective. \( \text{ID}_{50} \) = dose that inhibits 50% pseudovirus neutralization. Error bars represent standard error of mean. \( p \leq 0.0001 \).
Figure 8.6. Mice protected upon challenge with low, pathogenic viral strain. Body weight was observed for two weeks post-infection. All vaccinated mice maintained or gained weight similar to naïve, non-infected mice (A). Additionally, all mice demonstrated a reduction of viral load three days post-infection (B), which correlated with a significant reduction in inflammatory cytokines in comparison with saline immunized mice (C).
8.8 REFERENCES


CHAPTER 9:  
Conclusions and Ongoing/Future Research

9.1. CONCLUSIONS

Mucosal immunization is paramount for protection against respiratory pathogens because other routes of vaccination may not elicit mucosal immunity.\(^1\)\(^-\)\(^3\) Intranasal vaccination is an advantageous yet challenging strategy for advancing vaccine development against respiratory pathogens. Intranasal administration of vaccines can increase the availability of antigens due to the large, permeable surface area of the lung and can avoid the harsh environments of the gastrointestinal tract.\(^2\)\(^-\)\(^5\) Despite these advantages, antigen delivered alone is often not immunogenic and requires the use of an adjuvant.\(^4\) Polyanhydride nanoparticles have been shown to be a promising platform for intranasal immunization with many beneficial properties including sustained release, cell internalization, and immunomodulation which may be suitable for vaccines against respiratory pathogens such as H5N1 avian influenza.\(^6\)\(^-\)\(^9\)

To understand the early deposition kinetics of intranasal vaccination \textit{in vivo}, fluorescently tagged antigen was encapsulated in polyanhydride nanoparticles and administered to mice (Chapter 4). By utilizing flow cytometry and high throughput microscopy, polyanhydride nanoparticles were shown to deposit uniformly within the lungs, prolong antigen presence within the lungs, and increase internalization by antigen presenting cells. The prolonged persistence of antigen after the initial deposition and the nanoparticle chemistry were found to play important roles in controlling antigen release kinetics and the kinetics of the antibody response (Chapter 5). Finally, by utilizing an adoptive transfer model with ovalbumin-specific T cells, it was found that amphiphilic CPTEG:CPH nanovaccine formulations enhanced early CD8\(^+\) T cell expansion and differentiation into effector memory phenotypes (Chapter 6).

The ability of polyanhydride nanoparticles to release stable trimeric hemagglutinin antigen (H5\(_3\)) was investigated in Chapter 7. These studies showed the preservation of protein
structure as well as antigenicity upon release from three polyanhydride nanoparticle formulations, which demonstrated that these particles can serve as suitable adjuvants/carriers for influenza vaccination. In addition, the immune responses to H5₃ encapsulated in 20:80 CPTEG:CPH nanoparticles was examined over a period of 9 weeks (Chapter 8). All formulations were found to achieve robust neutralizing antibody titers that were protective against a low pathogenic, live viral challenge demonstrating that polyanhydride nanovaccines represent a promising platform for H5N1 influenza vaccines.

9.2. ONGOING/FUTURE WORK

While polyanhydride nanoparticles have so far been shown to be a promising platform for the delivery of H5₃, further studies are necessary to optimize an efficacious vaccine for H5N1 avian influenza. The H5₃ antigen was found to be highly immunogenic and a dose titration was performed for subsequent studies. Further in vivo experiments included the addition of different polyanhydride chemistries with or without additional excipients such as poly I:C. These optimized formulations will be examined for efficacy against a cross-clade, high pathogenic viral challenge (i.e., pandemic influenza) in contrast to the low pathogenic challenge described in Chapter 8. Finally, the inclusion of other viral components (such as matrix or nucleoproteins) as well as development of dry powder, intranasal formulations may lead to an efficacious and broadly protective influenza vaccine suitable for pandemics.

9.2.1. Dose titration of H5₃ antigen in vivo

Vaccine shortages can occur for a wide variety of reasons; however, in a pandemic scenario, a limited supply of immunizations could have a large impact on the spread, morbidity, and mortality of the disease. The initial in vivo studies presented in Chapter 8 demonstrated that H5₃ is a potent immunogen against influenza. It is possible that a lower dose of antigen would be within the therapeutic window and provide similar efficacy to previous immune responses,
and therefore, enhance vaccine availability and cost effectiveness. Polyanhydride nanovaccines have also been demonstrated to possess dose-sparing capabilities.\textsuperscript{10} In a recent study (see Appendix A), immunization with 25 μg of polyanhydride particle-encapsulated antigen elicited similar antibody titers and avidity in comparison with a 1600 μg dose of antigen alone, demonstrating a 64-fold dose sparing.\textsuperscript{10}

To identify an optimal dose of H5\textsubscript{3} antigen, BALB/c mice were immunized subcutaneously with 2, 4, or 8 μg of protein (in comparison with the 10 μg administered in Chapter 8). Each mouse received a total of three immunizations three weeks apart and was monitored for serum antibody over the course of 9 weeks. Consistent with previous work, little antibody was present 21 days post-immunization. The lowest antigen dose (2 μg) demonstrated fewer antibodies at 42 days post-initial immunization compared to the larger doses (Figure 9.1). However, with the addition of a third immunization, equivalent and robust antibody responses were demonstrated with all three doses at 63 days post-initial immunization.

9.2.2. Optimization of polyanhydride nanoparticle chemistry \textit{in vivo}

Chapter 8 demonstrated the immune response to and efficacy of 20:80 CPTEG:CPH nanovaccines encapsulating H5\textsubscript{3}. However, the data presented in Chapter 4 indicates that other polyanhydride nanoparticle chemistries also released stable H5\textsubscript{3}. The chemistry of individual polyanhydride copolymers has been demonstrated previously to have immunomodulatory capabilities that may play an important role in the \textit{in vivo} response to H5\textsubscript{3} immunization.\textsuperscript{8} To this end, the immune response to H5\textsubscript{3} encapsulated in 20:80 CPH:SA nanoparticles was evaluated.

BALB/c mice were immunized subcutaneously with H5\textsubscript{3}-loaded nanovaccine + sH5\textsubscript{3} (H5\textsubscript{3} NP) blank (i.e., no protein) nanoparticles + sH5\textsubscript{3} (Blank NP), sH5\textsubscript{3} alone, or saline (Table 9.1). Similar to the experiments performed in Chapter 8, two immunization regimens were used: single dose (Day 0 immunization) and prime/boost (Day 0 and 21 immunizations). While the single dose regimen generally displayed low levels of antigen-specific IgG, the addition of a
booster immunization led to an approximate 50-fold increase in antibody production at day 42 (Figure 9.2). Consistent with previous work, each formulation displayed similar levels of antibodies demonstrated in the dose titration experiment (Figure 9.1).

Additionally, all mice received a 5 µg intranasal dose of H53 co-delivered with 20 µg of poly I:C to mimic an antigenic challenge at day 42. After administering bromodeoxyuridine (BrdU) to detect proliferating cells, mice were euthanized 5 days post-challenge to examine T cell recall responses via flow cytometry. While all immunization formulations were not able to elicit proliferative CD8+ T cell responses (Figure 9.3), mice receiving the prime/boost regimen of the H53 NP formulation demonstrated significant and robust CD4+ T cell proliferation (Figure 9.3). It is important to note that immunization with H53-loaded nanovaccine displayed similar levels of T cell proliferation to the results presented in Chapter 8 despite a 2.5-fold reduction in antigen dose. In contrast, the reduced dose of sH53 antigen delivered alone did not induce any significant T cell proliferation in comparison to saline-administered mice.

Finally, examination of neutralizing antibody titers will aid in the identification of formulations with the most robust immune responses. These key formulations will move forward to protection studies against a highly pathogenic H5N1 influenza live viral challenge. Protection will be ascertained using a combination of survival, viral load, and cytokine analyses, as described in Chapter 8. These experiments will be performed in collaboration with Dr. Richard Webby at St. Jude Children’s Research Hospital in Memphis, TN.

9.2.3. Development of intranasal influenza vaccines with broad protection

The development of a single dose, intranasal vaccine that can elicit broad protection against several influenza strains is of paramount importance. The need for multiple doses or prime/boost immunization regimens to achieve protection is a large drawback, especially in pandemic or post-exposure situations. A single dose, intranasal vaccine is also easier to administer without significant training allowing for rapid deployment of vaccines during a
pandemic and permitting healthcare professionals to focus on responding to critical patients. In addition, intranasal delivery is advantageous in that it enables both mucosal and systemic immunity.

A unique aspect of the polyanhydride nanovaccine platform is the ability to create dry powder vaccines. Dry powder vaccines are useful in that they can be delivered via several different routes, including intranasal, and can generally maintain antigen stability better than liquid formulations. Lyophilization of influenza vaccines has been explored in the literature; however, these vaccines often require stabilizers and additives to maintain the potency of the antigen upon reconstitution. In contrast, polyanhydride nanovaccines have shown that antigen stability can be preserved without additional components for up to ten months at room temperature. The optimization of dry powder polyanhydride nanovaccines could provide effective single dose, intranasal vaccines that can be stockpiled in anticipation of an influenza pandemic.

Finally, influenza vaccines that are broadly protective would be valuable against both seasonal and pandemic strains. Neutralizing antibodies have been shown to be fully protective against antigenically matched viruses, however, the hemagglutinin protein (H5) typically is the most variable or mutation-prone of the virus. Thus, incorrect predictions of seasonal strains or rapid changes of epitope presentation in pandemic circumstances could render neutralizing antibodies against H5 useless. Although not usually protective on their own, the addition of conserved viral antigens to hemagglutinin vaccines have been shown to induce cross-protective immunity. Inclusion of conserved viral antigens, such as the surface protein neuraminidase (NA), ion channel matrix proteins (MP), and the internal nucleoprotein (NP), in subunit vaccines have been noted to enhance the expansion of CD8+ T cells leading to cross-protection. The addition of these conserved antigens to H5 polyanhydride nanovaccines could provide great benefit in terms of complementing the humoral (Chapter 8) and enhancing the cell-mediated
(Chapters 6 and 8) immune responses previously discussed, leading to the development of an efficacious, universal influenza vaccine.
9.3. TABLES

Table 9.1: H5 20:80 CPH:SA Nanovaccine Immunization Regimen

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>H5 NP (µg)</th>
<th>Blank NP (µg)</th>
<th>Total H5 Encap (µg)</th>
<th>Total Soluble H5 (µg)</th>
</tr>
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<td>H5 NP</td>
<td>300</td>
<td>0</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>2</td>
<td>Blank NP</td>
<td>0</td>
<td>300</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>3</td>
<td>sH5</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>4</td>
<td>Saline</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

9.4. FIGURES

Figure 9.1. Antibody response to H5 dose titration. Mice (n = 6) were immunized subcutaneously with 2, 4, or 8 µg H5 at days 0, 21, and 42. Total anti-H5 IgG of a 1:1000 serum dilution was quantified using a fluorescent multiplex method. Error bars represent standard error of mean.
Figure 9.2. H5₃-specific IgG antibody responses to 20:80 CPH:SA nanovaccine. Mice (n = 8) were immunized subcutaneously with a single dose or prime/boost immunization regimen. Antibody responses at 42 days post-immunization demonstrated an approximate 50-fold increase of antibody production with the prime/boost regimen. Error bars represent standard error of mean.
Figure 9.3. T cell responses to intranasal H53 antigenic challenge of prime/boost immunized mice. All mice received at 5 μg H53 + 20 μg poly I:C intranasal challenge 42 days post-initial immunization. Mice (n = 8) immunized with H53-loaded polyanhydride nanovaccine displayed robust CD4⁺ T cell proliferation five days post-challenge. Different letters indicate statistical significance. Error bars represent standard error of mean. p ≤ 0.0052.
9.5. REFERENCES


APPENDIX A:

Single Immunization with a Suboptimal Antigen Dose Encapsulated into Polyanhydride Microparticles Promotes High Titer and Avid Antibody Responses


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*These authors contributed equally to the design and performance of these studies.

Abstract

Microparticle adjuvants based on biodegradable polyanhydrides were used to provide controlled delivery of a model antigen, ovalbumin (Ova), to mice. Ova was encapsulated into two different polyanhydride microparticle formulations to evaluate the influence of polymer chemistry on the nature and magnitude of the humoral immune response after administration of a suboptimal dose. Subcutaneous administration of a single dose of polyanhydride microparticles containing 25 μg of Ova elicited humoral immune responses that comparable in magnitude to that induced by soluble doses of 400–1600 μg Ova. In contrast, the avidity of the Ova-specific antibodies was greater in mice administered the microparticle formulations in comparison to the higher soluble doses. Finally, the microparticle delivery system primed an anamnestic immune response as evidenced by the significant increases in Ova-specific antibody when mice were administered an antigenic challenge of 25 μg of Ova at 12 weeks post-vaccination. Together, these results indicate that encapsulation of antigens into

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polyanhydride microparticles facilitates isotype switching, establishes immunologic memory, and the humoral response was characterized by a higher quality antibody response.

1. **INTRODUCTION**

Many current subunit vaccine formulations consist of poorly immunogenic recombinant proteins that require adjuvants to induce humoral and cellular immune responses. Although beneficial, vaccine adjuvants do pose a risk of adverse reactions, which may, in part, explain why only three formulations (the aluminum salts Al(OH)$_3$ and AlPO$_4$, oil-in-water emulsion, and monophosphoryl lipid A) are currently licensed for human use in the United States.$^1$ Recombinant proteins are also often costly to produce. Reducing the amount of antigen needed to elicit protective immune responses could help eliminate vaccine production shortages similar to those observed during the 2009 H1N1 influenza pandemic.$^2$ Immunization with subunit vaccines can also fail to induce robust humoral immune responses in which naïve B cells differentiate into antigen-specific, long-lived plasma cells and memory B cells. Although these vaccines elicit measurable antibody titers, the quality (i.e., avidity) and kinetics of the antibody response may be less than optimal. Successful vaccines must induce antigen-specific memory B cells capable of rapidly proliferating upon antigen stimulation.$^{3,4}$ In addition, an avid antibody response must be developed through somatic hypermutation and positive selection of high affinity B cell clones. These attributes are all essential factors in determining the quality of antibody-mediated protection against a subsequent pathogen challenge.

To overcome many of the limitations associated with traditional vaccine regimens, antigens have been encapsulated into synthetic, biodegradable polymer micro- and nanoparticles.$^{5-8}$ The most extensively studied formulations include the polyesters poly(glycolic acid), poly(lactic acid), and their copolymers (i.e., poly(lactide-co-glycolide) or PLGA). Unfortunately, particles made of PLGA degrade by a bulk erosion mechanism that may negatively affect the stability of proteins susceptible to moisture-induced aggregation.$^{9,10}$ In
addition, the acidic microenvironment created by PLGA degradation products (i.e., lactic or glycolic acids) can lead to protein instabilities.\textsuperscript{11,12} Polyanhydrides, another class of well-studied biodegradable polymers, present an alternative for drug and vaccine delivery.\textsuperscript{13} These materials exhibit surface erosion characteristics and possess hydrolytically labile anhydride bonds.\textsuperscript{14} The most commonly studied polyanhydrides are based on the aliphatic sebacic acid (SA) and the aromatic 1,6-bis(\(p\)-carboxyphenoxy)hexane (CPH). Encapsulation of proteins into CPH:SA copolymers has been shown to preserve protein and antigenic epitope stability.\textsuperscript{9,15,16} These surface-erodible polymers also provide sustained protein release\textsuperscript{15-17} and possess immune-modulatory capabilities.\textsuperscript{18-21}

In this work, we extend our studies of polyanhydride particle-based vaccines by exploring the ability of this platform to enhance the immunogenicity of ovalbumin (Ova), a model vaccine antigen. We found that a single subcutaneous (SC) administration of a suboptimal dose of Ova encapsulated into polyanhydride microparticles induced an antibody response that was comparable in magnitude to that induced by 16-fold higher doses of soluble Ova or that induced by multiple doses of Alum-adjuvanted Ova.\textsuperscript{22} Moreover, this microparticle vaccine regimen successfully primed the humoral immune response for an anamnestic immune response. Together, these results indicate that encapsulation of vaccine antigens into polyanhydride microparticles provides a platform delivery system that can elicit a mature humoral memory response after a single administration.

2. MATERIALS AND METHODS

2.1. Materials and polymer synthesis

The chemicals needed for CPH monomer synthesis, 4-\(p\)-hydroxybenzoic acid, 1,6-dibromohexane, 1-methyl-2-pyrrolidinone, and SA (99%), were purchased from Sigma Aldrich (St Louis, MO); acetic anhydride, methylene chloride, and ethanol were purchased from Fisher Scientific (Fairlawn, NJ). CPH diacid was synthesized as described previously.\textsuperscript{23,24} Pre-polymers
of SA and CPH were synthesized by the methods described by Shen et al.\textsuperscript{25} and Conix.\textsuperscript{23} CPH:SA copolymers were synthesized by melt polycondensation as described previously.\textsuperscript{14} The purity and degree of polymerization of the copolymers was analyzed using $^1$H nuclear magnetic resonance (NMR) spectroscopy obtained from a Varian VXR-300 MHz NMR spectrometer (Varian, Palo Alto, CA). NMR spectra were consistent with previously published data and confirmed the synthesis of the desired copolymer compositions.\textsuperscript{24} In addition, polymer molecular weight was determined using gel permeation chromatography (GPC, Waters HPLC System, Milford, MA) using Varian, GPC columns.

\textbf{2.2. Microparticle fabrication}

To eliminate the endotoxin contamination of the Ova (Sigma Aldrich) and to prevent unintended enhancement of the immune response caused by contaminating endotoxin, affinityPak Detoxi-Gel endotoxin removal columns (Thermo Scientific, Rockford, IL) were used according to manufacturer's instructions. The recovered Ova contained <10 EU/mg protein and was lyophilized and stored at -20°C. Ova-loaded microparticles were fabricated using cryogenic atomization.\textsuperscript{5,9,26} The parameters used for each copolymer chemistry were previously specified by Torres et al.\textsuperscript{16} and Lopac et al.\textsuperscript{17} The obtained microparticles were collected by vacuum filtration and dried under vacuum. Using four replicative samples for each microparticle preparation, microparticle morphology and size distribution were analyzed by using images obtained by scanning electron microscopy (SEM) (JEOL 840 A, JEOL, Peabody, MA), and ImageJ image analysis software (National Institutes of Health, Bethesda, MD). An average of 200 particles per image was analyzed.

\textbf{2.3. \textit{In vitro} antigen release}

\textit{In vitro} Ova release kinetics were measured by suspending 15 mg of the Ova-loaded microparticles in 1 mL of phosphate buffered saline (0.1 \textit{M}, pH 7.4) with 0.01\% (w/v) sodium
azide, and incubating at 37°C on a shaker platform at 100 rpm. Aliquots of 750 μL were taken at prescribed time intervals and replaced with fresh buffer. Aliquots were stored at 4°C to measure protein concentration using micro-bicinchoninic acid (BCA) analysis at an absorbance of 570 nm. At least three replicates of each sample were analyzed. After 25 days, the remaining encapsulated protein was extracted by degrading the remaining particles in 17 mM NaOH. Protein concentration was determined with a micro-BCA assay. Total protein encapsulated in the particles was determined by calculating the protein released at each time point as described by Torres et al.\textsuperscript{16}

2.4. Mice and immunization procedures

Female C3H/HeNHsd (C3H) mice were purchased from Harlan Sprague Dawley (Frederick, MD). All animal procedures were conducted with the approval of the Iowa State University Institutional Animal Care and Use Committee. To evaluate the serum antibody response to nonadjuvanted Ova, 20 mg of endotoxin-free Ova was suspended in pyrogen-free saline and diluted to the indicated concentrations. Mice were subcutaneously (SC) immunized with soluble Ova alone at doses of 1600, 400, 100, or 25 μg in 100 μL pyrogen-free saline with seven mice per treatment group (n = 7). Control animals received 100 μL saline alone (n = 6). Blood samples were collected from the left saphenous vein before immunization and every 4 weeks thereafter. Serum was collected after centrifugation and stored at -20°C until assayed for Ova-specific antibody as described below.

Mice were immunized SC with 25 μg of Ova encapsulated into 0.5 mg of either 20:80 CPH:SA or 50:50 CPH:SA microparticles suspended in pyrogen-free saline with eight mice per treatment group (n = 8). Before administration, microparticles were sonicated briefly to generate a uniform suspension. A total volume of 100 μL was administered at the injection site. For the antigenic challenge studies, mice that had been immunized 12 weeks prior were immunized SC
with 25 μg of endotoxin-free Ova suspended in pyrogen-free saline. Serum samples were collected 5 days later in order to measure the anamnestic antibody response.

2.5. ELISA for Ova-specific antibody titer and avidity

ELISA plates (Costar Catalog # 3590, EIA/RIA high binding) were coated overnight with 0.5 μg/well Ova. Plates were washed with phosphate buffered saline containing 0.5% Tween 20 at a pH of 7.4 (PBST). Plates were blocked for 2 h with 2% gelatin (Difco) in PBST. Plates were washed and serial dilutions of individual serum samples in PBST with 1% heat inactivated normal goat serum (GIBCO) were incubated overnight at 4°C. Plates were washed again with PBST and alkaline phosphatase-conjugated goat anti-mouse IgG (H&L), IgG1, or IgG2a (Jackson ImmunoResearch Laboratories, West Grove, PA) diluted 1:1000 in 1% heat inactivated normal goat serum in PBST was incubated for 2 h. Plates were washed and p-nitrophenyl phosphate (Sigma) substrate (1 mg/mL) in 50 mM Na₂CO₃ and 2 mM MgCl₂ buffer (pH 9.3) was added to each well. Changes in optical density were measured at 405 nm using a spectrophotometer (Spectra Max 190, Molecular Devices, Sunnyvale, CA). Antibody avidity ELISA was performed as described previously to determine antibody binding strength in the presence of a chaotropic agent that disrupts antibody–antigen binding interactions. The molar concentration of NaSCN corresponding to the 50% loss of absorbance was designated as the relative avidity value reported.

2.6. Statistical analysis

Longitudinal data were analyzed using repeated measure analysis of variance (ANOVA) models (with SAS version 9.2). Treatment and time were fixed effects in the statistical model, whereas mouse was the subject of repeated measures. Cross-sectional data were analyzed using one-way ANOVA models with treatment as the explanatory variable. Differences in mean responses among treatments were compared by using Tukey’s T-test. Log₁₀ transformation was
applied to responses with skewed distributions before analyses. Statistical tests with \( p \leq 0.05 \) were regarded as significant.

3. RESULTS

3.1. Single, soluble doses of Ova require \( \geq 100 \) \( \mu \)g to elicit significant antibody titers and prime the humoral response to respond to an antigenic challenge

To demonstrate the capabilities of a single dose immunization regimen employing a suboptimal dose of Ova encapsulated into polyanhydride microparticles, we first examined the kinetics of the IgG antibody response of mice immunized subcutaneously once with varying doses of soluble Ova (Figure 1A). Groups of mice that were administered 1600 \( \mu \)g, 400 \( \mu \)g, or 100 \( \mu \)g of Ova elicited significantly higher antibody titers in contrast to mice immunized with 25 \( \mu \)g of Ova. The kinetics of the IgG response induced by a single, soluble dose of Ova demonstrated that the peak titer was obtained 4 weeks post-injection and then began to wane.

Antibody avidity was also assessed as a surrogate marker of vaccine efficacy. Serum samples from the mice immunized with a single dose of 100 \( \mu \)g, 400 \( \mu \)g, or 1600 \( \mu \)g of soluble Ova developed a peak antibody avidity at 4 weeks post-vaccination that was stable through 12 weeks post-immunization (Figure 1B). Serum samples from mice vaccinated with 25 \( \mu \)g of soluble Ova alone did not induce a sufficient antibody titer to measure antibody avidity. To ascertain the generation of antigen-specific memory responses, a 25 \( \mu \)g “antigenic challenge” was administered at 12 week post-vaccination. A measurable increase in the secondary humoral antibody response was observed in mice that had received \( \geq 25 \) \( \mu \)g antigen at the initial vaccination (Figure 1C). A soluble dose of 25 \( \mu \)g of Ova was not sufficient to induce a primary serum antibody (Figure 1C, open bar) response but was able to prime the mice for a secondary immune response (Figure 1C, closed bar), indicating that 25 \( \mu \)g was a suboptimal dose of immunogen.
3.2. Controlled antigen release by polyanhydride microparticles in vivo results in a primed humoral response

Ova-loaded 20:80 and 50:50 CPH:SA microparticles were characterized by SEM after fabrication (Figure 2A). Morphologies of the Ova-loaded microparticles were consistent with previous work describing blank microparticles. The diameter of the microparticles ranged between 5 and 21 μm for both 20:80 CPH:SA and 50:50 CPH:SA. Although there was a greater initial burst release from the 20:80 CPH:SA formulation (Figure 2B), the release profile of Ova from the two polyanhydride formulations was shown to be sustained and is consistent with previous work.

Using the polyanhydride microparticle vaccine delivery platform, we sought to demonstrate that a significant humoral response could be induced when a suboptimal dose of Ova (25 μg) was encapsulated into one or both of the microparticle formulations evaluated in this study. The IgG response induced by the administration of 500 μg of either 20:80 CPH:SA or 50:50 CPH:SA containing 25 μg of Ova (i.e., 5%, wt/wt) was evaluated for 12 weeks after administration (Figure 3A). Both polyanhydride formulations tested elicited similar Ova-specific antibody titers. Compared to mice receiving 25 μg of soluble Ova, significant antibody titers were demonstrable in microparticle vaccinated groups beginning at week 4 and were maintained through 12 weeks post-vaccination. Mice receiving either Ova-encapsulated microparticle vaccine formulation developed a more avid Ova-specific antibody response than did mice receiving much larger doses of soluble Ova (Figure 3B). These more avid antibody responses were sustained over the 12 weeks of the experiment (Figure 3B). After the antigenic challenge (i.e., booster immunization), greater Ova-specific serum antibody responses (i.e., titers ≥ 100,000) were observed in mice administered the Ova-loaded microparticles as compared to the antibody responses (i.e., titers ≤ 10,000) induced by the soluble doses of Ova (Figure 3C compared to Figure 1C). Statistical comparisons of the antibody responses induced after the antigenic challenge (Figures1C and 3C, solid histograms) demonstrated an adjuvant
effect associated with the administration of the microparticles that was consistent with the elevated titers and avidity presented in Figure 3A, B.

3.3. **Isotype switching of the serum antibody responses suggests immunological memory**

Characteristics of an immune response can be determined by examining the antibody isotype produced. For mice immunized with Ova-loaded microparticles, the secondary serum antibody response was characterized by the presence of both Ova-specific IgG1 and IgG2a (Figure 4A,B, respectively). For mice immunized with single doses of soluble Ova (100–1600 μg), there was no demonstrable Ova-specific IgG2a detected after the antigenic challenge (data not shown). Together, these results indicate that a mature, antigen-specific memory response was obtained with a priming dose of only 25 μg when encapsulated into polyanhydride microparticles.

4. **DISCUSSION**

Biodegradable polymers exhibit adjuvant properties, making them ideal delivery platforms for single dose vaccine regimens.\(^{27-29}\) Specifically, vaccine formulations based on PLGA, PLA, or PGA have been shown to induce immune responses to a variety of immunogens.\(^{30,31}\) Several of these studies incorporated monophosphoryl lipid A, a known adjuvant, into the polymer delivery device along with the immunogen, thereby complicating the ability to determine whether or not the polymer itself provides any immune enhancing activity.\(^{13,29,31}\) Other studies have included excipients and/or stabilizers to enhance the immunogenicity of encapsulated proteins.\(^{27}\) In this study, no additional immune enhancers were included during the fabrication of our polyanhydride microparticles loaded with endotoxin-free Ova or administered at the time of immunization. Therefore, any immunomodulatory properties observed were the direct result of the polymers themselves.
Previously, we have demonstrated that polymer chemistry differentially effects in vitro antigen presenting cell (APC) cytokine production, particle uptake, and cell surface marker expression. In contrast to these differential effects demonstrated in vitro on APCs, in vivo administration of Ova-loaded 20:80 or 50:50 CPH:SA microparticles induced similar serum antibody titers (Figure 3A), avidity (Figure 3B), and recall responses (Figures 3C and 4). This study was performed to demonstrate that immune responses could be induced with a single, suboptimal dose of an immunogen, and not to compare the adjuvant capabilities of the microparticles to other adjuvants. However, these studies do infer that polyanhydride delivery devices provide the adjuvant properties essential for effective implementation of subunit vaccines. Indeed, when a subimmunogenic dose (e.g., 25 μg) of Ova was encapsulated into the microparticles, both copolymer formulations were able to induce a robust immune response comparable to that induced by 400–1600 μg of soluble Ova. Furthermore, the titers of mice immunized with single dose microparticle formulations were similar to those reported for mice immunized multiple times with Alum-adjuvanted Ova over a range of doses comparable to the 25 μg dose used in this study. For example, Pollock et al. reported mean IgG1 titers of 40,000 after two administrations of 100 μg Ova adjuvanted with Alum. In another study, a single immunization with 50 μg Ova adjuvanted with Alum in C57BL/6 mice induced an IgG1 titer of approximately 100, which increased to approximately 10,000 after a second immunization. Sun and Pan observed similar antigen-specific IgG titers after administration of 100 μg Ova adjuvanted with Alum. In this work, the titer values (approximately 1000) obtained by immunizing mice once with 25 μg of Ova encapsulated in polyanhydride microparticles are similar to those induced by multiple doses of Alum-adjuvanted Ova described in the literature.

Recombinant protein antigens provide excellent purity and safety profiles for vaccines, but may sacrifice potency of the vaccine and lead to less than efficacious immune responses. In this regard, the avidity of the antibody response induced by the microparticle formulations was
greater than that induced by any of the soluble doses of Ova used in this study. These observations demonstrate the importance of evaluating the magnitude (i.e., titer) and the quality (i.e., avidity) of the antibody response in order to fully appreciate the benefits of novel vaccine delivery platforms.

Persistence of antigen is also known to be critical for inducing long-lived plasma cells and memory B cells. Companion studies from our laboratory indicate the persistence of polyanhydride particles at injection sites up to 12 weeks post-administration (manuscript in preparation). In this study, the encapsulation, controlled release and subsequent persistence of Ova *in vivo* likely contributed to the induction of long-lived plasma cells and induction of a more avid humoral immune response than that induced by higher doses of soluble Ova. It may be speculated that the increased avidity of the serum antibody response in mice vaccinated with the Ova-loaded microparticles results specifically from persistence of antigen in germinal centers, possibly mediated by follicular dendritic cells, in secondary lymphoid tissue. The single dose of 25 μg Ova encapsulated into the microparticles was sufficient to induce a demonstrable antibody response and to prime the host for a more robust secondary antibody responses after an antigenic challenge 12 weeks later (Figures 3 and 4). In contrast, there was little evidence of a recall response in mice initially administered 1600 μg Ova. Bioerodible microparticles may be performing similar actions as traditional emulsification vaccine adjuvants by providing an antigenic depot and creating particulate antigen that is more readily recognized by B cells and taken up by APCs. Experimental models employing micro- or nanoparticle vaccination regimen incorporating recombinant immunogens followed by pathogen challenge will demonstrate the full potential of this polymer delivery platform to induce protective immune responses.
5. CONCLUSIONS

In this work, we demonstrated that robust, long-lived immune responses can be induced by a single, suboptimal dose of a weak immunogen by encapsulation into surface eroding polyanhydride microparticles. The use of these biodegradable delivery devices to immunize mice induced an anamnestic antibody response and generated isotype switching, as evidenced by the induction of antigen-specific IgG2a, an antibody isotype indicative of memory T cell development. Lastly, the avidity (i.e., quality) of the serum antibody induced by 25 μg of Ova encapsulated into microparticles was greater than that induced by 1600 μg of soluble Ova. Collectively, the data demonstrate that the use of surface-erodible polyanhydride microparticles as a vaccine delivery platform may enhance the magnitude and quality of the immune response to subunit or recombinant proteins, and thus broaden the arsenal of recombinant immunogens that can be safely, efficaciously, and cost effectively employed in vaccine formulations.
Figure 1. Single doses of soluble ovalbumin (Ova) required ≥100 μg to elicit significant antibody titers and prime the humoral response to respond to an antigenic challenge.

Separate groups of C3H mice were immunized with a titrating dosage of soluble Ova: 1600 μg (open diamonds), 400 μg (open squares), 100 μg (open circles), 25 μg (open triangles), or saline alone (x). Ova-specific serum antibody titers (A) and antibody avidity (B) were measured over 12 weeks. (C) At 12 weeks post-immunization, the Ova-specific serum antibody titer was measured before (open histograms) and 5 days after (closed histograms) an antigenic challenge administered subcutaneously in the form of 25 μg Ova. All data are presented as the mean ± SEM and are representative of three independent experiments. For panel A, * represents a statistically significant difference from the 25 μg group at p < 0.05. For panel C, * indicates a statistically significant difference from treatments before boost at p < 0.05. Treatments with different letters are significantly different from one another at p < 0.05.
Figure 2. Morphology and *in vitro* release kinetics of Ova-loaded polyanhydride microparticles. Scanning electron photomicrographs of Ova-loaded 20:80 CPH:SA (mean diameter ± SD, 10 ± 5 μm) and 50:50 CPH:SA (12 ± 7 μm). (A) Scale bar: 20 μm. (B) Release kinetics of Ova from 20:80 CPH:SA and 50:50 CPH:SA microparticles. Data are presented as the mean ± SEM and are representative of two independent experiments with duplicate samples analyzed in each experiment.
Figure 3. Enhanced serum antibody response in mice immunized with Ova-loaded polyanhydride microparticles. Ova-specific serum antibody titers (A) and antibody avidity (B) for C3H mice immunized with 20:80 CPH:SA Ova-loaded microparticles (closed circles), 50:50 CPH:SA Ova-loaded microparticles (closed squares), 25 μg soluble Ova (open triangles), or saline alone (x). (C) At 12 weeks post-immunization, the anamnestic Ova-specific serum antibody titer was measured before (open histograms) and 5 days after (closed histograms) a subcutaneously administered dose of 25 μg Ova (i.e., antigenic challenge). All data are presented as the mean ± SEM and are representative of three independent experiments. For panel A, * represents a statistically significant difference from the 25 μg soluble Ova group at p < 0.05. For panel C, * indicates a statistically significant difference from treatments before boost at p < 0.05. Treatments with different letters are significantly different from one another at p < 0.05.
Figure 4. Mice immunized with Ova-loaded polyanhydride microparticles develop an anamnestic humoral response characterized by IgG1 and IgG2a, indicating antibody isotype switching. Ova-specific (A) IgG1 and (B) IgG2a serum antibody titer in mice administered a subcutaneous antigenic challenge of 25 μg Ova in saline 12 weeks after initial immunization with Ova-loaded polyanhydride microparticles. Data are presented as the mean ± SEM and are representative of three independent experiments. Treatments with different letters are significantly different from one another at p < 0.05.
7. REFERENCES


APPENDIX B:
Evaluation of Biocompatibility and Administration Site Reactogenicity of Polyanhydride-Particle-Based Platform for Vaccine Delivery

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Abstract

Efficacy, purity, safety, and potency are important attributes of vaccines. Polyanhydride particles represent a novel class of vaccine adjuvants and delivery platforms that have demonstrated the ability to enhance the stability of protein antigens as well as elicit protective immunity against bacterial pathogens. This work aims to elucidate the biocompatibility, inflammatory reactions, and particle effects on mice injected with a 5 mg dose of polyanhydride nanoparticles via common parenteral routes (subcutaneous and intramuscular). Independent of polymer chemistry, nanoparticles more effectively disseminated away from the injection site as compared to microparticles, which exhibited a depot effect. Using fluorescent probes, the \textit{in vivo} distribution of three formulations of nanoparticles, following subcutaneous administration, indicated migration away from the injection site. Less inflammation was observed at the injection sites of mice-administered nanoparticles as compared to Alum and incomplete Freund’s adjuvant. Furthermore, histological evaluation revealed minimal adverse injection site reactions and minimal toxicological effects associated with the administration of nanoparticles at 30 days post-administration. Collectively, these results demonstrate that polyanhydride nanoparticles do

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not induce inflammation as a cumulative effect of particle persistence or degradation and are, therefore, a viable candidate for a vaccine delivery platform.

1. **INTRODUCTION**

Next generation vaccine design aims to recapitulate the positive effects of enhancing immune responses to the immunogen of interest while avoiding the detrimental side-effects often caused by adjuvants. While efficacy is an essential outcome of vaccine design, biocompatibility is critical for ensuring patient compliance and ultimately developing protective immunity. Adverse injection site reactions that cause either pain or tissue damage are major hurdles in the development and licensure of vaccines containing immunostimulatory adjuvants. These adjuvants are often necessary vaccine components, as they enhance immune responses against poorly immunogenic antigens. Specifically, adjuvants often comprise particulate material that is readily taken up by antigen presenting cells, activates innate immunity, and provides an antigenic depot to sustain immune responses. The most common adjuvants used in human and veterinary medicine consist of aluminum salts, modified Toll-like receptor ligands, oil-water emulsions, or combinations thereof. The majority of adjuvanted vaccines approved for human use contain potassium aluminum sulfate (Alum), MF59 (a squalene oil-in-water emulsion), or ASO4 (monophosphoryl lipid A and Alum). Unfortunately, administration of vaccines containing these adjuvants often induces clinical signs of pain, redness, rash, swelling, and fever.

Polyanhydrides are a class of biodegradable polymers that have been studied for more than three decades as carriers for drugs, proteins, and vaccines. The degradation products of polyanhydrides are metabolized and either released as carbon dioxide or excreted through urine and feces as carboxylic acids and were found to have no significant impact on kidney or liver functions. Additionally, polyanhydride wafer implants comprised of 1,3-bis-(p-carboxyphenoxy)pentane (CPP) and sebacic acid (SA) have been successfully used in humans to deliver chemotherapeutic drugs to treat glioblastoma multiforme. Recently, amphiphilic
polyanhydride nanoparticles based upon copolymers of SA, 1,6-bis-(p-carboxyphenoxy)hexane (CPH), and 1,8-bis-(p-carboxyphenoxy)-3,6-dioxaoctane (CPTEG) have been explored as a vaccine delivery platform with inherent adjuvant and antigen stabilization properties.\textsuperscript{15,16} These particles provide amphiphilic environments for release of conformationally and functionally stable protein antigens\textsuperscript{16,17} and demonstrate a combination of bulk and surface erosion kinetics that create a controlled release of encapsulated payload, making them ideal candidates for delivery of single-dose vaccines. Recent studies have shown that these polyanhydride nanoparticles exhibit pathogen-mimicking properties in terms of their effect on immune activation, cellular uptake, and cellular persistence.\textsuperscript{18–22} Moreover, single dose vaccination with these particles has induced long-lasting, high titer, and avid antibody responses against multiple immunogens. For example, administration of a single-dose of CPH:SA microparticles encapsulating tetanus toxoid to mice created antibody titers that persisted for at least 26 weeks post-vaccination.\textsuperscript{23} Additionally, immunization of mice with a single dose of ovalbumin-loaded polyanhydride microparticles elicited humoral responses comparable to those induced by a 40-fold greater dose of soluble ovalbumin.\textsuperscript{24} We have also demonstrated the ability of the polyanhydride nanoparticle vaccine platform to induce, in a single intranasal administration, long-lived protective immunity in mice for up to 280 days after an otherwise lethal challenge by \textit{Yersinia pestis}, the causative agent of pneumonic plague.\textsuperscript{21,22}

However, the toxicological effects of these novel polyanhydride nanoparticles, especially when administered via various routes, need to be determined. To complement our previous findings of efficacy, we demonstrate here that polyanhydride nanoparticles result in deposition characteristics similar to those of traditional adjuvants. However, in contrast to traditional adjuvants, immunization with polyanhydride nanoparticles induced minimal inflammatory reactions and little to no tissue damage at sites of injection. Together with previous results, these data demonstrate the biocompatibility and limited reactogenicity of this nanoparticle delivery platform.
2. MATERIALS AND METHODS

2.1. Synthesis and Characterization of Copolymers

CPH and CPTEG monomers were synthesized using the chemicals listed: 4-p-hydroxybenzoic acid, 1,6-dibromohexane, 1-methyl-2-pyrrolidinone, and tri-ethylene glycol. All these chemicals and sebacic acid (99%) were purchased from Sigma Aldrich (St. Louis, MO); 4-p-fluorobenzonitrile was obtained from Apollo Scientific (Cheshire, UK); potassium carbonate, dimethyl formamide, toluene, sulfuric acid, acetic acid, acetonitrile, acetic anhydride, methylene chloride, and petroleum ether were purchased from Fisher Scientific (Fairlawn, NJ). Synthesis of CPH and CPTEG diacids was performed as previously described\textsuperscript{16,25} and prepolymers of SA and CPH were synthesized using previously described methods.\textsuperscript{25,26} Copolymers (20:80 and 50:50 CPH:SA and 20:80 and 50:50 CPTEG:CPH) were synthesized using a melt polycondensation process as detailed by Kipper et al. and Torres et al.\textsuperscript{16,23} respectively. The degree of polymerization, molecular weight, chemical structure, and polymer purity were determined using \textsuperscript{1}H nuclear magnetic resonance (NMR) spectroscopy (Varian VXR-300 MHz, Palo Alto, CA).

2.2. Fabrication and Characterization of Particles

Microparticles were fabricated using a cryogenic atomization method as described in Kipper et al.\textsuperscript{23} Blank and dye-loaded (Kodak X-Sight 640 LSS Dye, NHS Ester Carestream Health, Rochester, NY) nanoparticles (0.5% w/w loading) were fabricated using the anti-solvent nanoencapsulation method outlined in Ulery et al.\textsuperscript{20} Briefly, the copolymer was dissolved in methylene chloride at a concentration of 25 mg/mL at 4°C. The dye was added to the dissolved copolymer and the solution was sonicated for uniform dispersal of the dye within the copolymer. The dissolved dye and copolymer (dye-loaded) or copolymer (blank) solution was rapidly transferred into chilled pentane (−20°C) at a non-solvent to solvent ratio of 80:1, and this solution was vacuum filtered to recover the nanoparticles. Shape and size of the resulting
nanoparticles were characterized using scanning electron microscopy (SEM) (JEOL 840 A, JEOL, Peabody, MA). The particle size distribution was obtained from SEM images using Image J version 1.44 image analysis software. An average of 200 particles per image was analyzed.

2.3. Mice

Female BALB/c mice were purchased from Harlan Laboratories (Indianapolis, IN). Female SKH1-E (hairless) mice were purchased from Charles River Laboratories (Wilmington, MA). All mice were housed under specific pathogen-free conditions where all bedding, caging, water, and feed were sterilized prior to use. Animal procedures were conducted with the approval of the Iowa State University Institutional Animal Care and Use Committee.

2.4. Mouse Treatments

2.4.1. Microparticle and Nanoparticle Biodistribution

Separate groups of BALB/c mice received a subcutaneous (SC) injection of either 0.5 mg of microparticles or 0.5 mg nanoparticles loaded with LSS 640 in 250 μL of saline at the nape of the neck (Figure 1).

2.4.2. Nanoparticle and Adjuvant In Vivo Imaging

To avoid the auto-fluorescence often associated with animal fur, immunocompetent, hairless SKH1-E mice were employed for these studies and assigned to one of six treatment groups: 1.5 mL of sterile saline, 5 mg of nanoparticles (of three different chemistries) loaded with LSS 640 suspended in 1.5 mL of sterile saline, 200 μL of 1:1 sterile saline batched with Imject Alum (Pierce Rockford, IL) or 200 μL of 1:1 sterile saline emulsified in incomplete Freund’s adjuvant (IFA). To administer the same amount of fluorescent dye as present in the polyanhydride particles, LSS 640 was solubilized in saline (6.25 μg/mL). Each mouse was injected SC at the nape of the neck with 2.5 mg of particles in 330 μL saline, 1.75 mg of
particles in 230 μL of saline in the left rear flank intramuscularly (IM) and 0.75 mg in 100 μL of saline IM in the right rear flank. The three nanoparticle formulations used for these studies were 20:80 CPH:SA, 50:50 CPTEG:CPH, and 20:80 CPTEG:CPH. IFA and Alum injections consisted of 100 μL SC at the nape of the neck, 75 μL IM in the left rear flank, and 25 μL IM in the right rear flank. The regimen for the administration of sterile saline with LSS 640 employed the same volumes as the nanoparticle injections. Rotational image capture was performed daily for 7 days after the injection with a Multimodal Animal Rotation System (Carestream Multispectral FX, Rochester, NY). In vivo images were captured while mice were anesthetized with 2% isoflurane in 100% O₂, at 2.5 L/min. Images were captured using 60-second exposures with an excitation filter of 630 nm and emission filter of 790 nm. Rotational images consisted of exposures taken every 25° from 0° to 400° to provide sufficient overlap and create a full 360° rotational image of each mouse. All image analysis was performed using Image J software version 1.44. Raw images were inverted and background subtracted via a rolling ball radius of 150 pixels. The ImageJ lookup table “thal” was applied to the data in Figures 1 and 2. A region of interest (ROI) was constructed and used to analyze mean fluorescence intensity (MFI) at the injection site of all mice for every image. The MFI of the ROI at the injection site of mice receiving particles was compared to the MFI of the ROI at the injection site measured immediately after administration of nanoparticles and the data is expressed as percent of initial fluorescence intensity.

2.4.3. Nanoparticle and Adjuvant Inflammation

SKH1-E mice were injected with blank nanoparticles as outlined in the treatment groups described above. To evaluate the in vivo inflammatory response, mice were intravenously administered 2 nmol of ProSense 750 (VisEn Medical, Woburn, MA) 8 h prior to imaging on days 3 and 7 after the administration of the nanoparticles. ProSense 750 is an activatable fluorescent reagent that is optically visible when the dye is cleaved by degradative enzymes, including cathepsin B, L, S, or plasminogen, that are common at sites of inflammation. After 8
h, *in vivo* images were captured while mice were kept under anesthesia with 2% isoflurane in 100% O₂, at 2.5 L/min. Images were captured using 30-second exposures with an excitation filter of 730 nm and an emission filter of 790 nm. All image analysis was performed using ImageJ version 1.44.²⁷ Raw images were inverted and background subtracted via a rolling ball radius of 150 pixels. The ImageJ lookup table “smart” was applied to the data in Figure 3. ROI analysis of injections sites was performed and mean fluorescence intensity (MFI) was quantified.

### 2.4.4. Nanoparticle and Adjuvant Histological and Biomarker Examination

BALB/c mice were injected with a total of 5 mg of nanoparticles as described above. Other treatments included mice injected with Alum, IFA, or saline. Serum samples were collected from all mice prior to the administration of the nanoparticles and on days 3, 7, and 28 after administration. Serum samples were obtained via saphenous vein bleeds. Serum, tissue, and urine samples were collected at necropsy.

### 2.5. Serum Cytokine Analysis

Serum samples obtained from BALB/c mice were analyzed using a 22-plex chemokine and cytokine antibody array (Millipore, Billerica, MA) measuring the following analytes: G-CSF, GM-CSF, IFN-γ, IL-10, IL-12p70, IL-13, IL-15, IL-17, IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-9, IP-10, KC, MCP-1, MIP-1α, RANTES, and TNF-α. Assays were performed according to the manufacturer’s recommendations; data were acquired and analyzed using a Bio-Plex 200 (Bio Rad, Hercules, CA).
2.6. **Biomarker Analysis**

Serum and urinary biomarkers of kidney and liver function were analyzed using Vitros 5.1 Chemistry Analyzer. Toxicological biomarker values were compared to those reported in Mazzaccara et al.\textsuperscript{20}

2.7. **Histological Evaluation**

Formalin-fixed tissues from BALB/c mice were embedded, sectioned, stained with hematoxylin and eosin (H&E), and blindly evaluated by a board-certified veterinary pathologist (JM Hostetter) for indications of toxicity in liver and kidney and adverse injection site reactions in muscle as well as in the epidermal and dermal tissue. A twenty-point histopathological scoring system for adverse reactions at injection sites was created in which scores of 0–5 were assigned to four independent parameters: inflammation, distribution of inflammatory cell infiltrate, muscle degeneration, and fibrosis. A fifteen-point histopathological scoring system for toxicological damage in liver and kidneys was created in which scores of 0 to 5 were assigned to three independent parameters: inflammation, distribution of inflammatory cell infiltrate, and tissue necrosis.

2.8. **Statistical Analysis**

Cytokine values were $\log_{10}$ transformed and analyzed using repeated measure Analysis of Variance (ANOVA) models. Treatment and time were fixed effects in this model, while mouse was the subject of repeated measures. Histological, weight, and biomarker data were analyzed using one-way ANOVA models with treatment as an explanatory variable. Differences in mean responses among treatments were tested with an overall F-test followed by a post-hoc Tukey's t-test. Statistical tests with $p$ values $\leq 0.05$ were regarded as significant.
3. RESULTS

3.1. Nanoparticles Disseminated Away from the Injection Site Whereas Microparticles Formed a Depot

The estimated mean diameter of the LSS 640 fluorphore-loaded microparticles was 10 μm (±400 nm) (Figure 1A) and that of the LSS 640 fluorphore-loaded nanoparticles was 400 nm (±100 nm) (Figure 1B). These sizes were consistent between particles of all chemistries and with previously published studies.\(^ {19-21,23,30-34} \) The polymers were characterized by \(^ {1} \)H NMR and the molecular weights were determined to be within published ranges.\(^ {32} \)

The effect of size on particle biodistribution was investigated following SC administration of 50:50 CPH:SA nanoparticles or microparticles using live animal in vivo imaging. At four days post-administration, nanoparticles were more widely disseminated in contrast to the microparticles (Figure 1C). Specifically, three dimensional image analysis revealed a single, more intense peak of fluorescence at the injection site of mice receiving microparticles (Figure 1C). In contrast, multiple sites of discrete fluorescence were detected at tissue sites distal to the site of administration for mice receiving nanoparticles. This pattern of distribution observed following administration of the nanoparticles is not associated with the release of the tracer dye because at no time point after administration of the soluble tracer dye was a discrete pattern of fluorescence detected.

3.2. In Vivo Persistence of Nanoparticles Mimicked That of Traditional Adjuvants When Administered Via Parenteral Routes

Traditional vaccine adjuvants, including Alum, are thought to elicit immune-enhancing effects partly by forming antigenic depots.\(^ {3} \) In these studies, live animal in vivo imaging was employed to compare the deposition and persistence of polyanhydride nanoparticle adjuvants to those of traditional adjuvants. SKH1-E mice were administered a total of 5 mg of nanoparticles at three different injection sites as outlined in the methods (Figure 2A). Control mice were
administered saline containing the fluorescent dye (LSS 640), and other mice were administered the LSS 640 dye adsorbed onto Alum or emulsified into IFA. Rotational images of mice were captured at 7-day intervals; a representative image of a mouse from each treatment group is shown in Figure 2B. Mice administered Alum or IFA exhibited depot effects as demonstrated by the focal fluorescence present at the injection site after 7 days (Figure 2B). Each of the nanoparticle formulations provide a depot, however, there were lower amounts of fluorescence emanating from the injection sites, suggesting dissemination and/or erosion of the nanoparticles. Image analysis of injection site regions of interest (ROI) revealed chemistry-dependent effects on particle persistence (Figure 2C). Fluorescence intensity diminished most rapidly (by 2 weeks post-injection) for 20:80 CPH:SA nanoparticles. Based on the fluorescence intensity, 50:50 CPTEG:CPH nanoparticles persisted for 6 weeks post-injection, while the fluorescent signal was detectable at the injection site for 12 weeks post-administration for the 20:80 CPTEG:CPH nanoparticle formulation.

3.3. Polyanhydride Nanoparticle Formulations Did Not Induce Deleterious Injection Site Reactions

To assess the biocompatibility and phlogistic properties of polyanhydride nanoparticles, the magnitude of the host inflammatory response to each nanoparticle formulation was compared to that induced by Alum or IFA. Local tissue inflammation was visualized in SKH1-E mice using ProSense 750, a fluorescent probe activated by enzymes released by inflammatory cells. At 3 days post-injection, the magnitude of the inflammation induced at the SC (nape of the neck) site of administration was significantly greater in IFA treated mice than in mice administered Alum or any formulation of polyanhydride nanoparticles (Figure 3). The IM injection of Alum (left flank) induced a more severe inflammatory reaction than that induced by nanoparticles or IFA. At 7 days post-injection, the relative intensity of the local inflammatory reactions at sites of nanoparticle administration was significantly lower (p ≤ 0.05) than that
observed in mice receiving Alum or IFA. These results indicate that the nanoparticles did not induce inflammation as a cumulative effect of particle persistence or degradation (see Supporting Information (SI), Figure S2). Furthermore, there was little to no evidence of local inflammation at 30 days post-injection for any of the treatment groups, even though the nanoparticles persisted in vivo for up to twelve weeks post-injection (data not shown).

The systemic inflammatory response of mice administered Alum, IFA, or nanoparticles was examined by assaying for inflammatory chemokines and cytokines in the serum at 3, 7, and 30 days post-injection. At 3 days post-injection, levels of the monocyte-recruiting chemokines IP-10 and MCP-1 were elevated in the serum of mice administered CPTEG:CPH nanoparticles, Alum, or IFA compared to saline controls (Figure 4A–B). Of note, the MCP-1 and IP-10 levels remained elevated in mice administered Alum on day 7 post-injection while the levels for mice receiving other treatments were similar to the saline controls (Figure 4C-D). At 30 days post-immunization, all cytokine levels were basal (data not shown).

Because polyanhydride nanoparticles persist in vivo, tissues from SC and IM injection sites were harvested at 30 days post-injection and evaluated for microscopic evidence of tissue damage and inflammation. The highest dosages of adjuvant were administered at the SC immunization site, and it was observed that the tissue response induced by Alum was histopathologically more severe than that induced by the nanoparticles or IFA (Figure 5A). Statistical differences were observed in only the 20:80 CPH:SA groups for both IM tissues sites indicating more inflammation associated with CPTEG:CPH chemistries (Figure 5B–C). Mice immunized with Alum had marked injection site reactions, characterized by muscle degeneration, inflammatory cell infiltrate, and fibrosis. Macrophage infiltrate was observed in all groups at the SC injection site; however, polymorphonuclear cell infiltrate was only noted in tissue recovered from mice treated with Alum, IFA, or 50:50 CPTEG:CPH nanoparticles. Granuloma formation was recorded in five of the six Alum treated mice at the SC injection site.
In contrast, only one mouse (50:50 CPTEG:CPH) was found to have granuloma formation across all the nanoparticle-treated mice.

3.4. Minimal Toxicological Effects on Kidney and Liver Function Following Immunization with Polyanhydride Nanoparticles

Although enhanced tissue distribution of polyanhydride nanoparticles may provide immunological benefits, accumulation of CPTEG:CPH or CPH:SA nanoparticles in the liver or kidney could prove detrimental to normal physiological processes. Therefore, separate groups of mice were administered one of the three nanoparticle formulations, Alum or IFA and the appearance of serological biomarkers of liver or kidney damage was subsequently evaluated.

To measure liver function, total bilirubin, alanine aminotransferase (ALT) aspartate aminotransferase (AST) ratios, albumin, and lactate dehydrogenase were assayed using serum from mice at seven and 30 days post-treatment. Kidney function was evaluated by measuring changes in blood urea nitrogen (BUN), serum creatinine, and albumin in serum samples. For all parameters evaluated, no statistical differences were observed between nanoparticle treated mice in comparison to saline controls (Table 1). Similar results were observed at both 7 and 30 days post-injection, indicating that there was no evidence of adverse responses at acute or chronic times post-treatment (see SI, Table S1).

Liver and kidney tissues were also examined both macroscopically and microscopically to identify structural or histological damage. No statistical differences in liver and kidney weights were observed for any adjuvant-treated mice as compared to controls (Figure 6A and B). Histopathological analysis revealed no significant indications of toxicity, inflammation, or necrosis in livers and kidneys from nanoparticle-treated mice (Figure 6C and D). While one mouse administered 20:80 CPTEG:CPH particles did present with focal signs of hepatic necrosis, the pathologist interpreted this lesion as being consistent with commonly observed tissue changes within the liver of BALB/c mice. Altogether, tissues analyzed from all mice,
regardless of treatment group, revealed normal histological features similar to those observed in the saline treated controls.

4. **DISCUSSION**

Vaccine adjuvants must maintain a delicate balance between eliciting a robust antigen-specific immune response and inducing an adverse reaction at the site of administration.\(^3,4,36–39\) When administered subcutaneously, polyanhydride nanoparticles persisted at the injection site, providing an antigenic depot similar to that provided by Alum and IFA (Figure 2). However, unlike the traditional adjuvants, the nanoparticles provided the added advantage of disseminating throughout the body (Figure 1 and 2). Based on companion studies and the injection of soluble tracer dye, the distinct biodistribution observed was not a result of detecting tracer dye released from the nanoparticles but the dissemination of the nanoparticles themselves. Similar to the systemic spread of a pathogen, the capacity to distribute or disseminate throughout the body further supports the pathogen-mimicking potential of the polyanhydride nanoparticles.\(^18,22\) Measuring the presence of the dye-loaded nanoparticles over time demonstrated that polyanhydride nanoparticles persisted for up to 12 weeks *in vivo*, with polymer chemistry influencing the degree of persistence (Figure 2). Additional analyses evaluated the biocompatibility of the polyanhydride nanoparticle platform by comparing the inflammatory responses, injection site tissue responses, and liver and kidney functions from nanoparticle-treated mice to those of mice administered Alum or IFA (Figures 3 to 6). No indications of inflammation or adverse reactions associated with administration or degradation of the nanoparticles were observed.

In combination with our previous reports of nanoparticle-based vaccine potency and efficacy,\(^19–21,23,32\) the reactogenicity profile presented in this work further supports the *in vivo* use of polyanhydride nanoparticle adjuvants in vaccine formulations. Independent of chemistry, the nanoparticles presented unique biodistribution characteristics not observed with the
microparticles, including their ability to disperse from the injection site. We hypothesize that particle size and chemistry influences both dissemination to distal tissues and more efficient uptake by phagocytic cells that would contribute to tissue distribution and/or persistence of the nanoparticles.\textsuperscript{20,40} The observed persistence of the nanoparticles at injection sites resembles that of Alum and IFA; however, CPTEG:CPH nanoparticle chemistries appear to more readily disperse throughout the body and mimic the distribution patterns of pathogens.\textsuperscript{41–43}

To date, the majority of \textit{in vivo} immunization studies in murine models performed in our laboratories have employed a single 0.5 mg dose of the polyanhydride nanoparticles.\textsuperscript{21,23} The data presented herein demonstrated negligible inflammatory and adverse biological responses in mice subsequent to the administration of 5.0 mg (i.e., 10-fold higher amount) of polyanhydride nanoparticles as compared to Alum or IFA. IP-10, thought to be the chemokine primarily responsible for the efficacy of the yellow fever vaccine,\textsuperscript{44} was elevated in the nanoparticle-treated mice, indicating monocytic recruitment. Collectively, our results indicate that although polyanhydride nanoparticles may mimic pathogens with respect to tissue distribution and phagocytic uptake,\textsuperscript{18–20} they do not, however, induce detrimental tissue destruction caused by extensive inflammatory cell infiltration.

The surface erosion characteristics of the polyanhydride nanoparticles enables them to persist \textit{in vivo} as long or longer than other vaccine formulations. It is, therefore, critical that polyanhydride delivery systems be evaluated for the induction of both acute and chronic adverse toxicological reactions. In the present study, we observed no significant elevation of physiological biomarkers of liver or kidney damage in mice administered a 5 mg dose of nanoparticles (Table 1), suggesting that neither the nanoparticles nor their degradation products were adversely accumulating or altering liver or kidney functions. This finding is in agreement with previous studies where liver and kidney functions were fully preserved after implantation of a poly anhydride wafer used to treat human brain cancer.\textsuperscript{10,45} Together, these results demonstrate the non-toxic and biocompatible characteristics of polyanhydride nanoparticle-
based vaccine carriers and support the hypothesis that their inherent physicochemical properties (e.g., degradation kinetics and pathogen-mimicking ability, leading to dissemination and persistence) positively affect the immune response by inducing long-lived, protective immunity.  

5. CONCLUSIONS

Developing adjuvants that balance enhancing innate and adaptive immune responses while simultaneously limiting the induction of non-specific adverse events are a challenge for vaccinology. The results of this study indicate that administration of larger doses of polyanhydride nanoparticles are more biocompatible than nominal doses of traditional adjuvants such as Alum and IFA. The nanoparticles disseminated rapidly into tissues distal to the administration site, and persisted for as long as 12 weeks, potentially providing a basis for the induction of long-lived immunity. Furthermore, administration of a 5 mg dose of polyanhydride particles resulted in no adverse effects as evidenced by the lack of biomarkers of liver and kidney damage and/or dysfunction, affirming the biocompatibility of these materials. The data herein also outline the inherent adjuvant activity of polyanhydride nanoparticles that stimulates immune responses with minimal lesion development at the site of administration. These data offer an initial standard for translating polyanhydride nanomaterials into use as a vaccine delivery platform for humans and animals.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.
6. TABLES

Table 1. No elevated biomarkers of liver or kidney damage in serum of mice 30 days after injections with polyanhydride nanoparticles. Enzymatic tests, synopsis of test, and normal ranges are indicated in the first three columns. Values are presented as the mean ± SEM.

<table>
<thead>
<tr>
<th>Test</th>
<th>Synopsis</th>
<th>Normal Range$^9$</th>
<th>Treatment Results (Mean ± SEM)</th>
<th>Units</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Liver Function</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total Bilirubin</td>
<td>High levels of total bilirubin indicate diminished liver function due to liver being incapable of sufficiently removing bilirubin.</td>
<td>0.1–0.9</td>
<td>0.46 ± 0.08</td>
<td>Milligrams per deciliter (mg/dL)</td>
</tr>
<tr>
<td>Alanine Aminotransferase (ALT)</td>
<td>Used to detect liver injury and/or diagnose liver disease. Elevation of ALT levels may be elevated when exposed to substances that are toxic and/or decrease blood flow.</td>
<td>29–191</td>
<td>36.2 ± 6.50</td>
<td>International Units per Litter (U/L)</td>
</tr>
<tr>
<td>Albumin</td>
<td>Low albumin levels suggest liver disease.</td>
<td>2.5–4.8</td>
<td>2.49 ± 0.13</td>
<td>g/dL</td>
</tr>
<tr>
<td>Aspartate Aminotransferase (AST)/ALT Ratio</td>
<td>Used in conjunction with ALT measurement for diagnosis of diminished liver function. AST/ALT ratios can be calculated to detect liver disease or decreased function and AST/ALT &lt;1.0 may indicate damage.</td>
<td>&gt;1.0</td>
<td>5.25 ± 0.84</td>
<td>Ratio</td>
</tr>
<tr>
<td>Lactate Dehydrogenase (LDH)</td>
<td>High levels of total LDH may be an indicator of acute or chronic tissue injury.</td>
<td>843–3150</td>
<td>2224.7 ± 192.8</td>
<td>IU</td>
</tr>
<tr>
<td><strong>Kidney Function</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blood Urea Nitrogen (BUN)</td>
<td>Increased BUN is suggestive of impaired kidney function or excessive catabolism.</td>
<td>18–29</td>
<td>15.36 ± 0.59</td>
<td>mg/dL</td>
</tr>
<tr>
<td>Serum Creatinine</td>
<td>Used in conjunction with BUN to assess kidney function. Combination of blood and urine creatinine can be used to assess how well kidneys are filtering small molecules out of blood.</td>
<td>0.1–0.4</td>
<td>0.26 ± 0.02</td>
<td>mg/dL</td>
</tr>
</tbody>
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$^9$Normal range values are acquired from Mazzacaro et al.$^{26}$ and from the Veterinary Clinical Pathology laboratory in the College of Veterinary Medicine at Iowa State University.
7. FIGURES

Figure 1. Nanoparticles disseminated away from the injection site, whereas microparticles formed a depot. Scanning electron photomicrographs of 50:50 CPH:SA (A) micro- (scale bar 20 μm) or (B) nanoparticles (scale bar 2 μm). C) Representative 3D surface plot for a BALB/c mouse 4 days after SC administration of 500 μg of 50:50 CPH:SA micro- (left) or nanoparticles (right) graphed as a function of platform location (x- and y-axes) and fluorescent intensity (z-axis). x- and y-axes represent size (in millimeters) of field of view; z-axis represents fluorescence intensity.
Figure 2. *In vivo* persistence of nanoparticles mimicked that of traditional adjuvants when administered via parenteral routes. A) Schematic of dosing regimen for mice injected with either polyanhydride nanoparticles or traditional adjuvants. B) *In vivo* imaging of mice injected with a total of 5 mg of 20:80 CPH:SA, 20:80 CPTEG:CPH, or 50:50 CPTEG:CPH nanoparticles loaded with fluorescent dye or administered with the fluorescent dye adsorbed onto Alum or emulsified into incomplete Freund’s adjuvant (IFA). Images were captured at 7 days post-injection. Fluorescence intensity calibration bar is located in bottom right corner. C) Fluorescence extinction of dye at injection sites. Data depict the percent of fluorescence intensity of the SC region of interest (ROI) at the nape of the neck as compared to the fluorescence intensity of the initial image taken immediately after administration. n = 2 mice per group.
Figure 3. Polyanhydride nanoparticles induced less inflammation-associated enzymatic activity than traditional adjuvants. At 3 days post-injection, mice were administered ProSense 750, a protease activatable fluorescent imaging agent activated by cathepsins B, L, S, and Plasmin at sites of inflammation. ProSense 750 is optically silent in its inactivated state and becomes highly fluorescent following protease-mediated activation. A) Images were captured 8 hours after ProSense 750 administration. B) Data depict the mean of the fluorescence intensity of the ROI’s of the three injection sites. n = 2 mice per group.
Figure 4. Increased levels of the monocyte-recruiting chemokines IP-10 and MCP-1 in the serum of mice administered either nanoparticles, Alum, or IFA. Concentrations of (A–B) IP-10 and (C–D) MCP-1 in the serum of mice administered a 5.0 mg dose of 20:80 CPH:SA, 20:80 CPTEG:CPH, or 50:50 CPTEG:CPH nanoparticles, Alum, IFA, or saline at 3 (A and C) or 7 days post-injection (B and D). Treatment groups marked with # are significantly different from the Alum treatment group at p < 0.05. n = 6–7 mice per group.
Figure 5. Polyanhydride nanoparticle formulations did not induce deleterious injection site reactions.

Composite histopathological scores of injection site subcutaneous tissue (A), left muscle (B), and right muscle (C) from mice administered 20:80 CPH:SA, 20:80 CPTEG:CPH, 50:50 CPTEG:CPH nanoparticles, Alum, IFA, or saline at 30 days post-administration. Treatment groups marked with # are significantly different from the Alum treatment group at p < 0.05. n = 6–13 mice per group.
Figure 6. Minimal toxicological effects on kidney and liver function following immunization with polyanhydride nanoparticles. A,B) Livers and kidneys from immunized mice were weighed at 30 days post-injection; no significant differences among treatments were observed. C,D) Composite histopathological scores of liver and kidney tissues from mice administered 20:80 CPH:SA, 20:80 CPTEG:CPH, 50:50 CPTEG:CPH nanoparticles, Alum, IFA, or saline at 30 days post-administration; no significant differences from healthy controls were observed. n = 6–13 mice per group.
8. REFERENCES


27. Rasband WS. 1.44 ed., U.S. National Institutes of Health, Bethesda, Maryland, USA.


