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Polyanhydride nanovaccine platform against bacterial pathogens

Shannon Lee Haughney

Iowa State University

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Polyanhydride nanovaccine platform against bacterial pathogens

by

Shannon Lee Haughney

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

Major: Chemical and Biological Engineering

Program of Study Committee:
Balaji Narasimhan, Major Professor
Kaitlin Bratlie
Ian Schneider
Dennis Vigil
Michael Wannemuehler

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This thesis focuses on the design of novel strategies for the prevention and treatment of bacterial infections using polyanhydride nanoparticles as a vaccine delivery platform. The overall goal of this research is to design efficacious vaccines against the respiratory bacterial pathogens *Streptococcus pneumoniae* and *Yersinia pestis* using polyanhydride nanoparticles to elicit a protective immune response to the pneumococcal surface protein, PspA, and the *Yersinia* fusion protein, F1-V, respectively.

Polymers and copolymers based on the various anhydride chemistries (i.e., CPTEG, CPH, and SA) were investigated as nanovaccine formulations for antigen delivery. The mechanism of action of polyanhydride nanoparticles as vaccine adjuvants was investigated to better understand how these nanovaccines interact with immune cells at early time points (48 hours) and through the evaluation of the immune response at extended time points (~several months). Fluorescently-labeled antigen was delivered in 50:50 CPTEG:CPH nanoparticles and compared to soluble protein and protein adjuvanted with MPLA initially. Polyanhydride nanoparticle-encapsulated protein demonstrated enhanced persistence, cellular uptake and immune cell interactions at early time points compared to soluble protein, or MPLA-adjuvated protein. To investigate how prolonged antigen presence affected vaccine efficacy, several polyanhydride chemistries were tested and compared to MPLA at 14, 36, and 63 days after administration. The 50:50 CPTEG:CPH nanovaccine formulation elicited a robust humoral immune response, which significantly increased in titer and avidity at each of the time points investigated, suggesting the presence of long-lived plasma cells as a result of immunization with this polyanhydride nanovaccine.
Once a better understanding of the mechanism of action of polyanhydride nanoparticles was obtained, these findings were used to design efficacious nanovaccines against two respiratory pathogens, *S. pneumoniae* and *Y. pestis*. The encapsulation and release of PspA from polyanhydride nanoparticles was examined and it was demonstrated that PspA retaining its stability, antigenicity, and biological functionality upon release from both 50:50 CPTEG:CPH and 20:80 CPH:SA nanoparticles. Based on these results, the *in vivo* immune response to vaccination with PspA nanovaccine formulations was evaluated and a protective vaccine against lethal challenge with *S. pneumoniae* based on polyanhydride nanoparticles was designed. Additionally, the *in vivo* immune response to vaccination with F1-V nanovaccine formulations was examined to design a protective vaccine against lethal challenge with *Y. pestis* including novel small molecule adjuvants in nanovaccine formulations with the goal of inducing protective immunity against *Y. pestis* challenge at both early time points (~several weeks) as well as after extended periods of time (~several months). Overall, the work described in this thesis lays a platform for the use of polyanhydride nanoparticles for a combination vaccine against both influenza and pneumonia as well as for the delivery of antimicrobial drugs.
CHAPTER 1: INTRODUCTION

1.1 Introduction

Since the development of Edward Jenner’s smallpox vaccine in the early 19th century, science, technology, and medicine have progressed exponentially. Paradoxically, vaccine-preventable infectious diseases remain a significant cause of morbidity and mortality in both the developed and developing world. Acute lower respiratory infections such as pneumonia are the leading cause of death among infectious diseases and the third-highest global killer overall, behind heart disease and stroke\(^1\). Additionally the global burden of pneumonia is high, resulting in the greatest number of disability-adjusted life years lost of all medical conditions considered\(^2\). The world-wide incidence of pneumonia infections is increased by the synergistic effects of viral and bacterial infections. A major cause of morbidity and mortality during each of the major influenza events of the past century has been attributed to secondary pneumonia infections, of which the most common is pneumococcus\(^3^5\). Despite this, research on bacterial pneumonia is exceedingly sparse in comparison to the disease burden, as reported recently\(^6\). Therefore, a strong need exists for increased research on the pathogens that cause respiratory disease with the goal of increased worldwide vaccination against these preventable diseases.

Many microbes are responsible for deadly lower respiratory tract infections. In addition to bacterial infections caused by pathogens such as *Staphylococcus aureus*, *Yersinia pestis*, and *Streptococcus pneumoniae*, viral infections such as influenza and respiratory syncytial virus as well as some fungi contribute to the disease burden\(^7\). Community-acquired pneumonia remains the leading cause of lower respiratory infections, with *S. pneumoniae* being the most common causative agent\(^8\). *S. pneumoniae* is a gram-positive bacterium with greater than 90 known serotypes, which differ in their polysaccharide capsule. This pathogen can result in asymptomatic...
nasopharyngeal carriage, pneumonia, or systemic infection, which may be lethal\textsuperscript{9}. During colonization, \textit{S. pneumoniae} resides in the upper respiratory tract, specifically the naso-oropharynx region. If the bacteria are able to gain access to the sterile lower respiratory tract, the resultant inflammatory response results in the onset of disease, commonly referred to as pneumonia. The systemic spread of the organism, known as bacteremic pneumonia or bacteremia, is a complication that is associated with increased mortality\textsuperscript{9}. It is well documented that pneumococcal carriage is a precursor to systemic bacteremia with many factors that contribute to virulence, including capsular type and the presence of surface proteins such as choline binding protein A, phosphorylcholine, and pneumococcal surface protein A (PspA)\textsuperscript{10}.

Another bacterial pathogen of interest is \textit{Yersinia pestis}, a vector-born zoonotic pathogen transmitted from a rodent reservoir to humans by fleas, which is the etiologic agent of plague, and which over the course of recorded history has killed an estimated 200 million people\textsuperscript{11}. Historically, the most common form of plague infection in humans has been bubonic plague, transmitted through flea bites or direct contact with an infected individual. Septicemic plague may follow resulting in a mortality rate between 50 and 60\% for infections of bubonic plague\textsuperscript{11}. Worldwide plague pandemics have been made increasingly improbable through the introduction of modern sanitation. However, \textit{Y. pestis} remains enzootic in many animal populations worldwide and there is growing concern regarding the use of \textit{Y. pestis} as a bioterrorism agent\textsuperscript{12}. Current treatment for \textit{Y. pestis} infections includes the use of the antibiotics streptomycin and gentamicin, with doxycycline as an emerging alternative therapy. Fluoroquinolones such as ciprofloxacin have demonstrated success in research settings, but have not been widely tested in humans\textsuperscript{13}. Additionally, treatment must be administered rapidly after the onset of symptoms for optimal efficacy\textsuperscript{13}.
Respiratory infections are particularly common, in part due to the unique role the lungs play in interacting with the environment. The lungs must constantly intake air from the surrounding environment, which is more often than not, contaminated with microbial pathogens and other inert nano and micro-scale materials. The host defenses of the lung must therefore determine when a threat is present and respond accordingly. There are many defense mechanisms within the lung that can remove inert particulate matter and recognize pathogens such as bacteria. The first innate immune defense systems of the lung are non-specific factors such as the mucocilliary escalator and the cough reflex. The cellular component of the pulmonary innate immune response is mediated by resident and recruited cells including epithelial cells of the respiratory tract and leukocytes such as macrophages, dendritic cells, and neutrophils. The innate humoral component of the pulmonary immune system includes antimicrobial factors, including lysozyme, lactoferrin, collectins, and defensins, that are secreted into lung surfactant\textsuperscript{14}. It is often this innate immune response that leads to pneumococcal disease through the increased inflammation caused by the recruitment of immune cells. This unique immunological environment presents a number of challenges when designing vaccines against respiratory diseases such as \textit{S. pneumoniae} and \textit{Y. pestis}.

There are currently two widely used vaccine formulations against \textit{S. pneumoniae}: the 23-valent polysaccharide vaccine and the 7-valent pneumococcal conjugate vaccine\textsuperscript{15,16}. Both of these vaccines have limitations that affect their efficacy. The polysaccharide-based vaccine does not effectively provide protection in young children and the elderly\textsuperscript{16}. The 7-valent conjugate vaccine avoids this problem, but is not ideal because it does not provide cross protection across strains with capsular phenotypes that have not been included in the vaccine. This inadequacy has been observed to lead to serotype substitution within a few years of vaccine introduction\textsuperscript{17}. 
Furthermore, the polysaccharide-protein conjugate vaccine is difficult to produce leading to high cost and limited supply, precluding its use in developing countries. Next-generation vaccine research has focused on using \textit{S. pneumoniae} surface proteins as protective antigens. One such protein is pneumococcal surface protein A (PspA), a choline-binding protein found on the surface of all pneumococcal strains, which has been shown to be critical to the virulence of infections with \textit{S. pneumoniae}. Vaccination with PspA has been shown to be protective against a lethal challenge with \textit{S. pneumoniae} making it a promising candidate for use in an improved vaccine. In addition, PspA can be economically synthesized as a recombinant protein and the protein-based vaccine avoids the loss of antigenicity observed with the polysaccharide-based vaccine.

Despite the threat of antibiotic resistance, both naturally-occurring and engineered, and the potential for use as a biological warfare agent, there is no commercially licensed plague vaccine available in the United States and Europe. There is currently a live, attenuated strain of \textit{Y. pestis}, EV76, which has been in use as a vaccine in the countries of the former Soviet Union and Asia, but it is not commercially available in the United States and Europe. Due to the safety concerns of live, attenuated \textit{Y. pestis} vaccines, much of the emerging research has focused on the identification and testing of protective protein subunits of the \textit{Y. pestis} bacterium. A F1-V subunit based on polyanhydride nanoparticles has recently been demonstrated to provide protective immunity against lethal challenge with pneumonic plague. The polyanhydride nanovaccine formulation provided 100% protection against challenge with CO92 \textit{Y. pestis}, with both soluble protein alone and protein adjuvanted with MPLA failing to elicit a protective immune response. These results suggest the potential for an effective, easily synthesized vaccine against \textit{Y. pestis}. 
Unlike live attenuated vaccines, most non-replicating vaccines, including subunit vaccines based on recombinant proteins, such as PspA and F1-V, are poorly immunogenic when administered alone. Therefore, in order to generate protective immunity, an adjuvant must be used to safely and effectively boost the immune response against the protein. Currently, there are only two widely used licensed vaccine adjuvants: alum, a mixture of aluminum salts, and MF59, a squalene-based oil-in-water emulsion adjuvant approved for human use in Europe. Neither is approved for mucosal administration, an effective option against respiratory pathogens because of the induction of mucosal immunity, and other benefits such as improved patient compliance through needle-free administration. Recently, nano-carriers have been studied for their potential use in pulmonary drug delivery. The small particle size leads to increased surface area and the ability to evenly distribute the inhaled dose to the alveoli.

Polyanhydrides are a class of synthetic biodegradable polymers that can be formulated into nanoparticles for mucosal administration. These materials have many additional properties that make them ideal candidates for vaccine delivery, including the ability to stabilize and protect fragile protein antigens, provide sustained release of antigen, and modulate and enhance the immune response. These polymers have excellent biocompatibility and have been shown to have non-toxic, non-mutagenic degradation products. The Gliadel® wafer, a post-surgical implant composed of a copolymer of sebacic acid and 1,3(bis-p-carboxyphenoxy)propane, is FDA-approved for the treatment of brain cancer. Polyanhydrides are characterized by a backbone of monomeric dicarboxylic acids (diacids) connected by anhydride linkages which are hydrolytically labile and cleaved in the presence of water to form carboxylic acid moieties. Polymers and copolymers based on sebacic acid (SA) (Figure 1.1), 1,6(bis-p-
carboxyphenoxy)hexane (CPH) (Figure 1.2) and 1,8(bis-p-carboxyphenoxy)-3,6-dioxaoctane (CPTEG) (Figure 1.3) have been shown to be effective vaccine adjuvants\textsuperscript{24,34–36}.

Proteins, such as PspA and F1-V, are fragile molecules, comprised of intricate hierarchical structures that are prone to physical and chemical degradation\textsuperscript{37}. When using protein antigens, it is imperative to have a delivery device that can prevent antigen degradation and as a result preserve functionality and antigenicity upon release. Polyanhydride particles have been shown to successfully preserve antigenicity during fabrication, storage, and release\textsuperscript{38,39}. Synthesis of polyanhydride particles using a non-aqueous process can prevent protein degradation due to prolonged exposure to water, and also maintain high encapsulation efficiency, in contrast to aqueous processes\textsuperscript{37}. The penetration of water into the bulk of a polymeric device determines the erosion method, which can be classified as either bulk or surface erosion. Biodegradable polymers, including polyesters such as PGLA and polyethers such as poly(ethylene oxide), undergo bulk erosion, indicating that water is easily able to penetrate into the bulk of the device\textsuperscript{40}. This influx of water into the core of the delivery device has been shown to lead to protein degradation and a loss of antigenicity in some cases\textsuperscript{41,42}. The hydrophobic chemistry of polyanhydrides prevents this bulk penetration of water, both protecting the payload and leading to controlled surface erosion and zero-order payload release kinetics\textsuperscript{43,44}.

Polymeric vaccine adjuvants, such as polyanhydrides, also provide sustained release of antigens as the polymer erodes. The time scale and mechanism of polymer erosion depends on a number of factors including polymer chemistry, hydrophobicity, and molecular weight\textsuperscript{45}. The persistence of particles, and, by extension, antigen, may have an advantageous effect on vaccine efficacy. In non-replicating vaccines, such as protein subunit vaccines, persistence may serve to mimic the effect of a replication-competent vaccine, such as a live attenuated vaccine. Similarly,
polyanhydride nanoparticles can activate the immune response and act as a vaccine adjuvant\textsuperscript{28,46,47}. It has been shown that polyanhydride nanoparticles possess pathogen-mimicking properties and thus can activate the innate immune response by stimulating pattern recognition receptors (PRRs) on the surface of antigen presenting cells, specifically dendritic cells\textsuperscript{46}. In addition to the stimulation of the innate immune response, it has also been shown that vaccination using polyanhydride nanoparticles as an adjuvant can enhance the adaptive immune response as measured by antibody titer and avidity\textsuperscript{24}. Polyanhydride particles can also be used to modulate the immune response\textsuperscript{48}. There are a number of ways that polyanhydride particles can be modified in order to target specific immune cells including particle size, chemistry, and surface modification. It has been suggested that increased oxygen in the polymer backbone by using CPTEG-rich chemistries (Figure 1.3) leads to increased recognition by PRRs\textsuperscript{46}.

The overall goal of this research is to design efficacious vaccines against the respiratory pathogens \textit{S. pneumoniae} and \textit{Y. pestis} based on polyanhydride nanoparticles. Polymers and copolymers based on CPTEG, CPH, and SA were investigated as nanovaccine formulations for antigen delivery.

1.2 References


1.3 List of Figures

Figure 1.1. Chemical structure of sebacic acid (SA)

![Chemical structure of sebacic acid (SA)](image1)

Figure 1.2. Chemical structure of 1,6-(bis-p-carboxyphenoxy)hexane (CPH)

![Chemical structure of 1,6-(bis-p-carboxyphenoxy)hexane (CPH)](image2)

Figure 1.3. Chemical structure of 1,8-(bis-p-carboxyphenoxy)-3,6-dioxaoctane (CPTEG))

![Chemical structure of 1,8-(bis-p-carboxyphenoxy)-3,6-dioxaoctane (CPTEG))](image3)
CHAPTER 2: LITERATURE REVIEW

2.1 Abstract

Traditional vaccine design, as exemplified by the work of Edward Jenner, is centered on the principle of introducing or mimicking a mild pathogenic infection to train the immune system to protect against a subsequent severe pathogenic infection. As such, the optimal training regimen for the immune system against pathogens of different tissue tropisms is to mimic a pathogenic infection in that same tissue site. Respiratory pathogens remain a significant cause of morbidity and mortality because of their ease of transmission, population dynamics of infection, and the social and economic impact. There is an unmet need for highly effective vaccines against respiratory pathogens. However, the mucosal surface of the lungs presents a unique and tightly regulated immunological environment that is highly adverse to inflammation. Therefore, when designing a pulmonary vaccine to elicit a mucosal immune response in respiratory tissues, minimizing reactogenicity is paramount. Adjuvants can provide enhanced immune responses against the pathogens of interest but must straddle the line between engaging the immune system and avoiding detrimental immunopathology. Insights into immunological mechanisms of innate and adaptive immunity have led to myriad applications in pulmonary adjuvant design. Herein we attempt to outline, albeit non-exhaustively, and review advances in pulmonary adjuvant technologies by combining information about lung biology at the cellular and molecular levels with advances in nanotechnology and polymer chemistry.
2.2 Introduction to Pulmonary Immunology

The human body constantly encounters infectious pathogens and non-infectious particulate matter. It is incumbent upon the body’s natural defenses to determine when a true threat is present and respond appropriately. The epithelium, shown in Figure 1, is typically the first surface to have contact with an infectious pathogen. This may be the external epithelium, or skin, in the case of pathogens transmitted through physical contact, such as some fungi or insect bites. The internal epithelium, which consists of mucosal surfaces such as the respiratory, reproductive, and gastrointestinal tracts, acts as the first line of defense against pathogens transmitted through aerosolized droplets, sexually, or through tainted water or food, respectively. Both the external and internal epithelia have unique properties that make these surfaces effective barriers against invading pathogens. Made up of tightly bound epithelial cells, both the surface and internal epithelia secrete numerous antimicrobial factors. Internal mucosal surfaces are covered in a layer of mucosa that may help prevent the deposition of microbes onto the epithelium itself. The respiratory tract, for example, contains what is known as the mucociliary escalator, which expels microbes through the beating of epithelia associated cilia.

Few microbes are able to pass through these epithelial barriers and replicate in the host. Those that do are encountered first by phagocytic innate immune cells and a chemically mediated system of proteins known as complement. These initial encounters release a chemical signaling cascade in which cytokines and chemokines recruit additional innate immune cells in a process known as recruited innate immunity. This process is able to eliminate many pathogens that are able to evade the initial defenses of the body’s epithelial tissues. However, some microorganisms are able to overwhelm or evade the initial and recruited innate immune responses and persist as a replicating infection. This is where the adaptive immune system has
evolved to mount a highly specific cellular response based on the clonal selection of lymphocytes. This is a delayed process which may take 4-7 days to develop, during which time the resident and recruited immune cells play a crucial role in directing the adaptive immune response and controlling the infection.

Because of the unique interaction of the lungs with the outside environment, infections within the mucosal surfaces of the respiratory tract pose a unique set of challenges for the immune system, which must distinguish between countless particulates aspirated through normal breathing and potentially dangerous invading microorganisms. The lungs provide mechanisms of defense against pathogens beginning with resident innate immune defenses. Then, if necessary, they recruit additional innate immune defenses, which serve to direct the development of microbe-specific adaptive immunity. The lungs contain a delicate environment that is highly susceptible to inflammation-induced damage, which can often be a product of dysregulated innate immune responses to infections. Because of this, the development of mucosal immunity to pathogens is often an important factor in preventing infection-associated pathology. In this section, the development of the immune response to an infection within the unique pulmonary environment from initial or resident innate immune responses to recruited innate immune responses, the development of adaptive immunity, and the generation of a memory response are discussed.

2.2.1 Resident innate immune response

Innate immunity represents the earliest stages of the host response against an infection. Germ line-encoded and constantly present in all healthy individuals, innate immunity does not increase or develop with repeated exposure to a pathogen, but rather discriminates based on common
features of a group of pathogens, using microbe associated molecular patters (MAMPs). Innate immune responses are particularly important in the context of the lung because of the constant interface between the lungs and the environment. The lungs inhale as many as 15 breaths per minute containing up to 500 mL of air each, which is ubiquitously contaminated with inert particulates and potentially dangerous microbes, at over 1000 colony forming units (CFUs) of bacteria per cubic meter in a university auditorium. The upper respiratory tract can become asymptomatically colonized with a number of commensal bacteria, typically resulting in the formation of a biofilm. These inert infections may become serious if the pathogen is able to reach the sterile lower airspaces of the lungs through the aspiration of air from the upper airways, which is common during sleep, or due to pathogen-associated virulence factors.

When a microbe is able to reach the lower airspaces, the lungs present a number of resident defense mechanisms, including mechanical, antimicrobial, and cellular responses to infectious organisms. The first line of defense against invading organisms is the epithelial layer itself, which separates the lung lumen from the basolateral surface. The epithelial layer is made up of many different cell types (up to eight), which have been classified into three categories: basal, ciliated and secretory, with ciliated columnar epithelial cells being the most common (up to 50%). Basal cells are thought to be a common progenitor, giving rise to secretory cells such as mucus cells, goblet cells, serous cells and Clara cells, and to terminally-differentiated ciliated cells. Immune cells such as inflammatory mast cells and phagocytic cells such as dendritic cells (DCs) and macrophages can also reside within the epithelium. Mechanical defenses within the lung include the cough reflex and the mucociliary escalator, which uses ciliated columnar epithelial cells to expel inhaled particulates. This network of cells extends from the larynx to the terminal bronchioles. Ciliated airway epithelial cells are punctuated by secretory cells such as
goblet cells, serous cells, and Clara cells at a ratio of about five to one, which cover the epithelial layer with a film of mucus. The cilia beat together at a rate of 700-1000 beats per minute to expel particulate matter to the upper respiratory area, where it is swallowed. Mucociliary clearance is further augmented by the cough reflex.

As mentioned above, the respiratory tract is coated by a layer of mucus that has been shown to have inherent antimicrobial activity due to its slightly acidic composition (pH 6.4-7.3) and the presence of a number of antimicrobial peptides and proteins. Pulmonary secretions are generated by airway and alveolar epithelial cells, sub-mucosal glands and phagocytic cells such as neutrophils and macrophages. Antimicrobial proteins found in the pulmonary surfactant include lysozyme, lactoferrin, secretary leukoprotease inhibitor, and human defensins and cathelicidins. Of these molecules, lactoferrin and lysozyme are the most abundant.

Lactoferrin is secreted by both serous cells and neutrophils, and binds iron, reducing the ability of bacteria to replicate. Lysozyme is secreted by serous cells and neutrophils as well as macrophages and leads to the hydrolysis of peptidoglycan, a component of gram-positive bacteria. It is also important here to note the role played by the complement system, which opsonizes pathogens and leads to increased phagocytosis by immune cells.

In addition to the mechanical and antimicrobial defenses within the lung, cell-mediated innate immunity plays an important role in the clearance of pathogens as well as in generating an inflammatory response to recruit additional immune cells. Macrophages are present in both the interstitial tissue and in the alveolar spaces and play an important role in the pulmonary immune response through the phagocytosis of pathogens or particulates, and when necessary through the secretion of inflammatory cytokines and antimicrobial compounds such as reactive oxygen and nitrogen species (ROS and RNS, respectively). Mast cells are located at epithelial surfaces...
throughout the body and express Toll-like receptors which can detect pathogens. These cells also release inflammatory molecules to recruit additional cells and can serve to direct the adaptive immune response. Other important resident cell types within the lung include DCs, which are professional antigen presenting cells (APCs) that internalize and present pathogens to lymphocytes along with providing activation signals to stimulate the adaptive immune response. Neutrophils phagocytize and kill invading pathogens and eosinophils are associated with allergic inflammation.

2.2.2 Recruited innate immunity

When the resident pulmonary defenses are not able to clear a replicating infection, additional immune cells are recruited. The epithelial cells lining the respiratory tract have been shown to be responsive to the presence of microorganisms, leading to the induction of a recruited innate immune response. Though not typically thought of as immune cells, airway epithelial and endothelial cells have been shown to interact with MAMPs through a number of pattern recognition receptors (PRRs) including CD14, Toll-like receptors, and intracellular adhesion molecules (ICAMs). Airway epithelial cells can also be stimulated by cytokines through tumor necrosis factors (TNFs) and interleukin 1 (IL-1) receptors. Activated epithelial cells are able mount an inducible response to an infection in two ways: by increasing production of antimicrobial polypeptides, such as lysozyme and lactoferrin, and through the secretion of cytokines and chemokines to recruit additional immune cells to the site of infection, resulting in an inflammatory response. These cells have been shown to increase levels of CCL2 (MCP-1) in response to infection with *Burkholderia pseudomallei*, leading to the recruitment of monocytes, lymphocytes, and DCs to the site of infection.
In addition to the airway epithelial cells that make up the mechanical structure of the respiratory tract, other resident cells play an important role in the recruited innate immune response to pulmonary infection, including macrophages, mast cells, and DCs. Activated macrophages play an important role in recruiting additional phagocytic cells to the lungs. Macrophages within the lung may be classically or alternatively activated by the presence of IFN-γ and TNF or IL-4, respectively\textsuperscript{22}. Classically activated pro-inflammatory macrophages play an important role in the innate immune response to infection and are stimulated by IFN-γ produced by natural killer (NK) cells and TNF\textsuperscript{23}. These cells secrete cytokines, including IL-1, IL-6, and IL-12, IL-23, and TNF\textsuperscript{22,23}, and have been demonstrated to recruit monocytes, lymphocytes, and eosinophils to the site of infection through CCL15 and NK and T cells through CXCL9, 10 and 11\textsuperscript{24}. Alternatively activated macrophages are activated by IL-4 released by basophils or granulocytes and play an important role in wound healing, though they have been shown to be associated with the development of a T\textsubscript{H}2 adaptive immune response, in comparison to classically activated macrophages, which have been shown to direct to a T\textsubscript{H}1 type adaptive response\textsuperscript{23}.

Recruited innate immunity is an especially important consideration in infections such as pneumonia, where disease occurs as a result of an increased inflammatory response within the lung. Additionally, the cytokines released and cell types recruited during this phase of the immune response play a large role in informing and directing the adaptive immune response to the infection. As stated above, the polarization of activated macrophage phenotype can play a large role in the type of adaptive immune response with classically activated macrophages inducing a more T\textsubscript{H}1-type response through the increased secretion of IL-12 and alternatively activated macrophages recruiting T\textsubscript{H}2 cell types, which has been attributed to CCL22.
expression\textsuperscript{23,25}. In addition, the antimicrobial and phagocytic activity of resident and recruited innate immune cells plays an important role in controlling infection during the development of an adaptive immune response, which is highly specific and relies on a process of clonal selection which may take days or a week to become functional.

2.2.3 Adaptive response

Few pathogens are able to evade or overwhelm the innate immune responses. Those that do persist lead to the induction of an adaptive immune response. This process is mediated by chemical signals from the resident and recruited innate immune responses to an infection. Adaptive immunity is a time-dependent process that can take days to a week to reach its full potential\textsuperscript{14}. There are two interrelated branches of adaptive immunity that both play important roles in the response to a pulmonary infection: cell-mediated and humoral (or antibody-mediated) immunity.

Cell-mediated immunity within the lung focuses on the interactions of T cells, NK cells, and DCs. Although NK cells do not exhibit RAG-dependent receptors, meaning adaptive immune receptors, like B and T lymphocytes, this cell type plays an important role in the development of the pulmonary adaptive immune response, specifically to viral pathogens\textsuperscript{26}. The pulmonary adaptive immune response begins with immature myeloid DCs (mDCs), which transport antigen from the lung to the lung-associated lymph nodes. Immature DCs reside in the healthy lung and are able to phagocytize microorganisms, but do not typically display antigen on the cell surface. Two phenotypes of these resident DCs are especially important in the pulmonary immune response: mDCs and plasmacytoid DCs (pDCs), which are derived from bone-marrow monocyte precursors and lymphoid precursors, respectively\textsuperscript{27}. The distinction between these two cell types
is important due in large part to the different TLR expression of each, with TLRs 1, 2, 3, and 4 expressed on mDCs, responding to viral RNA and LPS, and TLRs 7 and 9 expressed in pDCs, responding to bacterial DNA. In order to develop into a professional APC, DCs must be stimulated through TLR ligation or by the secretion of cytokines, at which point the cells will migrate to the lymph node and mature. DCs play the important role of instructing the immune response through interactions with T cells, with possible outcomes of a T_H1, T_H2 or regulatory T cell-type response, as classified by the CD4 cell subset elicited.

After entering the lymph nodes through the afferent lymphatics, DCs encounter naïve T cells, which have been recruited by the secretion of cytokines from activated DCs. These T cells recognize antigen displayed on the surface of DCs in the context of either major histocompatibility complex (MHC) class I or II, based on their co-receptor phenotype with CD8+ T cells recognizing the former and CD4+ T cells the latter. In this way, the innate immune response of the APC serves to direct the adaptive immune response based on the properties of the pathogen, with intracellular pathogens being degraded in the cytosol and presented on MHC class I and extracellular pathogens being degraded in endocytic vesicles and presented on class II. CD8+ or cytotoxic T cells act to seek out and kill APCs or other cells of the body which display antigen presented in MHC class I. This type of cellular adaptive immune response is considered to be of increased importance in viral and intracellular infections. CD4+ T cells function to activate other effector cells such as B cells and macrophages. These cells are classified into two types, T_H1 and T_H2 cells, which serve to activate macrophages and initiate an inflammatory response and activate B cells to produce antibodies, respectively.

In addition to directing the T cell immune response to infection, DCs play an important role in the activation of the humoral immune response through interactions with B lymphocytes.
In an adaptive immune response, there are two types of antigen-specific cellular receptors, T cell receptors and immunoglobulins, which act as B cell receptors. As discussed above, DCs activate and direct the T\textsubscript{H}2 helper cell response, which then induces B cell expansion and the production of antibodies. Antibodies serve a number of functions in the adaptive immune response, including opsonization and the activation of complement, activation of effector cells, and neutralization to block an essential function of a pathogen. There are five classes of immunoglobulins that differ in their C regions, IgA, IgD, IgE, IgG, and IgM. Of these, the most important molecules from the perspective of the pulmonary immune response are IgG, typically associated with antibody-mediated immunity, and IgA, which is found in mucosal areas such as the respiratory tract and may prevent colonization through the ability to cross the epithelial layer\textsuperscript{29}.

The adaptive immune response takes place in the lymph nodes, a specialized tissue which provides an environment for APCs to interact with T cells and B cells. These organs are organized with sub-sections for T cells and B cells around a germinal center. B cell follicles are located near the outer capsule with T cell zones located on the interior in the paracortical areas. Lymphocytes home to the lymph nodes from the blood, entering through high endothelial venules (HEVs) into the T cell zones. B cells also enter through this T cell zone and migrate to B cell follicles, where they encounter antigen and become activated\textsuperscript{2}. In addition to the draining lymph nodes (i.e., the mediastinal lymph nodes in the case of pulmonary environment), epithelial tissue such as the respiratory tract often has associated lymphoid tissues. This highly organized area is known as the mucosal associated lymphoid tissue or MALT\textsuperscript{29}. MALT is organized similarly to a lymph node, with germinal centers of T cell areas and a B cell follicle. This
specialized pulmonary tissue plays an important role for the endogenous presentation of antigen to lymphocytes driving the adaptive immune response.

2.3 Review of Licensed Pulmonary Vaccines and Adjuvants

The vaccinia virus, the causative agent of smallpox, was a ubiquitous pathogen believed to originate around 10,000 BC, and has been discovered to have been present in a number of early cultures including India, Egypt and China and frequently affected Europe during the middle ages. During this time it was common knowledge that survival of the disease conferred immunity against future infections. This understanding led to the practice of inoculation (or variolation), where material from an infected individual was injected subcutaneously into a naïve patient. Believed to have originated in Turkey and championed in Europe by Lady Mary Wortley Montague, this practice had a number of risks, including transfer of other blood borne diseases and the possibility of dissemination of the subcutaneous injection of virus. The next major development in the fight against smallpox came at the end of the 18th century, when Edward Jenner hypothesized that deliberate infection with cowpox could confer immunity against subsequent infections with smallpox. Jenner’s discovery is widely credited to be the first instance of vaccination, and two hundred years later in 1980, the World Health Assembly announced the successful eradication of smallpox

Since its introduction, vaccination has proved to be one of the greatest human health triumphs of modern times. After the introduction of the smallpox vaccine in the late 1700’s, the 19th century saw the introduction of vaccines against rabies, typhoid, cholera and plague, with the introduction of vaccines against many more diseases in the 20th century, including tuberculosis, pertussis, diphtheria toxoid, influenza, polio, measles, mumps and rubella, to name
a few. Despite the many technological advances during the 19th and 20th centuries, the field of vaccine design has not changed much since the introduction of the smallpox vaccine. Most vaccines rely on a timely process of trial-and-error to either remove the virulence factors or improve the immune response to a killed pathogen. This section will attempt to summarize current vaccine technologies and discuss recent advances in the field of vaccine design, specifically the use of biodegradable polymer particles for vaccine delivery, which may lead to improved efficacy, reduced production time, and lower cost.

2.3.1 Vaccine classifications

Most similar to the original use of the cowpox virus to vaccinate against smallpox, live attenuated vaccines rely on using living organisms from which the virulence factors have been selected against. These vaccines are able to replicate in the host, and therefore, often able to successfully confer protective immunity without the need for an adjuvant or a multiple-dose regimen. Currently used commercially available live attenuated vaccines include the measles, mumps and rubella vaccine (MMR combined vaccine) consisting of live attenuated strains of the three pathogens, the varicella (chicken pox) vaccine, the Sabin oral polio vaccine, the Mycobacterium bovis (BCG) vaccine against Mycobacterium tuberculosis, and the live influenza vaccine (Flu-mist nasal spray). The success of the Sabin oral polio vaccine is a prototypical example of the benefits of a live vaccine. The attenuated organism is delivered orally, and like a live infection, it is replicated in the gut for a period of several weeks. Additionally, the fecal-oral transmission route led to the passive immunization of persons in close contact with the vaccinated patient. While these live attenuated vaccines have been effective, this strategy is costly and inefficient, relying predominantly on a trial-and-error approach to vaccine design. In
addition, live attenuated organisms may not produce an effective immune response against chronic pathogens, which have evolved to avoid detection by the host immune system\textsuperscript{33}. Conversely, in immune-compromised patients, an attenuated strain, cleared quickly in healthy patients, may lead to a persistent infection or cause disease. Additionally, even with the ability to replicate in a host, live attenuated vaccines have been reported to require multiple doses to induce long-lived immunity. A single dose of the MMR combination vaccine has been reported to lead to outbreaks of measles and mumps among highly vaccinated populations with most countries implementing a compulsory second dose\textsuperscript{34}.

Killed vaccines consist of a whole non-attenuated pathogen that has been killed or otherwise inactivated through the use of heat, radiation or chemicals. Due to the retention of the surface PAMPs of the organism, killed vaccines are often able to induce a strong humoral immune response without the associated risks of administering a live pathogen to patients. Additionally, killed vaccines do not require the multiple in vitro passes to eliminate virulence as live attenuated vaccines do and have been shown to be less sensitive to storage and temperature fluctuations than their live counterparts. Killed/inactivated vaccines in current use include the Salk polio vaccine, the trivalent seasonal influenza vaccine, the typhoid, cholera and pertussis vaccines and the current vaccine against plague. While these vaccines maintain the antigen in its native state, i.e., not attenuated, the organisms are not able to replicate in the host like their live counterparts, often leading to reduced immunogenicity and the need for multiple doses or the use of an adjuvant. Despite being potent initiators of humoral immunity, killed vaccines are often poor at inducing a cellular response, making them ineffective against many intracellular pathogens. Finally, like live vaccines, the microbial components of killed vaccines such as LPS
and other inflammation-associated MAMPs remain intact, which has been shown to lead to
discomfort and adverse reactions associated with vaccination\textsuperscript{35}.

Subunit vaccines involve identifying a specific antigen from an infectious microorganism that is
able to elicit protection. Subunits may be a polysaccharide, protein, or DNA. Current subunit
vaccines that are available to the public include the 7 and 13-valent pneumococcal conjugate
vaccines, the meningococcal and the \textit{Haemophilus influenza} type B vaccines that utilize the
bacterial polysaccharide capsule conjugated to a protein, the hepatitis B vaccine, and vaccines
against human papilloma virus, which are composed of self-assembling recombinant proteins
which make up the viral capsid. Subunit vaccines have the advantage of being able to be
delivered through a number of different routes which may mimic the route of infection.
Additionally, by using only a component of a pathogen, subunit vaccines, avoid exposure to
microbially associated compounds such as LPS, which may cause adverse reactions through
innate immune triggering of inflammatory responses. Like killed vaccines, subunits from
microorganisms such as proteins or DNA are typically poorly immunogenic and require the use
of an adjuvant to be effective\textsuperscript{36,37}. In addition, these vaccines, often based on recombinant
proteins, can be expensive to produce\textsuperscript{38}.

2.3.2 Vaccine adjuvants

Vaccines that cannot replicate in the host such as killed vaccines and subunit vaccines are often
poorly immunogenic, and therefore require the use of an adjuvant to boost the immune response
to the antigen. Adjuvants have been added to vaccines to improve efficacy since the early 20\textsuperscript{th}
century\textsuperscript{39}. It has been proposed that adjuvants can act to improve the immune response by five
mechanisms\textsuperscript{7}. The first, immunomodulation, upregulates the immune response through the
cytokine network and may skew the immune response to a T\textsubscript{H}1 or T\textsubscript{H}2 immune phenotype. The second, preservation or presentation, refers to adjuvants that can protect the payload and that are able to display intact antigen to cells in its native state. Thirdly, adjuvants can act to induce cell-mediated or cytotoxic T lymphocyte (CTL) responses. Fourthly, vaccine adjuvants can be designed to target effector immune cells. For example, carbohydrate adjuvants target C-type lectin receptors on macrophages and DCs\textsuperscript{40}. Finally, vaccine adjuvants may improve the immune response through the generation of a depot for antigen, leading to the potential for a single-dose vaccine regimen\textsuperscript{39}.

### 2.3.2.1 Aluminum salts

The first, and most commonly used, licensed vaccine adjuvant is based on aluminum salts, or Alum, and has been used in vaccines for over 70 years\textsuperscript{39}. First reported to successfully boost the immune response to diphtheria toxoid in 1926, this adjuvant is characterized as aluminum oxyhydroxide (AlOOH) and aluminum hydroxyphosphate (Al(OH)\textsubscript{x}(PO\textsubscript{4})\textsubscript{y})\textsuperscript{41}. It has been suggested that aluminum salt adjuvants the immune system by two mechanisms: the generation of an antigen depot and immune stimulation\textsuperscript{41}. The mechanism of the depot effect is explained via the trapping of soluble antigen in the aluminum matrix through adsorption, which is thought of to extend the ability of antigen to drive the immune response\textsuperscript{42}. Additionally vaccines adjuvanted with aluminum salts have been shown to stimulate the complement system and activate macrophages and eosinophils\textsuperscript{43}. These adjuvants tend to induce only a modest T\textsubscript{H}2 phenotype immune response, making for ineffective vaccines against viruses and intercellular bacteria. While generally considered safe and widely used in human vaccines, the production of IgE antibodies, associated with allergic responses, have been reported with the use of vaccines
formulated with mineral salts. Toxic buildup of aluminum has also been reported in patients who are unable to metabolize the mineral effectively, resulting in neurological symptoms. Additionally, mineral salts are only effective in intramuscular delivery applications, making it ineffective against mucosal-associated pathogens which may require vaccine delivery to the site of infection for optimal efficacy. Calcium, iron and zirconium have also been investigated for the adsorption of antigens, with far less use than aluminum, despite the advantages of reduced neurological and allergic effects. However due to the increased recruitment of monocyte and granulocyte cell populations, aluminum salts may not prove an appropriate adjuvant for the delicate immune environment of the lung.

2.3.2.2 Adjuvant emulsions

The next vaccine adjuvant to be approved for human use since the introduction of alum is the oil-in-water emulsion adjuvant, MF59. MF59 consists of squalene, a steroidal precursor to cholesterol in humans, in citrate buffer stabilized by polyoxyethylene sorbitan monooleate and sorbitan trioleate, commonly known as Tween 80 and Span 85, respectively. MF59 has been extensively studied as an adjuvant for vaccines against the influenza virus. MF59 has been used clinically in patients over the age of 65 and has been shown to produce statistically higher anti-hemagglutinin titers than the traditional seasonal influenza vaccine. MF59 has also been shown to be an effective adjuvant for use against pandemic strains of influenza in naïve populations. It was used in the 2009 H1N1 pandemic influenza vaccine. It has also been shown to outperform alum in trials with the H5N1 avian influenza virus. MF59 creates a localized inflammatory response at the injection site, but unlike other adjuvants, it does not provide a depot effect for antigen. The mechanism of action of MF59 has been
shown to be resultant from increased uptake by dendritic cells\textsuperscript{44}. Seubert et al.\textsuperscript{44} further elucidated this mechanism by proving that MF59 stimulates differentiation of lymphoid precursors into dendritic cells and also enables the release of chemokines to attract other immune cells to the injection site. The ability to recruit innate immune cells is attributed to the up-regulation of ICAM-1, as it has been shown that MF59 loses this ability in ICAM-1-deficient mice\textsuperscript{53}. Caproni et al. recently reported that MF59 induces an IFN-independent immune response to both influenza and tetanus toxoid\textsuperscript{47}. While MF59 has been studied extensively for safety, data from numerous clinical trials indicate an increased risk for both local and systemic reactions\textsuperscript{44}. These data present a reason for concern when considering MF59 for use in a pulmonary vaccine, because increased inflammation in the lung could pose serious health risks.

In addition to squalene-based oil-in-water emulsion adjuvants, other oils, such as grapeseed, soybean, and sesame, have been investigated for their adjuvant properties. Though the triglycerides tested had similar particles sizes and stability to squalene, they produced significantly lower IgG titers when administered with Fluzone\textsuperscript{54}. Additionally, AS03, a proprietary squalene-based oil-in-water emulsion containing α-tocopherol has been investigated for use in pandemic influenza vaccines\textsuperscript{55}.

Microemulsions and water-in-oil-in-water emulsions have also been investigated as potential vaccine adjuvant systems. Leclercq et al. recently reported improved serum protection against rabies challenge with both microemulsion and water-in-oil-in-water emulsions as compared to alum and complete Freund’s adjuvant\textsuperscript{56}.
2.3.2.3 Biologically-derived vaccine adjuvants

There are a number of microbially derived compounds currently being studied for use as vaccine adjuvants. Most of this research relies on isolating specific MAMPs that interact with immune cells through pattern recognition receptors (PRRs) on the cells. The adjuvant effect of these compounds comes from the stimulation of the innate immune response, which can be used to direct the phenotype of the resultant adaptive immune response. TLR-agonists based on lipopolysaccharide (LPS), a component of cell walls of gram-negative bacteria have been extensively studied as immune-boosting vaccine additives. LPS and LPS-derived compounds are known to interact with immune cells through the TLR4 endotoxin receptor. However, LPS has toxic effects and is not an appropriate adjuvant for human use. Monophosphoryl lipid A, a compound derived from LPS, has been shown to provide immune-stimulating properties without the toxicity of LPS. The TLR9-agonist, CpG DNA has also demonstrated promise as a vaccine adjuvant through stimulation of the innate immune response. Poly(I:C) or polyinosine-polycytidylic acid has also been studied as a potential vaccine adjuvant. This compound interacts with TLR3, which is found in human mDCs and T cells, making it a good candidate for vaccines that require a cellular immune response.

2.3.2.4 Cytokines as vaccine adjuvants

The ability to modulate the immune response to a desired phenotype may be beneficial when designing vaccines. This has been studied through the inclusion of various cytokines in vaccine formulations. As reviewed in Section 2.2, the cytokine profile of the innate immune response to a pathogen, or an adjuvant, plays a large role in directing the adaptive immune response. By including cytokines in vaccine formulations, or by using cytokines as adjuvants in
vaccines, it is possible to design vaccines with the desired immune phenotype(s)$^{61}$. A large body of work is available using cytokines to adjuvant experimental, or even commercial, vaccines$^{60}$. Cytokines that have been tested include IL-1, IL-2, IL-3, IL-6, IL-12, TNF and GMCSF$^{60}$. The addition of these proteins has been studied as vaccine additives or through fusion to a protective antigen such as pneumococcal surface adhesion A (PsaA) and IL-2 or IL-4 constructs in vaccines against $S.~pneumoniae$$^{62}$. For example, in order to adjuvant other vaccines against $S.~pneumoniae$, the cytokines IL-12 or IL-17 have been added to vaccine formulations to improve performance$^{63}$.

2.4. Current Vaccine Technology Against Pulmonary Infections

In this section, we summarize the current state of vaccine technologies against several important pulmonary infections, including pneumonia, influenza, and pneumonic plague.

2.4.1 Streptococcus pneumoniae

Since its first isolation in the 1880s, $Streptococcus~pneumoniae$ (shown in Figure 2), often referred to as the pneumococcus, has been found to be endemic globally in all areas which have been tested$^{64}$. This bacteria is so prevalent that it is considered to be part of the commensal bacteria flora of the human nasopharynx, with rates of colonization peaking in those of 1-2 years of age and declining thereafter$^{65}$. The bacteria may colonize the upper respiratory tract, specifically the nasopharynx, asymptotically for anywhere from several to upwards of 30 weeks depending on the immunogenicity of the serotype$^{65,66}$. When the pneumococcus spreads from the colonized nasopharynx to the sterile lower respiratory tract or systemically to the bloodstream, the result is what is known as pneumococcal disease$^{10}$. Clinical diseases resulting from $S.~pneumoniae$ range from upper respiratory symptoms including otitis media and sinusitis
to lower respiratory tract infections such as pneumonia to systemic bacteremia and meningitis\textsuperscript{10,65}. Despite having a higher disease burden in children\textsuperscript{67}, pneumococcal pneumonia can affect individuals in any age range, and if left completely untreated has been reported to have a case fatality rate upwards of 30\%\textsuperscript{68}. There are over one million pneumococcal infections each year resulting in significant global morbidity and mortality\textsuperscript{65}. \textit{S. pneumoniae} however is largely vaccine-preventable, with the introduction of the heptavalent pneumococcal conjugate vaccine in 2000 leading to a significant reduction in cases of invasive pneumococcal disease in both the United States and Europe\textsuperscript{69,70}. The introduction of the 13-valent pneumococcal conjugate vaccine (PCV13) in the United States in 2010 included the 13 most prevalent serotypes, responsible for over 90\% of childhood cases of invasive pneumonia\textsuperscript{69}. Analysis of the results of the introduction of the PCV13 is still ongoing, and it remains to be seen whether serotype substitution occurs within a few years of introduction, as observed after introduction of the PCV7 vaccine.

A considerable amount is known about the colonization and pathogenesis of \textit{S. pneumoniae}. Much of this information has been thoughtfully used to discover protective antigens, design novel vaccines, and reduce pneumococcal colonization and disease. Herein, we outline the classification strategies for \textit{S. pneumoniae}, review current literature on the pathogenesis of the organism from colonization to clinical disease, and discuss current and emerging vaccine technologies.

2.4.1.1 Classification

\textit{S. pneumoniae} bacteria are gram-positive, alpha-hemolytic diplococci\textsuperscript{69}. There are over 90 known serotypes of pneumonia which differ in their polysaccharide capsule, specifically the
sugars making up the capsule and the linkages between those sugars, and are classified based on its interaction with anti-sera\textsuperscript{10,71}. The capsular type has been demonstrated to have a large impact of the ability of \textit{S. pneumoniae} to cause invasive disease, with serotypes lacking a capsule exhibiting greatly attenuated properties\textsuperscript{72}. Medically-relevant \textit{S. pneumoniae} serotypes include 19 A, the most common serotype causing invasive pneumococcal disease (IPD) in children (before inclusion in the PCV13 vaccine), with these serotypes accounting for a significant portion of the disease burden. Before the introduction of the 23-valent polysaccharide vaccine or the PCV7 or 13, serotypes 6, 14 and 18 accounted for over half of cases of systemic bacteremia and meningitis, with the majority of otitis media infections caused by serotypes 3, 19, and 23\textsuperscript{73}. Capsular serotype has also been shown to have an effect on the development of antibiotic resistance. Resistance to penicillin was first noted in \textit{S. pneumoniae} in 1965. Since that time highly resistant strains, as well as β-lactam resistance, and multi-drug resistance, defined as resistance to antibiotics of at least three different classes, have been reported with increasing frequency\textsuperscript{74}.

2.4.1.2 Pathogenesis

Unlike many circulating diseases, humans provide the main reservoir for \textit{S. pneumoniae}, making person to person transmission a crucial step in the survival and spread of the pathogen\textsuperscript{65}. While transmission from symptomatic patients is possible, the pneumococcus is most commonly spread through aerosolized droplets from healthy individuals with asymptomatic \textit{S. pneumoniae} colonization of the upper respiratory tract\textsuperscript{65}. It is estimated that at any given time up to 40\% of adults and children are colonized with \textit{S. pneumoniae}, with the most commonly found serotypes being 6, 14, 18, 19, and 23, which account for upwards of 60\% of pneumococcal infections. This
bacterial carriage in the upper respiratory tract or nasopharynx typically lasts from 4-6 weeks, but has been shown to persist for upwards of 30 weeks depending on the immunogenicity of the serotype\(^{65,66}\). Adherence to epithelial cells and the ability to colonize the nasopharynx is mediated by pili, filamentous structures decorating the outer surface of the pneumococcus\(^{75}\). How this bacteria disseminates from the nasopharynx to the lower respiratory tract has been the subject of numerous investigations, with a number of virulence factors identified which play roles in infection with \textit{S. pneumoniae}\(^{76}\).

\textit{S. pneumoniae} is a transient member of the commensal bacteria of the upper respiratory tract, often remaining asymptomatic for the duration of the infection. It is when bacteria reach the sterile lower airways that disease can occur, often due to inflammation associated with the innate immune response to infection, leading to pneumonia. Capsular polysaccharide (CPS) has been shown to be one of the main virulence factors in IPD. Other virulence factors include cell wall components, choline-binding proteins, and secreted factors such as hemolysin, pneumolysin, autolysin, and hydrogen peroxide\(^{77}\). Cytolysins such as pneumolysin are released by pneumococcus during infection, and contribute to lung inflammation and lung injury during pneumonia\(^{78}\). Systemic infection can occur when \textit{S. pneumoniae} disseminates from the lung into the bloodstream. These types of infections may be lethal. Virulence factors that have been demonstrated to have an effect of the ability of \textit{S. pneumoniae} to disseminate to the bloodstream include pneumococcal surface adhesin A (PsaA) and the first capsular gene, cpsa\(^{79}\). Additionally the cell surface protein pneumococcal adherence and virulence factor A (PavA), important in bacterial binding to fibronectin, has been shown to play an important role in the establishment of a septicemia in a murine model\(^{80}\).
2.4.1.3 Current vaccine strategies

*S. pneumoniae* is the leading cause of community-acquired pneumonia; it is also the causative agent of most cases of bacterial meningitis. Untreated bacteremia has a fatality rate of upwards of 70%. The use of antibiotics has greatly improved morbidity and mortality associated with *S. pneumoniae*, but with the increase in prevalence of antibiotic-resistant strains of *S. pneumoniae*, vaccination against this pathogen remains one of the most important strategies in combating pneumococcal disease. *S. pneumoniae* had long been a public health concern globally, and vaccination attempts against the pathogen have been reported since the 1940s, culminating in commercial availability of a 6-valent vaccine formulation of purified polysaccharide capsule, including types 1, 2, 3, 5, 7, 12. The drive for an effective vaccine against *S. pneumoniae* was abruptly ended after the introduction of effective antibiotics against the pathogen, the first, sulfapyridine, was introduced in 1939. After the introduction of the widespread use of antibiotics to treat these bacterial infections, both doctors and lawmakers abandoned support for new vaccination programs. It would not be until the 1990s that the increasing prevalence of antibiotic resistant *S. pneumoniae* strains and the lack of widespread pneumococcal vaccinations would lead to renewed public health awareness about the disease. Given the number of serotypes of polysaccharide capsule of *S. pneumoniae*, a universal vaccine based on the capsule polysaccharides has not yet been developed. Polysaccharide-based vaccines have been utilized including Pneumovax 23 (Merck) and Pnu-Immune 23 (Lederle Laboratories). These 23-valent polysaccharide vaccines against *S. pneumoniae* has been shown to protect against the serotypes included in the vaccine, 1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F, and 33F. These vaccines tend to induce antibody-based immunity against the serotypes included in the vaccines. Serotype-specific antibodies
induced by vaccination with the 23-valent polysaccharide vaccines have been shown to increase opsonization and induce complement-mediated phagocytosis by immune cells. Epidemiological studies have shown that these vaccines have been very effective at preventing infections and may also provide protection against serotypes closely related to those included in the vaccine, 6A, for example. In most patients a single vaccination has been shown to maintain antibody level for five years, although in elderly and immune-compromised patients, diminished results have been documented, though still conferring over 70% protection in these groups.

One drawback of the polysaccharide vaccine formulations is the T cell-independent nature of the immune response generated. These vaccines are not optimal for use in infants, and in fact, have demonstrated limited to no efficacy in this population in which rates of pneumococcal colonization are the highest. Polysaccharide-conjugate vaccines use a carrier protein for the delivery of the purified polysaccharide capsule, eliciting a T cell-dependent immune response.

The introduction of the 7-valent polysaccharide conjugate vaccine (PCV7) in 2000 in the US and 2001 in Europe, which includes serotypes 14, 4, 9V, 19F, 18C, 6B, and 23F, led to a significant decrease in circulation of the serotypes included in the vaccines. From 1995 (before the introduction of PCV7) to 2009 in one US city, there was a 10% reduction of the number of children colonized with pneumonia, with a decrease in colonization of the serotypes included in the vaccine from 83% to none in patients tested. In contrast, this led to an increase in prevalence of strain 19A, not included in the vaccine, from 5% to 49% of invasive pneumococcal disease (IPD) cases. In many countries the PCV7 vaccine has been replaced by the PCV 13 vaccine, introduced in 2010 in Europe, which includes 6 additional serotypes 1, 5, 7F, 19A, 6A, and 3. Though no comprehensive studies on the epidemiological effects of this vaccine have
been published yet, preliminary findings suggest a reduction of the incidences of the PCV13 only serotypes by at least 50%\textsuperscript{90}.

Antibody-mediated immunity against the pneumococcal polysaccharide capsule plays an important role in protection against infection. The successes of the 23-valent polysaccharide vaccine and the PCV7 and PCV13 vaccines have led to the assumption that anti-capsular antibodies are in large part responsible for clearance of infections, however this theory is challenged by the epidemiological similarities of infections across serotypes. Pneumococcal disease peaks and declines in infants according to near-identical patterns across serotypes, indicating another possible mechanism for protection other than capsule-specific antibodies, which would be dependent on exposure to specific serotypes\textsuperscript{91}. It has been proposed that other antigenic components of \textit{S. pneumoniae} are responsible for the generation of the natural immunity observed in infants\textsuperscript{91}; these protective antigens consist of surface proteins or cell wall components that are universally or near-universally present across serotypes, and include pneumococcal surface protein A (PspA), pneumococcal adhesin A (PsaA), pneumolysin, cell wall components and lipteichoic acid (LTA).

While it has long been assumed that antibody-mediated immunity to \textit{S. pneumoniae} polysaccharide capsule plays a role in protection, the induction of cell-mediated response may also be important in the generation of immunity. Age-specific acquired immunity to \textit{S. pneumoniae} appears to occur in a serotype-independent mechanism, indicating the possibility of a T cell-mediated response\textsuperscript{91}. Several reports have indicated that CD4\textsuperscript{+} T cells are important in generating protective immunity against pneumococcal carriage\textsuperscript{92}. It has recently been reported that monocytes play an important role in the generation of T\textsubscript{H1} and T\textsubscript{H17}-mediated immunity to \textit{S. pneumoniae} infection\textsuperscript{93}. 
2.4.1.4 Novel vaccine strategies

Many of the specific virulence factors reviewed above have been investigated for use as protective antigens in vaccine formulations, including PspA, PsA, pilus components, and pneumolysin. These antigens are summarized in Table 2.2. The most well-known and researched *S. pneumoniae* subunit for potential vaccines is PspA. This choline-binding surface protein is an important virulence factor in *S. pneumoniae* infections and is therefore present on all medically-relevant strains of the bacteria. PspA plays two different roles in invasive infection and nasopharyngeal carriage. In invasive, systemic infections with *S. pneumoniae*, PspA prevents the deposition of complement, an immune that assists antibodies and phagocytic cells. PspA also plays a role in carriage by binding the bactericidal glycoprotein, apolactoferrin. Vaccination with PspA has been shown to be protective against a lethal challenge with *S. pneumoniae* making it a promising candidate for use in an improved vaccine. In addition, PspA can be economically synthesized as a recombinant protein and the protein-based vaccine avoids the loss of antigenicity observed with the polysaccharide-based vaccine. Even though this protein exhibits a high degree of serological variance, it has been demonstrated to provide cross-protection across multiple capsular serotypes.

Another choline binding protein, pneumococcal surface protein C (PspC), also known as choline binding protein A (CbpA), has been studied for use in vaccines against *S. pneumoniae* as well. These proteins function to bind to human factor H and inhibit the activation of the alternative complement pathway and the binding of secretory IgA. PspC is present on approximately 75% of all serotypes. Antibodies against these proteins have been found in patients with pneumococcal carriage and invasive pneumococcal disease. These proteins have
been shown to be protective against carriage, meningitis, and sepsis when fused to pneumolysin toxin. Pneumolysin is a contributor to inflammation and lung injury during pneumococcal infection, it is also an important contributor to host recognition of pneumococci through interactions with TLR-4 and activating the classical complement pathway. Detoxified pneumolysin components have been shown to enhance protection in vaccines containing PspA.

PsaA has also been investigated as a potential protective antigen against *S. pneumoniae* and has demonstrated the ability to protect against lethal challenge. Thought to play a role in the dissemination of the bacteria to the bloodstream, PsaA is a metal-binding surface protein which has also been shown to be important for bacterial binding. Like PspA, this protein has also been shown to provide protection against heterologous challenge with *S. pneumoniae* with up to five different capsular serotype. Additionally, peptides of PsaA have been demonstrated to reduce carriage, an important consideration in the design of a *S. pneumoniae* vaccine.

The surface of some serotypes *S. pneumoniae* bacteria is decorated with pili. Surface pili are associated with bacterial adhesion in colonization and have been indicated in biofilm formation. These structures have been investigated as possible protective antigens against infection. Recombinant pilus proteins, RrgA, RrgB, and RrgC were demonstrated to be protective against challenge with piliated *S. pneumoniae*. Gene analysis, DNA sequencing, and advances in microbiology have led to the identification of a host of *S. pneumoniae* surface proteins and virulence factors which may be potential candidates for a cross-protective vaccine formulation. Recombinantly-expressed surface proteins such as PspA, PspA (CbpA), PsaA and pili proteins are typically poorly immunogenic when administered alone, requiring the use of an adjuvant to elicit a protective immune response.
*pneumoniae* toxins such as pneumolysin have been investigated not only as a protective antigen but also as a way to adjuvant other surface proteins such as PspA with resulting in protective immunity\(^{100}\).

Additionally, novel vaccine adjuvants including the use of cytokines and polymeric nano and microparticles have been investigated. Alginate microspheres containing PsaA have been shown to generate mucosal immunity when administered orally and provide protection against lethal challenge\(^{105}\). Polyanhydride nanoparticles encapsulating PspA have shown controlled release of antigen and the induction of measurable antibody titer against the protein\(^{110}\).

### 2.4.1.5 Review and future directions

With the increasing prevalence of multi-drug resistant *S. pneumoniae*, vaccination remains an important tool in reducing morbidity and mortality associated with pneumococcal disease. While two classes of vaccine are currently used, the 23-valent polysaccharide vaccine and the 7- and 13-valent pneumococcal conjugate vaccine, *S. pneumoniae*-associated disease remains the greatest global contributor to deadly acute lower respiratory infections. Capsular-dependent vaccine strategies have the significant limitation of not being able to induce universal protection, leaving vaccinated persons vulnerable to bacterial serotypes not included in the vaccine.

“Species-specific” antigens, based on surface proteins, cell wall components, and other virulence factors, rather than “serotype-specific” capsule have been suggested to be responsible for the acquired natural resistance to *S. pneumoniae* observed in infants and have the potential to generate a universal vaccine against the pathogen (ref needed). Many potential protective antigens have been identified, including PspA, PsaA, and pneumolysin to name a few, yet a universal vaccine based on one or more of these “species-specific” components remains elusive.
These protective antigens tend to be poorly immunogenic, requiring the use of an adjuvant. Future work on the development of a universal vaccine against \textit{S. pneumoniae} must include a safe, effective vaccine adjuvant capable of providing protection against pneumococcal carriage and disease.

2.4.2 \textit{Yersinia pestis}

Over the course of recorded history plague related disease has killed an estimated 200 million people. There are three major plague pandemics, beginning in the 6\textsuperscript{th} century and spanning the course of over a millennium. The causative agent, \textit{Yersinia pestis}, was not isolated until the late 19\textsuperscript{th} century\textsuperscript{111}. \textit{Y. pestis} is a vector-born zoonotic pathogen, transmitted from a rodent reservoir to humans by fleas\textsuperscript{112}. Historically, the most common form of plague infection in humans has been bubonic plague, transmitted through flea bites or direct contact with an infected individual. Septicemic plague may follow resulting in a mortality rate between 50 and 60\% for infections of bubonic plague\textsuperscript{112}.

Worldwide plague pandemics have been made increasingly improbable through the introduction of modern sanitation. However, \textit{Y. pestis} remains enzootic in many animal populations worldwide, including rabbits, squirrels, chipmunks, and prairie dogs in the American west, and marmots in China and Mongolia, with isolated cases documented globally\textsuperscript{113}. Current treatment for \textit{Y. pestis} infections includes the use of antibiotics. In murine models, intravenous and subcutaneous models of bubonic and septicemic plague responded well to quinolones and fluoroquinolones such as streptomycin, ceftriaxone, doxycycline, ciprofloxacin, and ofloxacin\textsuperscript{113}. Intranasal models of pneumonic plague were treatable by fluoroquinolones including ciprofloxacin\textsuperscript{113}. In humans, streptomycin and gentamicin are widely used, with doxycycline as
emerging alternative therapy. Fluoroquinolones such as ciprofloxacin have demonstrated success in research settings, but have not been widely tested in humans\textsuperscript{114}. Additionally, treatment must be administered rapidly after the onset of symptoms for optimal efficacy\textsuperscript{114}.

Though the likelihood for a modern plague pandemic remains low due to the introduction of enhanced sanitation and antibiotics for the treatment of infection, there is growing concern regarding the use of \textit{Y. pestis} as a bioterrorism agent. Beginning with the use of plague infected fleas by the Japanese in World War II to cause plague outbreaks in China\textsuperscript{115}, the ability of \textit{Y. pestis} to be used in biological warfare has been well-researched. Following World War II, research by the United States and the Soviet Union intensified with the US reportedly reaching production capabilities of up to 650 tons of at least seven classified agents\textsuperscript{116}. This research was purportedly abandoned after the ratification of the Biological and Toxin Weapons Convention (BWC) in 1969, with the Soviet Union reportedly continuing research until as late as 1992\textsuperscript{116}. Modern bioterror threats have been focused on the ability to formulate \textit{Y. pestis} into an aerosol form, eliminating the need for flea-born vector transmission, increasing the likelihood of widespread pulmonary infections, and as has been documented, greatly increasing the disease lethality\textsuperscript{117}. Additionally, it is possible to introduce antibiotic resistance into the bacteria, with a wild-type multi-drug resistant strain isolated in 1995\textsuperscript{118}. Further, it was shown that this antibiotic resistance plasmid was readily transferable to other strains of \textit{Y. pestis} and \textit{Escherichia coli}, where it remained stable\textsuperscript{118}. With the increased threat of natural and selected antibiotic resistance, current vaccine strategies, which have remained largely unchanged since the 1960’s may not provide the efficacy necessary to secure against emerging bioterror threats\textsuperscript{111}. In this section, we review the classification for \textit{Y. pestis}, assess current literature on the pathogenesis of
the organism from critical virulence factors to the manifestations of clinical disease, and analyze current and emerging vaccine strategies against *Y. pestis*.

### 2.4.2.1 Classification and pathogenesis

There are three *Yersinia* species that are commonly found in humans, *Y. pestis*, *Y. enterocolitica*, and *Y. pseudotuberculosis*. The later pathogens are food and water-born, causing limited infections of the gastrointestinal tract and intestinal lymphatics\(^\text{119}\). In sharp contrast, the vector-born *Y. pestis* causes highly transmissible, systemic, often lethal infection\(^\text{119}\). *Y. pestis* is considered to be an evolutionary product of *Y. pseudotuberculosis* and is classified as an intracellular, gram-negative bacteria\(^\text{119,120}\).

Three types of human infections with *Y. pestis* exist: bubonic, septicemic, and pneumonic\(^\text{121}\). Bubonic plague is characterized by edema, or swelling of the lymph nodes, known as buboes. Accompanying symptoms include fever and headache. Advanced bubonic plague and death occur when the bacteria disseminate from the lymph nodes and infect the liver and spleen causing necrotic lesions on the organs\(^\text{119}\). Septicemic plague occurs when the bacteria disseminate into the bloodstream, often leading to infection of the liver and spleen, resulting in necrosis\(^\text{122}\). Pneumonic plague occurs through the inhalation of the bacteria, where infection sets up in the lungs causing flu-like illness followed by pneumonia, and in nearly all untreated cases, death\(^\text{119,123}\).

As previously stated, *Y. pestis* is classified as an intracellular, gram-negative bacteria\(^\text{120}\). Upon infection the bacteria enters both neutrophils and macrophages through both phagocytosis and active entry. Typically killed in neutrophils, *Y. pestis* survives intracellularly in
macrophages, and develops antiphagocytic mechanisms, typically attributed to *Yersinia* outer proteins YopH and YopE, allowing it to survive extracellularly *in vivo*\textsuperscript{120,124}.

### 2.4.2.2 Current vaccine strategies

Despite the threat of antibiotic resistance, both naturally-occurring and engineered, and the potential for use as a biological warfare agent, there is no commercially licensed plague vaccine available in the United States and Europe. There is currently a live, attenuated strain of *Y. pestis*, EV76, which has been in use as a vaccine in the countries of the former Soviet Union and Asia, but is not commercially available in the United States and European countries\textsuperscript{125}. This spontaneous *pgm* deficient mutant conferred early (7 days) and long-lasting (up to 12 month) protection against bubonic, and to a lesser extent, pneumonic plague, however, there are concerns regarding safety as site reactions and incidence of disease in certain non-human primates have been reported\textsuperscript{121}. Additionally, the *pgm* mutant used in the EV76 vaccine retains virulence when administered intranasally or intravenously.

Previously, the United States has used killed whole cell vaccines to immunize military personnel travelling to areas where plague is endemic, with the majority of the data collected during the Vietnam War\textsuperscript{126}. Though protection through direct challenge in humans is not available due to ethical considerations, data collected of immunized US servicemen stationed in Vietnam shows a marked decrease in incidences of infection relative to expected exposure to plague\textsuperscript{126,127}. This killed whole cell vaccine required multiple immunizations and immunized individuals often experienced local site reactions. Additionally, the killed whole cell vaccine provided poor protection against pneumonic plague because parenteral administration resulted in poor mucosal
immunity, in contrast to the live, attenuated vaccine, which was shown to provide some protection against pneumonic plague. 

2.4.2.3 Novel vaccine strategies

While the EV76 live, attenuated vaccine is not widely considered suitable for human use, with up to 1% lethality observed upon vaccination in mice, research into creating suitable attenuated Y. pestis mutants has continued. Attenuation of live strains of pathogens for use as avirulent vaccines has proven successful with Salmonella typhimurium, Salmonella typhi, Shigella flexneri, leading to research into whether previously documented mutations may reduce virulence of Y. pestis. Research has shown that mutations in phoP and htrA reduced virulence of the Y. pestis strains from 30 to 75-fold. Additionally, it has been shown that administration of a sub-lethal dose of these mutant Y. pestis strains is able to confer protective immunity in certain animal models. With these promising results, research into live, attenuated vaccine candidates against plague continues.

Due to the safety concerns of live, attenuated Y. pestis vaccines, much of the emerging research has focused on the identification and testing of protective protein subunits of the Y. pestis bacterium. There are many experimental vaccines in development including live attenuated vaccines and a number of vaccines based on Y. pestis subunits, summarized in Table 2, especially F1, LcrV, and F1-LcrV (F1-V) fusion proteins. A F1-V subunit formulated with polyanhydride nanoparticles has recently been demonstrated to provide protective immunity against lethal challenge with pneumonic plague. The polyanhydride nanovaccine formulation provided 100% protection against challenge with CO92 Y. pestis, with both soluble protein alone
and protein adjuvanted with MPLA failing to elicit a protective immune response\textsuperscript{131}. These results suggest the potential for an effective, easily synthesized vaccine against \textit{Y. pestis}.

\textbf{2.4.2.4 Review and future directions}

The use of biological agents as bioterrorism weapons remains a considerable threat due to their relative ease of manufacture and the high mortality rates of many select pathogens\textsuperscript{132}. One pathogen that is considered to have the potential to be used as a biological weapon is \textit{Y. pestis}, the causative agent of plague-related diseases, due to its ability to spread through aerosolized droplets and the ability to easily introduce antibiotic resistance\textsuperscript{130}. Despite this, and the fact that plague is still endemic in many regions globally, no commercial vaccine against \textit{Y. pestis} is available\textsuperscript{130,133}. There are many experimental vaccines in development including live attenuated vaccines and a number of vaccines based on \textit{Y. pestis} subunits, especially F1, LcrV, and F1-LcrV (F1-V) fusion proteins\textsuperscript{130}. A F1-V subunit based on polyanhydride nanoparticles has recently been demonstrated to provide protective immunity against lethal challenge with pneumonic plague. The polyanhydride nanovaccine formulation provided 100\% protection against challenge with CO92 \textit{Y. pestis}, with both soluble protein alone and protein adjuvanted with MPLA failing to elicit a protective immune response\textsuperscript{131}. These results suggest the potential for an effective, easily synthesized vaccine against \textit{Y. pestis}.

\textbf{2.4.3 Influenza}

Although disease causing ultra-filterable agents were not identified until the beginning of the 20\textsuperscript{th} century, a high mortality disease that affected birds was defined in 1878 and referred to as “fowl plague”\textsuperscript{134}. It wasn’t until 1933 that the influenza virus was first isolated from swine. To this day,
the influenza virus continues to infect thousands of people each year while causing significant health and economic burdens\textsuperscript{135,136}. The most dramatic of these effects have been the cause of influenza pandemics. There have been three pandemics in the 20\textsuperscript{th} century: in 1918, 1957, and 1968. All three pandemics were caused by different subtypes of the influenza A virus and identified by their presumed sites of origin\textsuperscript{137}.

The “Spanish” influenza 1918 H1N1 pandemic has at times been referred to as the mother of all pandemics. An estimated one third of the world’s population was infected leading to 50 million deaths. Almost all cases of Influenza A globally are the cause of decedents from the 1918 virus. The “Asian” influenza 1957 H2N2 pandemic caused a million deaths worldwide from 1957 to 1968\textsuperscript{138,139}. This pandemic may have been the result of reassortment events in unknown mammalian hosts involving multiple avian viruses\textsuperscript{140}. The “Hong Kong” influenza 1968 H3N2 pandemic, like the previous pandemic, was also the result of antigenic shift and contained genes from avian influenza viruses. This was the first known outbreak of the H3N2 strain and resulted in a million deaths\textsuperscript{140}. The potential of a pandemic of several Influenza A viruses, including H5N1 and H9N2, is a public health priority for the 21\textsuperscript{st} century.

\textit{2.4.3.1 Classification}

Influenza consists of three of the four genera of the Orthomyxoviridae family: influenza A, influenza B and influenza C\textsuperscript{141}. Influenza is a negative sense RNA virus with single-stranded segmented genomes. The influenza B and C viruses primarily infect humans although Influenza C can occasionally infect pigs and dogs\textsuperscript{142}. Influenza A viruses infect a wide range of hosts in different geographical regions. This virus type is further classified into subtypes on the basis of the antigenic properties of the haemagglutinin and neuraminidase. This includes 15
hemagglutinin and 9 neuraminidase subtypes all of which can be found in wild birds. The influenza genome includes 8 segments that encode 11 viral genes. These viral genes include: hemagglutinin (HA), neuraminidase (NA), matrix 1 (M1), matrix 2 (M2), nucleoprotein (NP), non-structural protein-1 (NSP1), nuclear export protein (NEP) and polymerase proteins (PA, PB1, PB2, PB1-F2 (PB1-F2)). The influenza virus envelope is taken from the plasma membrane of the host cell during the budding process.

2.4.3.2 Pathogenesis

Influenza is an acute respiratory disease that replicates in epithelial cells in the lower and upper respiratory tract. It is characterized by a multitude of clinical signs including the onset of a high fever, cough, headaches and inflammation of the respiratory tract. Although this disease affects all age groups, the severity is typically increased among the young, elderly and sick. Complications that can arise as a result of infection include hemorrhagic bronchitis, pneumonia and death. The influenza A virus reaches its apex of replication at approximately 2 days during the inoculation of the throat. After this time, replication starts to slow down with little virus shed after approximately day 6. Influenza typically resolves within 1 – 2 weeks although a cough may persist for longer.

The hemagglutinin protein precursor, HA0, is composed of HA1 and HA2 subdomains which contain the receptor binding domain and the fusion peptide respectively. This protein interacts with sialic acid on the host cell for viral attachment and determines host tropism. The majority of linkages of the sialic acid residues on the host cell are α-2,6-linked and α-2,3-linked. Human and avian influenza viruses contain only one confirmation of sialic acid linkage while swine contain both. This makes swine an ideal vessel for viral RNA reassortment between human and avian
influenza viruses. After the influenza virus has attached to the host cell by binding to sialic acid residues, the virus enters via receptor-mediated endocytosis. The fusion peptide brings together viral and endosomal membranes allowing entry into the cytoplasm\textsuperscript{144}. The fusion peptide of the HA protein is exposed after cleavage of the HA0 precursor by trypsin-like enzymes. This limits the site of infection to the respiratory and intestinal tract. The HA0 cleavage sites of highly pathogenic avian influenza (HPAI) are cleaved by more ubiquitously present proteases, most likely furin, allowing these viruses to replicate throughout the host’s organs and tissues. For this reason the mortality rate of HPAI influenza viruses is far greater\textsuperscript{147}

Diagnostic methods of influenza include viral culture, the establishment of viral antigens or viral genetic material or through the monitoring of specific antibody titers. Infection can be treated with antiviral drugs or prevented with immunization which has been shown to be 70\% to 90\% effective in preventing influenza A infection in healthy young adults\textsuperscript{145}.

2.4.3.3 Current vaccine strategies

Protection against influenza is mediated by both mucosal and systemic immunity with antibodies elicited in the upper respiratory tract as a major contributor to resistance. Antibodies are elicited against all influenza proteins during infection but antibodies targeted to surface proteins HA and NA are most associated with resistance. Although humoral immunity plays a significant role in immunity, a cytotoxic T cell response directed against M1, M2 and NP proteins has an important role in clearing the virus and recover from illness. Natural infection can lead to long lasting immunity but due to the high mutation rate of influenza individuals may only be protected against new strains for only a few years which is why seasonal vaccination is essential to protection\textsuperscript{146}.
Inactivated influenza vaccines have been available since the 1940s. This vaccine type is less common today due to the reactogenicity of delivered inactivated whole virus to children.\(^{148}\)

In 2010, the Advisory Committee on Immunization Practices (ACIP) started recommending two different vaccine types based on age groups for universal seasonal influenza vaccination. The trivalent inactivated vaccine (TIV) for individuals 6 months or older, or the live attenuated influenza vaccine (LAIV) for healthy non-pregnant individuals between 2 and 49 years of age.\(^{149}\)

The dominant component in TIV is HA polypeptide purified from inactivated virions and contains at least 15 μg of HA for each strain of influenza. These vaccines generally function by offering hemagglutination-inhibition (HI) antibodies in previously influenza primed recipients.\(^{148}\)

The goal of LAIV is to confer protection through the natural route of viral infection. These vaccines can be administered intranasally via a spray device.\(^{146}\) A meta-analysis by Osterholm et al. reported that the LAIV and TIV immunizations had a pooled efficacy of 83% and 59% respectively when analyzing data from randomized controlled trials over multiple flu seasons.\(^{149}\)

### 2.4.3.4 Novel vaccine strategies

Recent focus on influenza vaccine research has focused on the use of subunit proteins as protective antigens,\(^{148,150,151}\) summarized in Table 3. A difficult obstacle for influenza vaccination is the need to protect against multiple HA and NA subtypes. There is a desire to develop a novel vaccine strategy that can offer cross protection against all Influenza A subtypes. One method is to target more highly conserved viral protein such as the NP, M1 and M2 proteins. An antibody response against the internal NP and M1 proteins does not offer protection because it does not neutralize the virus. However, there has been some effectiveness in eliciting antibody titers against the M2 surface protein in mice. Other research has focused on utilizing
DNA vaccination to establish a cell mediated immune response to the NP protein. The cell mediated immunity is a delayed response and may not offer protection against HPAI which can cause death as soon as 1 day after challenge\textsuperscript{143}.

2.4.3.5 Review and future directions

The high mutation rate and segmented genome of the influenza virus has made vaccination against this pathogen difficult. With the threat of a new pandemic from strains including H5N1 and H9N2, it’s increasingly important for government agencies to fund influenza based research. As we increase our knowledge of the pathogenicity of influenza we can continue to develop novel vaccine strategies against the virus. Newly developed vaccines will be tested against LAIV and TIV, which have a proven track record of safety and effectiveness.

2.5. Rational Design of Next-Generation Pulmonary Vaccine Nanoadjuvants

Traditionally used, off-the-shelf vaccine adjuvants differ greatly in their material properties and mechanisms of action. The goal of a vaccine adjuvant is to successfully stimulate the innate immune response to generate a long-lasting adaptive immune response. There is a unique opportunity to design efficacious vaccine adjuvants by exploiting knowledge of material properties from the many different approved vaccine adjuvants and combining them with polymer and particle synthesis methodologies. One of the benefits of the use of polymeric particle-based adjuvants for pulmonary delivery is the ability to tailor their interactions with respiratory tract fluid, which consists of pulmonary surfactant and surfactant proteins. The first interaction of inhaled particles with respiratory tract fluid occurs at the air-fluid interface. As inhaled particles come into contact with the pulmonary surfactant layer, they are introduced into
the fluid phase. This interaction can be modified through the particle surface chemistry, particle shape, and particle size. In addition, surfactants or other mucoactive compounds may be included to enhance particle distribution.

2.5.1. Route
The route of delivery is an important consideration in vaccine design. Routes of delivery that have been investigated for vaccines include oral, intramuscular, subcutaneous, and intranasal. The intramuscular route is the most common method of delivery for vaccines, being delivered via traditional syringe and needle. Vaccines delivered intramuscularly include the seasonal influenza vaccine, the PCV 7 and 13 vaccines, meningococcal vaccines, and all vaccines on the childhood vaccination schedule (e.g., MMR, dTaP, etc.). The BCG vaccine is delivered transdermally. This approach is often optimal for inducing an effective systemic response to the injection. Intramuscular and subcutaneous administrations have the limitation of widespread patient aversion for needles as well as the need for a medical professional to administer the shot.

Oral immunization has been known about for millennia with some early Bedouin tribes having been reported to consume the cooked meat of rabid animals as a vaccination strategy. The oral route provides some benefits for vaccine design, including a less invasive method of delivery and the ability to induce mucosal immune responses. Oral vaccines include the Sabin live oral polio vaccine, rotavirus, typhoid, and cholera vaccines. A prototypical example of oral immunization is the Sabin live oral polio vaccine, which has played an important role in the efforts to eradicate polio. This vaccine provided a number of advantages over Salk’s killed vaccine including ease of delivery, obviating the need for administration by medical workers,
reducing costs\textsuperscript{32}. One drawback of the oral route of vaccination is diminished immune responses in infants, children, and adults living in poverty and in developing countries, as observed with oral polio, rotavirus, cholera and \textit{shigella} vaccines\textsuperscript{156}. The mechanism of this disparity is poorly understood, but is hypothesized to be associated with vitamin deficiency, nutrition, or \textit{Giardia} infection\textsuperscript{156}. Additionally, many vaccines may have problems with stability associated with the gastrointestinal tract, particularly subunit vaccines based on proteins, which can be susceptible to acid-induced degradation\textsuperscript{153}.

The intranasal and pulmonary routes of administration have gained in interest in recent years due to the many advantages of this strategy, including needle-free administration and the high bioavailability and low metabolism of the respiratory tract. Vaccines delivered intranasally include the live attenuated influenza vaccine (LAIV, FluMist). Vaccines delivered intranasally and through aerosolization have the advantage of being able to elicit mucosal immunity in the nasal and respiratory tracts and be delivered via the route of natural infection for many respiratory pathogens\textsuperscript{153}. However, this route of vaccination has safety concerns because the delicate immune environment of the lung is particularly susceptible to inflammation-associated pathology, which may be problematic when immunizing with whole cell vaccine formulations\textsuperscript{153}.

2.5.2 Adjuvant chemistry

As stated above, the pulmonary route remains an attractive option for next-generation vaccine adjuvants due to high bioavailability, needle-free delivery, and the ability to induce mucosal immunity against respiratory pathogens such as influenza, pneumonia and plague\textsuperscript{157}. Obstacles to pulmonary delivery include delivery to the alveoli, poor uptake, and expulsion through mucociliary clearance\textsuperscript{158}. Biodegradable polymers have the ability to overcome many of the
obstacles presented by the pulmonary delivery route, however polymer interactions with the immune mechanisms of the lung are not yet fully understood. Herein we outline several classes of biodegradable polymers with the potential to improve pulmonary vaccine design, with each polymer class possessing unique chemical properties that may be used to rationally design protective vaccines against respiratory pathogens. Table 4 overviews the biodegradable polymer families that will be discussed in this review.

2.5.2.1 Polysaccharides

The polysaccharides that have been investigated to date include chitosan, dextran, and alginate. Particular attention has been given to the use of chitosan in vaccine formulations due to its ability to be delivered to mucosal surfaces and to generate IgA antibodies, due to its mucoadhesive properties\(^{159}\). Chitin and chitosan are polymers of D-glucosamine and N-acetylglucosamine with β-1,4 linkages, derived from glucose, found in the polysaccharide shells of crustaceans and insects\(^{160}\). Chitosan is formed from the deacetylation of chitin, using an excess of sodium hydroxide in water\(^{161}\). These polymers have mucoadhesive properties that have been shown to prolong residence time after intranasal administration\(^{162}\). In addition, one mechanism of action of these polymers is hypothesized to be through their ability to open the tight junctions between alveolar epithelial cells, facilitating transport of the vaccine\(^{163}\). Chitosan has demonstrated the ability to stimulate innate immune cells through interactions with TLR-2, macrophage mannose receptors, and C-type lectin receptors\(^{164}\). These interactions have been demonstrated to lead to the production of cytokines that recruit and activate innate and adaptive immune cells, resulting in the induction of a T\(_{H1}\), T\(_{H2}\), and T\(_{H17}\) adaptive immune phenotype\(^{165}\). Chitosan and chitin-based vaccines against influenza and diphtheria have been reported\(^{166,167}\).
Dextrans have also been investigated for use as vaccine adjuvants\textsuperscript{168,169}. These polysaccharides are $\alpha$-glucans comprised of D-glucose with $\alpha$-D (1,6) linkages produced by bacteria when grown in the presence of sucrose, purified by dissolving in water followed by precipitation in ethanol\textsuperscript{170}. These polymers have been investigated for use in vaccines against tetanus toxoid, 
\textit{Mycobacterium tuberculosis}, and cholera, with a mechanism of action proposed to be linked to its pro-inflammatory effects\textsuperscript{160,169}. Acetylated dextran microparticles have demonstrated success as a vaccine carrier against \textit{Bacillus anthracis}, however, the adjuvant, resiquimod, a TLR agonist, was also necessary to induce protection\textsuperscript{171}. Additionally, diethylaminoethyl-dextran (DEAE-dextran) has been investigated and shown to enhance the immune response to a Venezuelan equine encephalomyelitis virus vaccine in non-human primates\textsuperscript{160}.

In addition to glucans such as dextran and chitins, other carbohydrate-based vaccine adjuvants that have been investigated include fructans, mannans, and bacterially-derived compounds such as lipopolysaccharide and its derivatives\textsuperscript{160}.

\textbf{2.5.2.2 Polyesters}

Polyesters are the most extensively studied biodegradable polymers for drug and vaccine delivery. Degradable polyesters that have potential for vaccine delivery applications include poly(lactic acid) (PLA), poly(lactic-co-glycolic acid) (PLGA), poly(3-hydroxybutyric acid) (P3HB), poly(4-hydroxybutyric acid) (P4HB) and poly(ε-caprolactone) (PCL). Polyesters have been proposed to function as vaccine adjuvants by providing a depot for antigen and enable the controlled release of antigen over time. However, poly(glycolic acid) (PGA) by itself is limited as a vaccine delivery vector. Due to its hydrophilicity, it releases payload too rapidly and it is difficult to process using typical polymer solvents; instead it has mostly been used in degradable
sutures\textsuperscript{172–176}. In contrast, PCL has been shown to undergo extremely slow degradation with an in vitro and in vivo polymer weight loss of between 14 and 20\% in one year\textsuperscript{177–181}, release payload with high burst (40 – 70\%) followed by very slow remaining extended release, and possess agent-dependent loading efficiency (14 – 70\%) making it questionable for vaccine delivery applications\textsuperscript{182–188}. Among the most common polyesters investigated for use as adjuvants or for delivery are copolymers of lactic and glycolic acids. Poly(lactic-co-glycolic acid) (PLGA) degrades into biocompatible metabolites and is FDA-approved for human use\textsuperscript{189}. These polymers are often used as nano and/or micro particles in vaccine delivery systems. With nano and microparticles based on PLGA shown to interact with APCs and induce the release of cytokines, stimulating the immune response and resulting in a T\textsubscript{H}1 immune phenotype\textsuperscript{190–192}. These polymers have been investigated for use in vaccines against a number of pathogens including hepatitis B, tetanus toxoid, and \textit{Bacillus anthracis}\textsuperscript{193–195}. Even with the extensive use of polyesters in drug delivery applications, there still exist significant shortcomings with their use, including limited flexibility in tuning payload release, the formation of low pH microenvironments, and the constant moisture exposure of payload due to its bulk erosion profile. The bulk erosion of polyesters allows for significant residence time with water for encapsulated materials. These factors combine to allow for sensitive amino acids and proteins like insulin and uterocalin to aggregate, hydrolyze and change conformation\textsuperscript{196–198}. Another drawback of the use of polyesters for subunit vaccines is the report of protein instability during the manufacturing process and in the presence of acidic PLGA degradation products\textsuperscript{199}. The polyesters PLA and PLGA demonstrate significant lack of control in payload release. While the lactic acid component of PLGA can be easily modulated between 50\% and 100\%, the release profiles of encapsulated material do not significantly change\textsuperscript{200–202}. Coating the surface of PLGA
devices has been used to overcome this issue\textsuperscript{202,203}, but changing surface chemistry could drastically modulate function and interaction with the host and host cells. When these polymers degrade, their monomers (lactic acid and glycolic acid) significantly decrease the pH of their environment to as low as 1.5\textsuperscript{204,205}. Many drugs can be significantly affected by low pH, especially recombinant protein and subunit based vaccines (e.g., tetanus toxoid and diphtheria toxoid)\textsuperscript{206–208}. To mediate protein instability in the presence of lactic and glycolic acid, the inclusion of an antacid in the vaccine formulation as well as the use of the protein stabilizer, trehalose, have been investigated\textsuperscript{193}. While polyesters have been extensively used in drug and vaccine delivery, they possess significant limitations that must be accounted for in order to improve their function.

2.5.2.3 Polyanhydrides

Polyanhydrides, used in the textile industry since the 1920s and investigated for possible use as drug delivery systems since the 1980s, are a class of materials characterized by a backbone of monomeric dicarboxylic acids (diacids) connected by anhydride linkages\textsuperscript{209–211}. The anhydride linkages that join the monomer units are hydrolytically labile bonds that are cleaved in the presence of water to form non-toxic dicarboxylic acids\textsuperscript{212}. Polyanhydrides have many properties that make them ideal candidates for vaccine delivery including the ability to stabilize and protect fragile antigens such as proteins, provide sustained release of antigen, and the ability to modulate the immune response\textsuperscript{213–216}. These polymers have excellent biocompatibility and have been shown to result in non-toxic, non-mutagenic degradation products\textsuperscript{209}. The FDA-approved Gliadel\textsuperscript{®} wafer is a polyanhydride device composed of a copolymer of sebacic acid and 1,3-bis(\textit{p}-carboxyphenoxy)propane for the post-surgical treatment of brain cancer\textsuperscript{217}.
There are a number of classes of polyanhydrides, classified broadly as aliphatic, aromatic or unsaturated polyanhydrides, with sub-classes including copolymers of aliphatic, aromatic, polyester, and polyether polyanhydrides. Polymers and copolymers based on sebacic acid (SA), 1,6-(bis-p-carboxyphenoxy)hexane (CPH), and 1,8-(bis-p-carboxyphenoxy)-3,6-dioxaoctane (CPTEG) have been studied for use in drug and vaccine delivery and other biomedical applications. It has recently been shown that micro- and nanoparticles made from polyanhydrides can be effectively used as adjuvants in vaccine formulations. Nanoparticles comprised of polyanhydrides possess pathogen-mimicking properties and thus can activate the innate immune response by stimulating PRRs on the surface of APCs, specifically DCs and macrophages. In addition to the stimulation of the innate immune response, it has also been shown that vaccination using polyanhydride nanoparticles as an adjuvant can enhance the adaptive immune response. Antigen delivered in the context of polyanhydride nanoparticles has been shown to enhance the proliferation of cytotoxic T cells compared to antigen delivered alone or antigen delivered with alum. Additionally, shown that changing polyanhydride chemistry results in markedly different antibody responses after immunization. This information may suggest the ability to tailor polymer chemistry to the desired immune profile. Polyanhydride particles can also be used to tailor the immune response to the pathogen of interest. There are a number of methods by which polyanhydride particles can be modified in order to target specific immune cells, including modification of their size, chemistry, and surface charge. It has been suggested that the presence of oxygen and hydroxyl moieties in the polymer backbone leads to increased recognition by PRRs. In addition, carbohydrates have been attached to the surface of polyanhydride particles to target and stimulate selected immune
cells, leading to a change in surface charge, increased uptake, and a modification of serum adsorption profiles\textsuperscript{40,222,223}.

2.5.3 Safety

Of paramount importance to a vaccine candidate is an excellent safety profile. Particularly when administering to the delicate immune environment of the lung, biocompatibility, low to moderate inflammation, and a reduced recruitment of immune cells may provide the necessary adjuvant effects, while avoiding inducing immunopathology to the lung tissue. Many traditional vaccine adjuvants such as alum and squalene-based adjuvants such as MF59 have not been approved for intranasal delivery due to concerns of excess inflammation and pulmonary injury. Next-generation polymeric vaccine nanoadjuvants have the potential to meet these challenges by providing an optimal size and shape to reach and deliver antigen to the alveolar spaces\textsuperscript{224}. Additionally, these polymeric and particle-based vaccine delivery platforms have the potential to eliminate the need to deliver antigen in the form of a liquid suspension and transition to dry powder based formulations, potentially improving patient compliance and increasing ease of storage\textsuperscript{225}. Many polymers have been extensively tested for biocompatibility, including polyesters, polyanhydrides and polysaccharides, each demonstrating good safety profiles in early-stage testing\textsuperscript{226–228}.

Polyanhydrides have previously been shown to have an excellent safety profile when delivered subcutaneously and intramuscularly at up to 10X doses\textsuperscript{226}. Additionally polyanhydride nanoparticles have been delivered extensively intranasally without adverse effects. Compared to MPLA, polyanhydride nanovaccines induce less lung inflammation, as measured by ProSense\textsuperscript{®} 750 FAST, a fluorescent indicator activated by cathepsins produced by inflammatory cells such
as monocytes and macrophages. The nanoparticle based vaccine induced a higher neutrophil infiltration at early time points, as determined by H&E staining and examination by a veterinary pathologist, but this inflammation had resolved by 48 hours post administration (unpublished data). In contrast, the MPLA formulation induced less neutrophil infiltration at early time points, followed by a significantly higher presence of inflammatory cells, such as macrophages by 48 hours after vaccine delivery. Interactions between polyesters and lung cells have been studied in vitro, showing potential for safe delivery to lung cells, however surface modification with a hydrophilic polymer was necessary to cross the mucin barrier and increase intracellular accumulation.

2.5.4 Persistence

One of the benefits of polymeric vaccine adjuvants is that they provide sustained release of antigen as the polymer erodes, creating a depot effect. The time scale and mechanism of polymer erosion depends on a number of factors including polymer chemistry, crystallinity, hydrophobicity, and molecular weight. It has been proposed that the polymer erosion process occurs in three steps: the penetration of water into the polymer, degradation, and dispersion. The entry of water into the polymer is determined by the erosion method, which can be classified as bulk or surface. Some biodegradable polymers, including polyesters such as PGLA and polyethers such as poly(ethylene oxide), undergo bulk erosion. Bulk erosion is characterized by a diffusion-dependent release of payload since water will reach the encapsulated material much more quickly than the degradation and erosion of the polymeric device. In addition, once water infiltration has occurred, all hydrolytically labile bonds can be attacked simultaneously throughout the material. These types of materials tend to fissure and break into
smaller subunits before completing degradation, as shown in Figure 3. This erosion mechanism is not ideal for the delivery of fragile proteins because it allows the influx of water into the core of the delivery device leading to potential degradation and a loss of antigenicity\textsuperscript{235,236}. In addition, there is little control in payload release from bulk-eroding polyesters. The lactic acid component of PLGA can range from 50\% and 100\%, but the release profiles of encapsulated material do not significantly change\textsuperscript{200-202}.

In contrast, when water diffuses slowly compared to the rate of erosion, the material is said to be surface-eroding\textsuperscript{234}. These devices require the degradation of the surface polymer and dissolution of the resulting monomer to allow for encapsulated payload release. The hydrophobic chemistry of polyanhydrides prevents this invasion of water into the bulk of the device, leading to surface erosion and release kinetics\textsuperscript{231,232}. This surface erosion profile leads to the ability to tailor payload release both in vitro and in vivo based on the chemistry of surface eroding polymer chosen\textsuperscript{221,237}. Recent work by Haughney and Ross et al. has shown that the intranasal administration of three different polyanhydride nanoparticle chemistries with varying degrees of hydrophobicity modulated the availability of F1-V \textit{in vivo}. However, the persistence of antigen alone was not sufficient for driving an immune response, as soluble F1-V was shown to persist in the lung, but failed to elicit and antibody response. Therefore, when designing an intranasal vaccine differences in polymer hydrophobicity leading to the induction of inflammatory signals, polymer degradation rates and antigen availability must all be taken into account\textsuperscript{221}.

2.6 Consequences for Vaccine Design

The pulmonary immune response to infection is a complex process mediated by both chemical and cellular components. An understanding of how the immune system responds to an
infection can provide key insights into the design of a vaccine against a pathogen. Based on the above summary of immunological concepts of the pulmonary environment, there are a number of conclusions that can be drawn regarding vaccine design for mucosal immunization. First, an effective pulmonary vaccine must effectively bypass the intrinsic defenses of the lung mucosa in order to reach the effector cells associated with the immune response. This means that after aspiration into the airways, the vaccine components must not be exhaled through the cough reflex or be expelled through mucociliary clearance. There are several ways that this may be avoided for successful vaccine design. First, a vaccine may be designed to break up the surface tension of the mucosal layer to deposit onto the epithelium through the use of a surfactant. Second, a vaccine may be designed or modified to target or bind to alveolar epithelial cells, reducing clearance.

Additionally, an ideal vaccine formulation will avoid a high level of inflammation. Within the delicate immune environment of the lung, inflammation can lead to many unwanted side effects including damage to the epithelial layer and pneumonia. As reviewed above, there are a number of mechanisms that may lead to a recruited innate immune or inflammatory response, including ligation of certain TLRs on immune cells, leading to the release of pro-inflammatory cytokines. One way that vaccines may be designed to avoid activating these innate immune mechanisms is by coating them with polyethylene glycol, which has been shown to reduce the inflammatory response to adenoviral gene delivery vectors\textsuperscript{238}.

A good pulmonary vaccine will lead to the development of a highly effective adaptive immune response, inducing both systemic and mucosal immunity and a long-lasting memory response. The pulmonary route of vaccination itself has been demonstrated to be important for protection against colonization at mucosal surfaces, with the induction of secretory IgA (sIgA) as
the primary goal. The pulmonary route of delivery is patient friendly because it avoids the
discomfort caused by needles, leading to greater patient compliance and wider availability. It has
also been observed that patients exhibit fewer adverse effects to therapeutics delivered via dry-
powder aerosols as compared to delivery via injection\textsuperscript{239}. Pulmonary delivery also allows for the
distribution of the antigen directly to the site of infection for respiratory pathogens, allowing for
the targeting of the specific immune cells, such as alveolar macrophages, and a faster
development of mucosal immunity. Because the lungs have a relatively low metabolic rate,
pulmonary delivery avoids the first-pass metabolism that is often associated with therapeutics
delivered orally\textsuperscript{240}. The lung also offers a very high bioavailability, due to the large surface area
(\textgt;100 \text{m}^2) for drug deposition and action as well as for systemic absorption\textsuperscript{240,241}.

As discussed above, polymer chemistry can have a great effect on the immune response elicited
from a vaccine formulation. Affecting not only payload release and persistence, but also safety
and biocompatibility, polymer chemistry must be a careful consideration when designing next-
generation vaccine adjuvants. As described in detail above, the release profile and erosion
mechanism must be carefully considered. In addition, within a class of polymer, polyanhydrides
for example, the chemistry chosen may greatly affect the cellular uptake and recruitment\textsuperscript{242}, with
more hydrophobic chemistries eliciting danger signals\textsuperscript{218}. Polymer chemistry can also greatly
affect the in vivo release profile of antigen and can be used to modulate the immune response\textsuperscript{221},
introducing the possibility to design a vaccine to create the optimal immune profile for a given
pathogen rather than a trial-and-error method.

The antigen delivery vector is also an important consideration in designing next-generation
pulmonary vaccines. It is well known that the size of particles delivered to the lung will affect
both the distribution within the lung and the submersion of the particle within the respiratory
tract fluid. Modification of the size of polymeric delivery systems can be used to tailor the distribution of particles within the lung and target specific regions or immune cell populations. The relationship between particle size and deposition within the respiratory tract is well known. When considering the deposition of particles within the lung the aerodynamic diameter is an important variable, which can be manipulated through the particle density as well as geometric diameter. For example, as previously discussed, PLGA particles have a low density due to their porous structure and therefore have smaller aerodynamic diameters, allowing for deeper lung penetration. In addition, it has been shown that particle size may have an effect on the temporal recruitment of immune cells to the lungs. It has also been proposed that the modification of particle shape could have an effect on the biodistribution of inhaled particles. Huang et al. have shown that mesoporous silica nanoparticles administered intravenously travel to different locations in vivo depending on their aspect ratio. In addition, it has been shown that particle shape can have an effect on the adhesive properties of a particle. Decuzzi et al. have shown through mathematical modeling that vascular adhesion is affected by the aspect ratio and that an oblong particle shape will maximize both adhesion and payload, including upon pulmonary administration.

2.7 Conclusions
Respiratory pathogens are a significant contributor to the global disease burden. Despite this, many of these transmissible respiratory infections are vaccine-preventable. There is an urgent need for improved vaccine technologies based on increased knowledge of the unique immunological environment of the lung. Recent advances in the fields of medicine and engineering have led to an increased understanding of the immune system and its interactions
with invading pathogens. These breakthroughs have the potential to greatly improve the fields of vaccine design. Next-generation vaccines based on antigenic subunits may provide greater protection, wider availability, and the ability to induce cross-protection against multiple strains or serotypes. Despite their promise, antigenic subunits are rarely able to induce an immune response without the addition of an adjuvant, and can be prone to degradation in the presence of physiological environments. In order to maximize the benefit of subunit vaccines novel vaccine adjuvants and vaccine delivery systems must be developed which can protect the payload and deliver intact, functional antigen to the appropriate immune cells. These cells must then be suitably stimulated to drive the induction of an adaptive immune response.

Biodegradable polyanhydrides have the ability to meet all of the challenges presented by the increased use of protein subunits in vaccine formulations. These polymers may be formed into nanoparticles to encapsulate antigen in the polymer matrix and prevent degradation associated with exposure to water or heat. Additionally, these nanoparticles provide an optimal size range for the pulmonary delivery of vaccine formulations to APCs located in the alveolar regions of the lung. It has also been demonstrated that particles based on polyanhydrides have the ability to stimulate the immune system, acting as a vaccine adjuvant. These data support the use of polyanhydrides in next-generation subunit vaccine formulations.

2.8 References


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![Figure 2.1. Schematic of the airway epithelium interacting with bacteria (S. pneumoniae)](image)

associated immune mechanisms.
Figure 2.2. Schematic of *Streptococcus pneumoniae* bacterium, relevant surface antigens, and virulence factors.

Figure 2.3. Schematic of polymer degradation mechanisms. Blue-colored particles represent surface-eroding polyanhydride particles releasing payload, i.e., protein, DNA, or other pathogenic subunits (red particulates). Green-colored particles represent bulk-eroding polymers such as polyesters, which lead to an influx of water into the core of the particle and burst release kinetics.
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Table 2.1. Summary of *Streptococcus pneumoniae* subunit protective antigens as vaccine candidates.

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<th>Cross-Reactivity</th>
<th>Adjuvant(s)</th>
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<td>Polysaccharide Capsule</td>
<td>Function in virulence, inhibits phagocytosis</td>
<td>Licensed by Merck (Pneumovax), Lederle Laboratories (Pnu-Immune 23)</td>
<td>Protective against vaccine serotypes</td>
<td>None</td>
<td>81, 252</td>
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<tr>
<td>Polysaccharide Capsule-Protein Conjugate</td>
<td>Function in virulence, inhibits phagocytosis</td>
<td>Licensed by Pfizer (Prevnar, Prevnar\textsuperscript{13}), GlaxoSmithKline (Synflorix)</td>
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<td>PsaA, PiaA, PiuA</td>
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<tr>
<td>Pili</td>
<td>Bacterial adherence</td>
<td>Experimental</td>
<td>Cross-reactive, not present on all <em>S. pneumoniae</em> serotypes</td>
<td>Freund’s adjuvant</td>
<td>108,109</td>
</tr>
<tr>
<td>NanA</td>
<td>Neuraminidase, sialic acid residue</td>
<td>Experimental</td>
<td></td>
<td>Freund’s adjuvant</td>
<td>252,255</td>
</tr>
</tbody>
</table>
Table 2.2. Summary of *Yersinia pestis* subunit protective antigens as vaccine candidates.

<table>
<thead>
<tr>
<th>Protective Antigen</th>
<th>Biological Function</th>
<th>Vaccine Status</th>
<th>Recognized by Plague Survivor Serum (Murine)(^{256})</th>
<th>Adjuvant(s)</th>
<th>Selected References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fraction 1 antigen (F1)</td>
<td>Role in prevention of phagocytosis by macrophages</td>
<td>Experimental</td>
<td>Yes</td>
<td>PLGA microspheres, expression on live <em>Salmonella typhimurium</em></td>
<td>(^{124,257,258})</td>
</tr>
<tr>
<td>V antigen (LcrV)</td>
<td>Role in virulence, alters host cytokine response, mediates function of Yops</td>
<td>Experimental</td>
<td>Yes</td>
<td>Expression on live <em>Salmonella typhimurium</em>, Adenovirus vaccine vector</td>
<td>(^{259,260})</td>
</tr>
<tr>
<td>F1-V</td>
<td>Fusion protein of Fraction antigen 1 (F1) and V proteins</td>
<td>Interest in commercialization from FDA</td>
<td>N/A</td>
<td>Polyanhydride nanovaccines</td>
<td>(^{131,218})</td>
</tr>
</tbody>
</table>
Table 2.2 (continued)

<table>
<thead>
<tr>
<th>Protein</th>
<th>Function</th>
<th>Potential for Vaccine Applications</th>
<th>Formulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yersinia protein kinase A (YpkA)</td>
<td>Bacterial virulence factor, induces apoptosis</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>Yersinia Outer Proteins (YopH-YopK)</td>
<td>Modulate host response</td>
<td>Experimental</td>
<td>Yes, Oil-in-water emulsion</td>
</tr>
<tr>
<td>Plasminogen activator protease (Pla)</td>
<td>Role in spread of bacteria from subcutaneous to systemic infection</td>
<td>Potential for vaccine applications</td>
<td>Yes</td>
</tr>
<tr>
<td>pH 6 antigen (pH 6 Ag)</td>
<td>Prevents bacterial phagocytosis</td>
<td>Potential for vaccine applications</td>
<td>No</td>
</tr>
</tbody>
</table>
Table 2.3. Summary of Influenza subunit protective antigens as vaccine candidates.

<table>
<thead>
<tr>
<th>Protective Antigen</th>
<th>Biological Function</th>
<th>Vaccine Status</th>
<th>Cross-Reactivity</th>
<th>Adjuvant(s)</th>
<th>Selected References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hemagglutinin (HA)</td>
<td>Fusion with cellular membrane for viral entry</td>
<td>Liscenced by Novartis (Aggripal)</td>
<td>Cross-reactive across clades</td>
<td>Diphtheria toxoid protein conjugate, polyanhydride nanovaccines, MF59®</td>
<td>151,263-268</td>
</tr>
<tr>
<td>Neuraminidase (NA)</td>
<td>Role in virion release from infected cells through sialic acid cleavage</td>
<td>Liscenced by Novartis (Aggripal)</td>
<td>Some cross-reactivity across clades</td>
<td>MF59®</td>
<td>151,268</td>
</tr>
<tr>
<td>Nucleoprotein (NP)</td>
<td>Role in RNA transcription</td>
<td>Phase 1 clinical trials</td>
<td>Highly conserved</td>
<td></td>
<td>269</td>
</tr>
<tr>
<td>Matrix protein 2 (M2)</td>
<td>Proton channel</td>
<td>Phase 1 clinical trials</td>
<td>Nearly invariable across strains with potential for universal vaccine</td>
<td>Expressed on Hepatitis B virus, and Neisseria meningitidis outer membrane protein</td>
<td>270-272</td>
</tr>
</tbody>
</table>
Table 2.4. Chemical structures of polymeric vaccine adjuvants.

<table>
<thead>
<tr>
<th>Polymeric Vaccine Adjuvant</th>
<th>Chemical Structure</th>
<th>Selected References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alginate</td>
<td>$\alpha$-L-Guluronate $\beta$-D-Mannuronate</td>
<td>273,274</td>
</tr>
<tr>
<td>Chitosan</td>
<td></td>
<td>275,276</td>
</tr>
<tr>
<td>Dextran</td>
<td></td>
<td>277</td>
</tr>
<tr>
<td>Poly-$\varepsilon$-caprolactone</td>
<td></td>
<td>278,279</td>
</tr>
</tbody>
</table>
Table 2.4 (continued)

<table>
<thead>
<tr>
<th>Poly(glycolic-co-lactides)</th>
<th>lactic acid LA</th>
<th>glycolic acid GA</th>
<th>280,281</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image" alt="Poly(glycolic-co-lactides) structure" /></td>
<td><img src="image" alt="lactic acid LA structure" /></td>
<td><img src="image" alt="glycolic acid GA structure" /></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Polyanhydride</th>
<th>sebacic acid SA</th>
<th>282,283</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image" alt="Polyanhydride structure" /></td>
<td><img src="image" alt="sebacic acid SA structure" /></td>
<td></td>
</tr>
</tbody>
</table>

1,3-bis(p-carboxyphenoxy)propane CPP

1,6-bis(p-carboxyphenoxy)hexane CPH

1,8-bis(p-carboxyphenoxy)-3,6-dioxaoctane CPTEG
CHAPTER 3: RESEARCH GOALS AND THESIS ORGANIZATION

The overall goal of this research is to design innovative strategies for the prevention and treatment of bacterial infections using polyanhydride nanoparticles. Through this work, we hope to design efficacious pneumonia and plague nanovaccines to prevent infections through a protective immune response to the pneumococcal surface protein, PspA, and the Yersinia fusion protein, F1-V, respectively. The specific goals (SGs) of this research are:

SG1: Investigate the mechanism of action of polyanhydride nanoparticles as vaccine adjuvants through interactions with immune cells at early time points and through the evaluation of the immune response at extended time points;

SG2: Study the sustained release of structurally stable, antigenic, and functional PspA from polyanhydride nanoparticles;

SG3: Evaluate the in vivo immune response to vaccination with PspA nanovaccine formulations to design a protective vaccine against lethal challenge with S. pneumoniae; and

SG4: Evaluate the in vivo immune response to vaccination with F1-V nanovaccine formulations to design a protective vaccine against lethal challenge with Y. pestis.

The overall goal of this research is to design efficacious vaccines against the respiratory pathogens S. pneumoniae and Yersinia pestis based on polyanhydride nanoparticles. Polymers and copolymers based on CPTEG, CPH, and SA were investigated as nanovaccine formulations for antigen delivery. The mechanism of action of polyanhydride nanoparticles was investigated to better understand how these nanovaccines interact with the immune system both early and late. Fluorescently-labeled antigen was delivered in 50:50 CPTEG:CPH nanoparticles and compared to soluble protein and protein adjuvanted with MPLA initially (Chapter 4). To
investigate how prolonged antigen presence affects vaccine efficacy, several polyanhydride chemistries were tested and compared to MPLA at 14, 36, and 63 days after administration (Chapter 5). The bacterial surface protein PspA was found to retain stability and antigenicity upon release from 50:50 CPTEG:CPH and 20:80 CPH:SA nanoparticles (Chapter 6). Chapter 7 describes the design of a protective polyanhydride nanovaccine against *S. pneumoniae*. Chapter 8 details work done on including novel small molecule adjuvants in nanovaccine formulations with the goal of inducing protective immunity against *Y. pestis* challenge at early time points. Finally, Chapter 9 outlines ongoing and future work on the use of polyanhydride nanoparticles for the delivery of antimicrobial drugs and for a combination vaccine against both influenza and pneumonia.
CHAPTER 4: LUNG DEPOSITION AND CELLULAR UPTAKE BEHAVIOR OF PATHOGEN-MIMICKING NANOVACCINES IN THE FIRST 48 HOURS


Kathleen A. Ross¹, Shannon L. Haughney¹, Latrisha K. Petersen¹, Paola Boggiatto², Michael J. Wannemuehler², and Balaji Narasimhan¹*

¹Department of Chemical and Biological Engineering, Iowa State University, Ames, IA 50011

²Department of Veterinary Microbiology and Preventive Medicine, Iowa State University, Ames, IA 50011

*To whom all correspondences may be addressed

Keywords: antigen presenting cells, polyanhydride nanoparticles, vaccine, deposition, cellular uptake
4.1 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 immune responses with a single intranasal dose.

4.2 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\textsuperscript{5,6}. 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\textsuperscript{7,8}. However, compared to an approved adjuvant such as alum, polyanhydride nanoparticles are markedly less phlogistic\textsuperscript{8}. 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 nanoparticles provide pathogen-mimicking capabilities that enhance the activation of antigen presenting cells (APCs)\textsuperscript{8-10}. These nanovaccines induce robust immune responses and provide protective immunity without the damaging effects of disease\textsuperscript{7}. 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\textsuperscript{7,10}.

While it has been demonstrated that intranasally administered polyanhydride nanovaccines provide long-lasting protective immunity\textsuperscript{7,10}, 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\textsuperscript{11}. 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\textsuperscript{12}. 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\textsuperscript{13}. 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\textsuperscript{13}. It was also observed that sustained exposure of antigen enriched these interactions and enhanced T cell activation\textsuperscript{13}. 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 formulation compared to that associated with monophosphoryl lipid A (MPLA), providing new insights into the protective capabilities of pathogen-mimicking nanovaccines.
4.3 Experimental Section

4.3.1 Materials

Compounds for polymer synthesis included 1,6-dibromohexane, 1-methyl-2-pyrrolidinone, hydroxybenzoic acid, N,N-dimethylacetamide, sebacic acid, and triethylene 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).

4.3.2 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 ($M_w$~5100 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}. 
4.3.3 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% \( \text{w/w} \) 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).

4.3.4 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 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.
4.3.5 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 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. It is important to recognize that the methods described herein detect the deposition of the fluorescently labeled F1-V antigen, either delivered solubly or in the context of adjuvants such as MPLA and polyanhydride nanoparticles.

4.3.6 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. No F1-V was used in these experiments. 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 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.

4.3.7 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).

4.3.8 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).

4.3.9 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.

4.4 Results and Discussion

4.4.1 Encapsulation prolongs antigen presence

Mice received an intranasal administration of the vaccine formulations shown in Figure 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 1). Ex vivo analysis of lung tissues showed that the mice administered the nanovaccine formulations (i.e., encapsulated or soluble + encapsulated) showed prolonged presence of antigen. Figure 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 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\(^7\), 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.

4.4.2 Reduced pulmonary inflammation after deposition of polyanhydride nanoparticles

Because inflammation may affect the disappearance of antigen from the site of deposition, the inflammatory response induced by MPLA and the nanoparticles was evaluated. It is known that MPLA induces a marked inflammatory response\(^{16}\) 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\(^8\), the inflammation induced by intranasally delivered polyanhydride nanovaccines is unknown. Mice that were previously administered blank polyanhydride nanoparticles or MPLA alone (i.e., no antigen) intranasally were injected with ProSense® 750 FAST, a fluorescent imaging agent that is activated by cathepsins produced by inflammatory cells such as monocytes and macrophages\(^{20}\). While both polyanhydride nanoparticles and MPLA displayed similar magnitudes of inflammation at 6 hours post-administration (Figure 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 1.

Thus far, our data indicate that polyanhydride nanovaccines prolonged the presence of F1-V in the lungs (Figure 1) while inducing mild inflammation (Figure 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\(^7\). 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\(^7,10\). 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\(^13\).

4.4.3 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\(^13\). 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 3). This observation is consistent with the results in Figure 1. While soluble F1-V is rapidly internalized by APCs, antigen delivered in the context of the nanovaccine formulations is continuously released and can be 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 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\(^7,10\). It is hypothesized that soluble F1-V alone likely failed to induce an inflammatory response and 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 nanovaccine formulations 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.

4.4.4 Cellular uptake of antigen 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 (aMΦs): CD11c- CD11b+ F4/80+; and epithelial cells: CD11c- CD11b- CD324+ (Figure 4A). While the percentage of different cellular populations associated with antigen was independent of formulation (Figure 4B), the MFI or amount of F1-V within the cell populations varied among the different treatment groups (Figure 4C). Consistent with the data presented in Figures 1 and 3, the amount of antigen in the F1-V+ DCs and aMΦs was reduced significantly to background by 48 hours for cells recovered from mice administered MPLA-adjuvanted F1-V (Figure 4C). This is likely associated with the more rapid clearance of antigen (Figure 1) induced by inflammation (Figure 2). In contrast, mice administered nanovaccines or soluble antigen alone displayed stable or increased MFI of antigen associated with DCs or aMΦs and was comparable to cells recovered from mice that received only the soluble antigen (Figure 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.
4.5 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.

4.6 Acknowledgements

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CHAPTER 5: EFFECT OF NANOVACCINE CHEMISTRY ON HUMORAL IMMUNE RESPONSE KINETICS AND MATURATION

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Shannon L. Haughney†, Kathleen A. Ross†, Paola Boggiatto, Michael J. Wannemuehler, and Balaji Narasimhan*

†Department of Chemical and Biological Engineering, Iowa State University, Ames, IA 50011

‡Department of Veterinary Microbiology and Preventive Medicine, Iowa State University, Ames, IA 50011

†These authors contributed equally to this work.

* To whom all correspondence should be addressed
5.1 Abstract

Acute respiratory infections represent a significant portion of global morbidity and mortality annually. There is a critical need for efficacious vaccines against respiratory pathogens. To vaccinate against respiratory disease, pulmonary delivery is an attractive route because it mimics the route of natural infection and can confer both mucosal and systemic immunity. We have previously demonstrated that a single dose, intranasal vaccine based on polyanhydride nanoparticles elicited a protective immune response against *Yersinia pestis* for at least 40 weeks after immunization with F1-V. Herein, we investigate the effect of nanoparticle chemistry and its attributes on the kinetics and maturation of the antigen-specific serum antibody response. We demonstrate that manipulation of polyanhydride nanoparticle chemistry facilitated differential kinetics of development of antibody titers, avidity, and epitope specificity. The results provide new insights into the underlying role(s) of nanoparticle chemistry in providing long-lived humoral immunity and aid in the rational design of nanovaccine formulations to induce long-lasting and mature antibody responses.

5.2 Introduction

Acute respiratory infections represent a significant portion of global morbidity and mortality annually\(^1,2\). With the alarming increase in multi-drug resistant pathogens and the threat of aerosolized bioterrorism agents, there is an urgent need for the development of safe and efficacious vaccines against respiratory pathogens\(^3,4\). To vaccinate against respiratory disease, intranasal and pulmonary delivery are attractive routes because intranasal or inhaled vaccines can confer both mucosal and systemic immunity, and deliver antigen to immune inductive sites within the lung that can result in 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\(^7,8\). It is often noted, however, that antigen delivered alone does not induce protective immunity, and therefore, requires the use of adjuvants\(^9,10\).

Polyanhydride nanoparticle-based vaccines (i.e., nanovaccines) have previously been demonstrated to be a safe\(^11\) and efficacious delivery platform for protein antigens\(^12,13\). These biodegradable polymers demonstrate pathogen-mimicking properties that adjuvant poorly immunogenic subunit proteins and enhance the immune response\(^12\). Additionally, particle chemistry may be tailored to stabilize labile proteins as well as to control the rate of protein release\(^14\). A single intranasal dose of a polyanhydride nanovaccine formulation based on F1-V, a recombinant fusion protein of Yersinia pestis that has been shown to be a protective antigen against pneumonic plague\(^15\), was demonstrated to provide protective immunity in mice up to 40 weeks after vaccination\(^12\).

Previously, we have described the deposition, distribution, and prolonged presence of antigen delivered in the context of polyanhydride nanoparticles at early time points (i.e., 2 to 48 hours) after intranasal administration.\(^16\) These nanoparticles were based on 1,6-bis(p-carboxyphenoxy) hexane (CPH) and 1,8-bis(p-carboxyphenoxy)-3,6-dioxaoctane (CPTEG). While the initial interactions between antigen and antigen presenting cells (APCs) are important in laying a foundation for vaccine efficacy, the chemistry of the nanovaccine formulation plays an important role in the continual recruitment of APCs and for the development of high antibody titers with high avidity. In this work, we systematically analyzed the effect of nanoparticle chemistry on antigen availability, sustained internalization of antigen by immune cells, and the kinetics and maturation of the humoral immune response.
5.3 Materials and Methods

5.3.1 Materials

The materials used for monomer synthesis include sodium hydroxide, hydrobenzoic acid, dibromohexane, 1-methyl-2-pyrrolidinone, triethylene glycol, and sebacic acid (SA) (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 synthesis 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-conjugated anit-CD11c, biotinylated anti-CD324, streptavidin eFluor 710 (eBioscience, San Diego, CA), PE-CF594-conjugated anti-CD11b (BD Bioscience, San Jose, CA), and PE-Cy7-conjugated anti-F4/80 (BioLegend, San Diego, CA).

5.3.2 Polymer synthesis

The CPH and CPTEG monomers were synthesized as described previously\textsuperscript{17–20}. Pre-polymers of CPH and SA were synthesized from monomers as described previously\textsuperscript{18,19}. Copolymers based on CPH, CPTEG, and SA were synthesized using melt condensation as
described by Kipper et al. and Torres et al.\textsuperscript{17,18}. Polymer purity and molecular weight were determined using \textit{1H} NMR (Varian VXR300).

5.3.3 Nanoparticle fabrication

The Vivo Tag 680 fluorescent label was conjugated to F1-V fusion protein according to manufacturer instructions (Perkin Elmer). Briefly, 10 µL of Vivo Tag 680 was added per mg of F1-V and incubated at room temperature for 1 h. Excess unconjugated Vivo Tag 680 was 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 described previously.\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 solvent:non-solvent ratio of 1:250 at room temperature for CPH:SA formulations or at -40°C for CPTEG:CPH copolymers due to the lower glass transition temperature of the CPTEG-containing copolymers\textsuperscript{17}. 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{20,21}.

5.3.4 Mice

Five to six week old C57BL/6 mice were obtained from Harlan Laboratories (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.
5.3.5 Immunization protocol

Mice (between 7 and 9 weeks of age) were anesthetized with a 100 µL injection of a solution containing 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, or 20:80 CPH:SA nanoparticles with 40 µg of soluble F1-V delivered concurrently in 50 µL of PBS, 50 µg of F1-V delivered with 10 µg MPLA derived from Salmonella enterica serotype Minnesota Re 595 (Sigma Aldrich), or 50 µg of F1-V alone. While the animals were anesthetized, saline suspensions of the polyanhydride nanovaccines, protein alone, or protein with MPLA were delivered to the mice using a 100 µL pipettor fitted with a pipet tip to deliver a total of 50 µL of fluid to the animal. Approximately 25 µL was delivered through each nare of the nose. Sterile PBS was administered to all control animals. Mice were euthanized at 14, 36, or 63 days post-immunization. Samples were collected from four mice per group per time point and the experiment was repeated resulting in a total of eight mice per group per time point. The soluble protein alone and PBS control groups contained a total of four animals per treatment group.

5.3.6 Ex vivo lung imaging

Mice were euthanized to measure the amount of protein remaining in the lung at 14, 36, and 63 days after intranasal immunization. A lung perfusion with 5 mL of sterile PBS was performed to reduce background autofluorescence caused by red blood cells and the lungs were then excised. An in vivo live animal 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 with 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 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.

5.3.7 Multi-spectral imaging flow cytometry

After imaging, lung samples were incubated in Hank’s balanced salt solution (HBSS) with 1 mg/mL of collagenase D and 60 U/mL of DNase II for 20 min at 37°C. Tissue was then homogenized to a single cell suspension using a gentleMACSTM dissociator (Miltenyi Biotec, Cambridge, MA). The cellular suspensions were centrifuged at 250 rcf for 30 s to remove large debris. The supernatants were then passed through a 40 µm filter and the filtrate was then centrifuged at 250 rcf for 10 min at 4°C to pellet the cells. Remaining red blood cells in the lung homogenates 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 of 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 to measure the internalized F1-V using IDEAS® software (Amnis).
5.3.8 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. Cells were surface-labeled with specific monoclonal antibodies against CD11c, CD11b, CD324, and F4/80 for 30 min. After washing, the labeled 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).

5.3.9 Anti-F1-V serum antibody titers and avidity assays

Antibody titers were determined using an enzyme-linked immunosorbent assay (ELISA) as described elsewhere\textsuperscript{13}. 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 three-fold serial dilutions were performed. 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 of 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 the background (i.e., saline) value. 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 in a 0.1 M sodium phosphate buffer was added to the first well and serially diluted two-fold five times (i.e., for a final dilution of 1:32). Six control wells were used per sample and received sodium phosphate buffer alone. The solution was incubated for 15 min before washing thoroughly. The remainder of the assay followed the steps described above for ELISA. A relative avidity index was calculated by using an exponential fit of sodium thiocyanate serial dilutions to determine the concentration at which the OD is 50% of that of the non-treated wells containing the 1:200 diluted serum sample.

5.3.10 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.

5.3.11 Statistics

Statistical significance in Figures 1 and 3 was determined by one-way analysis of variance (ANOVA) followed by Tukey's post-test using GraphPad Prism software (Version 6.01, GraphPad Software, Inc., La Jolla, CA). A logarithmic transformation was performed on the antibody titer data presented in Figure 2 before determining statistical significance by ANOVA with a Bonferroni correction. In cases where the antibody titer was undetectable, a value of one-half the limit of detection was used. P-values less than or equal to 0.05 were considered significant.
5.4 Results

5.4.1 Immunization with polyanhydride nanovaccines induced prolonged availability of antigen in the lung for up to 63 days after administration

In order to compare the antigen availability and the kinetics of the humoral immune response induced by the different nanoparticle chemistries, a 50 µg dose of F1-V was used in all the vaccine formulations. A comparison of the nanoparticle sizes and polydispersity is shown in Table 1. The use of nanoscale particles is motivated by previous work from our laboratories on the deposition and clearance kinetics of micron-sized vs. nm-sized particles in the lung\textsuperscript{22}. These studies suggest optimum pulmonary deposition and cellular uptake of particles in the 300-500 nm size range, without inducing any adverse tissue responses. Vaccination of mice using a combination of 40 µg soluble F1-V along with 50:50 CPTEG:CPH nanoparticle-encapsulated F1-V (10 µg) in a single intranasal dose has previously been demonstrated to induce protection against lethal challenge with Y. pestis\textsuperscript{12,13}. The rationale of using a soluble bolus (40 µg) of antigen along with a portion (10 µg) encapsulated within the nanoparticles is that the soluble bolus rapidly primes the B cell response, while the encapsulated portion is released slowly over several weeks to sustain that immune response. In the present studies, F1-V was fluorescently labeled with Vivo Tag 680 in order to track the persistence of the protein in the lung after intranasal administration. Anesthetized C57B/6 mice were administered a single, intranasal dose of a given vaccine formulation and separate groups of mice were euthanized at 14, 36, and 63 days in order to quantify the remaining fluorescence in the excised lungs followed by flow cytometric assessment of F1-V associated with or internalized by dissociated lung cells.

Figure 1 demonstrates the persistence of F1-V within the lung following its administration in soluble form or encapsulated into polyanhydride nanoparticles. The data also
demonstrate the differential effect of nanoparticle chemistry on antigen availability. Consistent with our previous work, F1-V adjuvanted with MPLA was essentially cleared from the lung by day 14. In contrast, F1-V administered in the context of the polyanhydride nanovaccine formulations continued to persist in the lung as a function of nanoparticle chemistry. The decay of fluorescence associated with nanoparticle clearance kinetics from the lung is qualitatively consistent with previous work examining antigen release kinetics from polyanhydride nanoparticles in vitro. 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 availability of antigen within the lung. In contrast, nanoparticles based on the SA-rich 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.

Quantification of antigen internalization within the lung was used to investigate the combined effects of nanoparticle chemistry and antigen availability. Nanoparticles based on SA-rich chemistries were demonstrated previously to be more readily internalized by APCs in comparison to particles based on CPH-rich chemistries. However, SA-rich chemistries have faster erosion and release kinetics than CPH-rich chemistries, and, therefore, result in reduced antigen availability at later time points. Consistent with these observations, mice immunized with the 20:80 CPH:SA nanovaccine formulation demonstrated a qualitatively higher, though not statistically significant, percentage of lung cells that internalized antigen on day 14 post-immunization (Figure S1). However, by 36 and 63 days post-immunization, the percentage of cells containing internalized F1-V delivered in the 20:80 CPH:SA nanovaccine formulation had further decreased. In contrast, lung cells from mice administered the
hydrophobic and CPH-rich 20:80 CPTEG:CPH nanovaccine continued to demonstrate the presence of internalized antigen for at least 63 days, albeit at low levels. This observation can be attributed to the slower erosion profile of CPH-rich chemistries26, which enables sustained antigen release and availability.

5.4.2 Polyanhydride nanovaccines elicit sustained high titer antibody responses

The data in Figure 1 provides evidence regarding the kinetics of F1-V availability within the lung after intranasal immunization with polyanhydride nanovaccines. These data also suggest that F1-V is continuously present within lung cells for at least 36 days regardless of the formulation administered. Next, new insights are presented on the combined effects of polymer chemistry and antigen availability on the serum antibody response to F1-V. The data in Figure 2 demonstrates that the kinetics of the resultant anti-F1-V IgG antibody titer was affected by the choice of polymer chemistry, while antigen availability had less of an impact on the antibody titer over time. The antibody titer elicited by all the formulations studied was statistically significant in comparison to that induced by the soluble F1-V alone at each time point. Other than the IgG responses induced by 50:50 CPTEG:CPH, the serum antibody response for mice immunized with all other formulations demonstrated serum antibody titer ≥ 50,000 by day 36; however, the day 63 titer in these groups indicated that the F1-V-specific IgG response began to wane. However, the mice that received the 50:50 CPTEG:CPH nanovaccine showed progressively increasing serum antibody titer through the nine weeks of this study. While detectable in the lung for 63 days (Figure 1), the animals that received soluble F1-V alone did not develop a measurable antibody titer demonstrating that the presence of immunogen alone is not sufficient and that an appropriate adjuvant is necessary to induce the development of a F1-V-specific antibody response.
5.4.3 Antigen availability and release kinetics affects antibody avidity

The availability of antigen may not only facilitate sustained antibody titer, but drive the development of avid antibodies as well. Similar to antibody titer, the evolution of the avidity of F1-V-specific IgG was found to be a function of the nanovaccine polymer chemistry (Figure 3). Nanovaccines based on 20:80 CPTEG:CPH and 20:80 CPH:SA nanoparticles induced antibody avidities similar to MPLA. Although these formulations developed highly avid antibodies by 36 days post-immunization, there were no further increases in avidity at later time points. In contrast, the serum of mice that received the amphiphilic 50:50 CPTEG:CPH nanovaccine showed a significant increase in avid F1-V-specific IgG antibody at each time point. Only the mice administered the 50:50 CPTEG:CPH nanovaccine formulation showed continued maturation of the IgG avidity, as evidenced by the fact that the avidity was statistically different at each subsequent time point. These differences in avidity may be attributed in part to the differences in the antigen release kinetics from these polymers and, therefore, antigen availability. It is likely that the 20:80 CPH:SA nanoparticles release antigen too rapidly to stimulate the continual affinity maturation (i.e., sequentially higher avidity) of the F1-V-specific antibody response. Despite a similar antibody response induced by 20:80 CPH:SA nanovaccine, the most hydrophobic 20:80 CPTEG:CPH nanoparticles may release antigen too slowly such that there may not be sufficient F1-V to elicit an ever maturing antibody response. In contrast, the 50:50 CPTEG:CPH nanoparticles must exhibit a release rate of antigen that results in a sequential improvement of the avidity of the F1-V-specific IgG response.

5.4.4 Polyanhydride nanovaccines result in broader epitope specificity

The ability of vaccines to induce responses towards a broad spectrum of epitopes may enhance protection. Because the antibody avidity (Figure 3) is the summation of the antibody
response towards all epitopes, an overlapping peptide array of the F1-V antigen was used to
determine the antibody response to specific epitopes induced by each immunization regimen as
shown by the heat map in Figure 4. Consistent with the antibody titer and avidity responses
depicted in Figures 2 and 3, the responses to specific epitopes were highly dependent upon the
nanoparticle chemistry and the kinetics of antigen release. The number of peptides recognized by
serum IgG (i.e., peptides which showed an optical density (OD) value above background, or OD
value ≥ 0.5) at each time point is shown across the bottom of the heat map. Serum antibodies
from mice receiving the 20:80 CPTEG:CPH nanovaccine showed increased recognition in terms
of the number of epitopes recognized by serum antibody between days 14 and 36 post-
immunization; however, this response saturated by 36 days post-immunization. Likewise, the
number of recognized epitopes, while initially broad, waned over time in the case of serum from
the mice that received the 20:80 CPH:SA nanovaccine. Interestingly, the 50:50 CPTEG:CPH
nanovaccine induced an IgG response that continued to expand the epitopes recognized over
time, which corroborates the increases in antibody titer and avidity discussed previously.

The ability to induce antibodies specific for a broad spectrum of epitopes is no doubt
important for long-term protection; however, the V1 and V2 epitopes in particular have been
shown to be important for protection against Y. pestis in C57BL/6 mice\textsuperscript{27,28}. The data in Figure 5
compares the immune response to these two dominant epitopes in response to each of the
formulations, with an OD value with a greater than 1.5-fold change from saline controls defined
as epitope recognition\textsuperscript{29}. The immune response to these dominant epitopes induced by the 50:50
CPTEG:CPH nanovaccine exhibited a time dependent evolution and the optical density
increased over time as the immune response narrowed to these epitopes. This type of kinetics
was not observed with any of the other nanovaccine formulations nor with MPLA. Similar
responses were observed for the F18 epitope (data not shown), noting that the addition of responses to the F1 antigen enhanced the protection provided by the V antigen\textsuperscript{15}.

5.5 Discussion

In this work, different polyanhydride nanovaccine formulations were systematically examined for their ability to affect in vivo antigen availability and the resultant induction of a humoral immune response. While all the formulations examined produced similar antigen-specific antibody titers, the kinetics of the F1-V-specific immune response varied when antigen was delivered in the context of the different polymer nanovaccine chemistries. Building upon our previous work\textsuperscript{12,13,16}, the studies presented herein shed new light upon the complex relationship(s) between polyanhydride nanoparticle chemistry and the kinetics and maturation of the induced humoral immune response.

Of the three nanoparticle formulations evaluated, the 20:80 CPH:SA chemistry is the least hydrophobic. As a result, the 20:80 CPH:SA nanoparticles have the fastest polymer degradation kinetics, leading to the release of a vast majority of the protein within one month\textsuperscript{26,30,31}. In agreement with the above in vitro observations of release kinetics, the fluorescently-labeled F1-V was nearly undetectable in the lung by 36 days post-immunization indicating that the 20:80 CPH:SA nanoparticles had eroded and released its payload (Figure 1). In addition to faster antigen release, the clearance of F1-V encapsulated within the 20:80 CPH:SA nanoparticles can also be attributed to the enhanced internalization of these particles (Figure S1). As it is the antigen that is labeled in this work (rather than the particle), it is important to state that relative to the disappearance of the soluble protein alone, it is clear that the persistent F1-V in this group is due its encapsulation into 20:80 CPH:SA nanoparticles, which is in turn was cleared most rapidly among the polyanhydride chemistries tested. These observations
support previous work in which nanoparticles composed of SA-rich chemistries were rapidly internalized in comparison to CPH-rich chemistries\textsuperscript{20,24,25}.

While the 20:80 CPH:SA nanovaccine formulation more rapidly induced the development of antibody titers compared to the other nanoparticle formulations,(Figure 2), both the titer as well as the avidity of the antibody reached their peak values at 36 days post-immunization (Figures 2 and 3, respectively). The number of F1-V epitopes recognized by the antibody from mice administered the 20:80 CPH:SA nanovaccine decreased with time (Figure 4). This observation can be linked to the kinetics of the antibody titer and avidity of the response to the 20:80 CPH:SA nanovaccine formulation, which remain unchanged between the two later time points. The rapid release and clearance of antigen, and therefore decrease in antigen availability, may affect the ability of the 20:80 CPH:SA nanovaccine formulation to sustain long-term protective immune responses. In addition, the decreased hydrophobicity and rapid degradation of this polymer, leading to a similarly rapid waning of the pro-inflammatory cytokine response induced after implantation, may present fewer danger signals to the immune system in comparison with more hydrophobic chemistries\textsuperscript{32}. We hypothesize that the reduced danger signal elicited by this formulation, coupled with its rapid clearance from the lung, may lead to the induction of short-lived plasma cells\textsuperscript{33}, which is consistent with the waning antibody titers and avidity as observed in Figures 2 and 3. Thus, it is likely the 20:80 CPH:SA nanovaccine formulation may be suitable for rapid induction of immunity, but not for induction of long-lived antigen-specific IgG, as suggested by the data.

In contrast, the CPH-rich nanovaccine formulations (i.e., 20:80 CPTEG:CPH and 50:50 CPTEG:CPH) are more hydrophobic and degrade more slowly, providing slower protein release kinetics\textsuperscript{17} and prolonging the presence of antigen in the lung for at least 63 days as shown in
Figure 1. However, the CPH-rich chemistries were also less readily internalized by lung cells than the SA-rich chemistries as shown in Figure S1 and in previous work\textsuperscript{12,20}. The combination of the reduced internalization and slower degradation kinetics due to increased hydrophobicity enabled the CPH-rich nanovaccine formulations to provide sustained antigen presence. Together, these observations raise an important question on the effect of nanoparticle degradation and antigen availability on the generation and maintenance of humoral immune responses over time\textsuperscript{32}.

The antibody titer and avidity responses in mice administered the 20:80 CPTEG:CPH nanovaccine formulation reached their maximum by 36 days post-immunization (Figures 2 and 3). The kinetics of these responses were similar to those observed in mice administered the 20:80 CPH:SA nanovaccine formulation, albeit with higher titers. We hypothesize that the hydrophobicity of the 20:80 CPTEG:CPH nanovaccine formulation may promote a more inflammatory environment, possibly leading to an increased response to the initial soluble bolus of antigen, which is consistent with previous observations\textsuperscript{11}. We propose that the lower availability of antigen at later time points, after the depletion of the soluble portion of the vaccine, from this slowly degrading formulation is offset by the greater inflammatory milieu (i.e., enhanced adjuvanticity) provided by this more hydrophobic formulation. This combination of hydrophobicity-induced inflammation and slow yet continuous release of antigen may lead to the induction of longer lived plasma cells, which are important for long-term maintenance of the antibody titers\textsuperscript{33-35}. In addition, mice receiving this formulation demonstrated a greater breadth in the epitope specificity of the humoral immune response in terms of the ability to recognize more antigen-specific epitopes over time (Figure 4), possibly due to the prolonged exposure to F1-V in the lung. Together, these data suggest that highly hydrophobic nano-carriers can be used to
formulate vaccines that promote the maturation of potent and long-lived humoral immune responses.

The 50:50 CPTEG:CPH nanovaccine formulation has been demonstrated in our previous work to possess pathogen-mimicking abilities in terms of its internalization by APCs and its activation and induction of long-term protective immunity. The antibody titers and the avidity of the antigen-specific IgG responses of mice that received the 50:50 CPTEG:CPH nanovaccine formulation continued to increase at each successive time point (Figures 2 and 3). In contrast to the other formulations, the mean levels of circulating antibody increased between 36 and 63 days after immunization only for the mice that received the 50:50 CPTEG:CPH nanovaccine formulation. We propose that this observation suggests that the immune response induced by the 50:50 CPTEG:CPH nanovaccine and the persistent presence of antigen would facilitate continual differentiation of B cells into long-lived plasma cells. It is also important to note that memory B cells induced in early germinal centers typically develop broader antibody repertoires (i.e., greater avidity and increased epitope spread) as suggested by the data in Figures 3 and 4. There are several other factors to consider when analyzing these observations. First, CPTEG-rich polymers have lower glass transition temperatures compared to the other polyanhydrides studied herein that likely contributes to particle agglomeration within the lung, leading to a reduced surface area to volume ratio and resulting in slower release of antigen and prolonged presence of F1-V1. Second, the alternating pattern (i.e., -CPTEG-CPH-CPTEG-CPH-CPTEG-) provided by the 50:50 CPTEG:CPH copolymer (because of its equal monomer reactivity ratios) may be recognized by immune cells as a microbe-associated molecular pattern. As the nanoparticles erode, an increased number of oligomeric chains are generated with molecular patterns that might mimic an increasing microbial burden as with a replicating
pathogen or a replication-competent, live, attenuated vaccine. This is supported by previous work demonstrating protective immunity 40 weeks after a single intranasal immunization\textsuperscript{12,13}. In light of these observations, we suggest that the pathogen-mimicking 50:50 CPTEG:CPH nanovaccine formulation more effectively primes the immune response and induces long-lived immunity.

5.6 Conclusions

We have herein demonstrated the importance of polymer chemistry in the design of a 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.

5.7 Acknowledgements

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5.8 References


2. G. Nm, Epidemiol Rev, 1989, 12, 149–78.


Figure 5.1. Polyanhydride nanovaccines persisted in the lung for up to 63 days. Images represent fluorescence of remaining Vivo Tag 680 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 among formulations at one time point (e.g., comparing the MFI of the 50:50 CPTEG:CPH nanovaccine at day 36 to the 20:80 CPTEG:CPH nanovaccine at day 36). Asterisks represent statistical differences between time points within a single formulation (p ≤ 0.05).
Figure 5.2. 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 (day 63). The antibody titers elicited by all the formulations studied were statistically significant in comparison to that induced by the soluble formulation at each time point. Asterisks represent statistical differences between time points within a single formulation (p ≤ 0.0077).
Figure 5.3. 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 (day 63). Asterisks represent statistical differences between time points within a single formulation (p ≤ 0.0084).
Figure 5.4. Nanovaccine formulations resulted in enhanced epitope recognition by antibodies. The responses to eighty F1 and V peptides were evaluated by ELISA and are shown as a heat map, beginning at the top with the amino-terminal peptide and then moving down sequentially through the F1-V protein. The optical density of each peptide is indicated by a range of color from blue (no response) to red (maximum response). The number of reactive peptides (i.e., optical density (OD) value above background, or OD value ≥ 0.5) induced by each vaccine formulation at each time point is indicated below the heat map. Data presented is the average of eight individual mice for days 14 and 36, and four individual mice for the day 63 time point.
Figure 5.5. Evolution of the immune response to the immunodominant V1 and V2 peptides is affected by nanoparticle chemistry. The data is shown as a fold-change over saline. Data was collected from groups of animals for days 14 and 36 in two individual experiments and from four animals for day 63 in one experiment. Epitope recognition is defined as 1.5-fold or higher over saline. The dashed line represents a 1.5-fold change from saline controls for each peptide.
Supplemental Figure 5.1. 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. Asterisks represent statistical differences between time points within a single formulation (p ≤ 0.0082).

5.10 List of Tables

Table 5.1. Size and polydispersity of polyanhydride nanoparticles

<table>
<thead>
<tr>
<th>Chemistry</th>
<th>Size (nm)</th>
<th>PDI</th>
</tr>
</thead>
<tbody>
<tr>
<td>20:80 CPTEG:CPH</td>
<td>120 +/- 55</td>
<td>0.17</td>
</tr>
<tr>
<td>50:50 CPTEG:CPH</td>
<td>151 +/- 63</td>
<td>0.15</td>
</tr>
<tr>
<td>20:80 CPH:SA</td>
<td>324 +/- 162</td>
<td>0.17</td>
</tr>
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</table>
CHAPTER 6: RETENTION OF STRUCTURE, ANTIGENICITY, AND BIOLOGICAL FUNCTION OF PNEUMOCOCCAL SURFACE PROTEIN A (PSPA) RELEASED FROM POLYANHYDRIDE NANOPARTICLES

Shannon L. Haughney¹, Latrisha K. Petersen¹, Amy D. Schoofs², Amanda E. Ramer-Tait², Janice King³, David Briles³,⁴, Michael J. Wannemuehler², and Balaji Narasimhan¹*


¹ Department of Chemical and Biological Engineering, Iowa State University, Ames, IA 50011

² Department of Veterinary Microbiology and Preventive Medicine, Iowa State University, Ames, IA 50011

³ Department of Microbiology and ⁴Pediatrics, University of Alabama Birmingham, Birmingham, AL 35233

⁵ Department of Food Science and Technology, University of Nebraska-Lincoln, Lincoln, NE 68583

*To whom all correspondences may be addressed

Keywords: Pneumococcal surface protein A, polyanhydride, nanoparticle, Streptococcus pneumoniae, vaccine
6.1 Abstract

Pneumococcal surface protein A (PspA) is a choline-binding protein which is a virulence factor found on the surface of all *Streptococcus pneumoniae* strains. Vaccination with PspA has been shown to be protective against a lethal challenge with *S. pneumoniae*, making it a promising immunogen for use in vaccines. Herein, the design of a PspA-based subunit vaccine using polyanhydride nanoparticles as a delivery platform is described. Nanoparticles based on sebacic acid (SA), 1,6-bis-(p-carboxyphenoxy)hexane (CPH) and 1,8-bis-(p-carboxyphenoxy)-3,6-dioxaoctane (CPTEG), specifically 50:50 CPTEG:CPH and 20:80 CPH:SA, were used to encapsulate and release PspA. The protein released from the nanoparticle formulations retained its primary and secondary structure as well as its antigenicity. The released PspA was also biologically functional based on its ability to bind to apolactoferrin and prevent its bactericidal activity towards *Escherichia coli*. When the PspA nanoparticle formulations were administered subcutaneously to mice, the animals elicited a high titer and high avidity anti-PspA antibody response. Together, these studies provide a framework for the rational design of a vaccine against *S. pneumoniae* based on polyanhydride nanoparticles.

6.2 Introduction

The World Health Organization estimates that pneumonia causes 1.6 million deaths every year, with the majority occurring in children in developing countries1,2. *Streptococcus pneumoniae* is the leading cause of bacterial pneumonia worldwide, especially in children3. Current prophylactic options against *S. pneumoniae* include a 23-valent soluble polysaccharide vaccine (pneumovax) and 7 and 13-valent pneumococcal conjugate vaccine (PCV)4. The polysaccharide-based vaccine has been shown to induce humoral immunity in immune-competent patients, but fails to stimulate a cellular immune response, making the vaccine
ineffective in high-risk groups such as infants, the elderly, and immune-compromised individuals\textsuperscript{5}. Following the introduction of the multivalent, polysaccharide-protein conjugate pneumococcal vaccine into childhood immunization regimens, incidence of community-acquired pneumonia in children was reduced by 18\%\textsuperscript{6}. Although the PCV has been shown to be effective in reducing cases of pneumonia, it has several limitations. The vaccine is expensive and complicated to manufacture, leading to limited availability in developing countries; it does not provide cross-protection across pneumococcal serotypes; and while it reduces capsular type-specific carriage it has not been shown to reduce nasopharyngeal carriage of pneumococci in general. Additionally, the PCV requires a three-dose vaccination regimen and the 7-valent vaccine leads to increased prevalence within the community of strains not included in the vaccine (i.e., serotype substitution) within several years of introduction\textsuperscript{6, 7}.

Subunit vaccines against pneumonia using non-capsular antigens, specifically protein-based vaccines, have been extensively studied in recent years\textsuperscript{5, 8}. Of particular interest in this regard is pneumococcal surface protein A (PspA), which is a choline-binding protein found on the surface of all pneumococcal strains and a critical \textit{S. pneumoniae} virulence factor\textsuperscript{9}. PspA plays two different roles in invasive infection and nasopharyngeal carriage. During invasive, systemic infections with \textit{S. pneumoniae}, PspA prevents the deposition of complement on the surface of the bacterium, thus inhibiting the opsonization and killing of \textit{S. pneumoniae}\textsuperscript{9, 10}. PspA also inhibits bactericidal activity mediated by apolactoferrin (ALF) found on mucosal surfaces and in sites of inflammation\textsuperscript{9, 11-13}. Vaccination with PspA protects mice against a lethal challenge with \textit{S. pneumoniae} via the generation of anti-PspA serum antibodies that are highly cross reactive to other strains\textsuperscript{14-18}. However, PspA is poorly immunogenic and not capable of inducing a productive immune response without the addition of an adjuvant\textsuperscript{19-21}. In fact, a
vaccine regimen based on the inclusion of aluminum hydroxide, a commonly used adjuvant, required three doses to provide protective immunity in a murine model\textsuperscript{19-21}. Therefore, there is a need to design novel adjuvants and/or delivery vehicles for the formulation of efficacious vaccines that can protect against multiple strains of \textit{S. pneumonia} and enhance patient compliance by utilizing an acceptable dose regimen.

Because of the promise of PspA as a protective antigen against \textit{S. pneumoniae}, it has been the subject of numerous studies to evaluate novel vaccine delivery systems. Several research groups have shown the induction of immune responses through delivery of PspA with live attenuated bacteria such as \textit{salmonella}\textsuperscript{22,23} and through co-delivery with a whole-cell pertussis vaccine\textsuperscript{4}. Additionally, other novel, nanoscale delivery systems containing PspA have been evaluated, including gold nanoparticles\textsuperscript{24} and nanogel-based vaccine formulations\textsuperscript{25}. In this work, we demonstrate that biodegradable polyanhydride nanoparticles can successfully encapsulate and release stable, antigenic PspA.

Polyanhydrides have a number of benefits compared to other vaccine delivery systems. Their tunable polymer chemistry can allow for the modulation of the immune response and enable tailoring antigen release kinetics\textsuperscript{26,27}. Additionally, encapsulation into polyanhydride particles has been shown to protect fragile protein antigens from degradation\textsuperscript{28,29}. Polyanhydrides can be fabricated into nanoparticles for administration via inhalation or injection and have shown much promise as vaccine adjuvants and delivery vehicles\textsuperscript{26,30-33}. These polymers exhibit excellent biocompatibility and have been shown to degrade into non-toxic, non-mutagenic products\textsuperscript{34}. Polyanhydride particles have also been shown to stabilize fragile proteins throughout the manufacture, storage, and release steps and elicit immune responses \textit{in vitro} and \textit{in vivo}\textsuperscript{28-30,32,35,36}. In particular, amphiphilic polyanhydrides, which degrade through a
combination of bulk and surface erosion, provide a sustained release of protein, while maintaining protein structure and function upon release\textsuperscript{28, 37}. For example, encapsulation of the recombinant F1-V protein into nanoparticles made from a copolymer of 1,6-bis(\textit{p}-carboxyphenoxy)hexane (CPH) and 1,8-bis(\textit{p}-carboxyphenoxy)-3,6-dioxaoctane (CPTEG) stabilized the protein\textsuperscript{28} and provided protective immunity against a lethal challenge with \textit{Yersinia pestis} that persisted at least 23 weeks post-immunization using a single dose vaccine regimen\textsuperscript{32}.

In this work, we describe the encapsulation and release of stable PspA from polyanhydride nanoparticles. The released PspA retained its primary and secondary structure and preserved both its antigenicity and biological functionality. When the nanoparticle-based vaccine formulations consisting of soluble and encapsulated PspA were administered subcutaneously to mice, the animals developed and sustained high anti-PspA IgG titers that were also characterized by high avidity. These studies provide a framework for the rational design of an anti-\textit{S. pneumoniae} vaccine based on PspA-containing polyanhydride nanoparticles.

6.3 Materials and Methods

6.3.1 Materials

The materials used for monomer synthesis including sodium hydroxide, hydrobenzoic acid, dibromohexane, 1-methyl-2-pyrrolidinone, and triethylene glycol were purchased from 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); and 4-\textit{p}-fluorobenzonitrile was purchased from Apollo Scientific (Cheshire, UK). Sebacic acid monomer was purchased from Sigma Aldrich. The following chemicals used for acetylation and polymerization were purchased from Fisher Scientific: acetic anhydride, ethyl ether, petroleum ether, chloroform, methylene chloride, and
hexane. Deuterated chloroform and dimethyl sulfoxide for $^1$H NMR analysis of the polymers and monomers, respectively, were purchased Cambridge Isotope Laboratories (Andover, MA).

The K-12 MG1655 and the LPS-deficient K-12 NR688 *Escherichia coli* strains were provided by Dr. Gregory Phillips (Iowa State University).

The following chemicals used in the development of the PspA functional assay were purchased from Sigma Aldrich: ALF, lactoferricin, erythromycin, deferoxamine, and 2,2'-bipyridine. The following materials for bacterial culture were purchased from Becton Dickinson (Franklin Lakes, NJ): Luria-Bertani (LB) agar, trypticase soy broth, yeast extract, and tryptone. Agar and sodium chloride were purchased from Fisher Scientific. Sterile phosphate-buffered saline was purchased from Mediatech Inc. (Manassas, VA).

6.3.2 Polymer synthesis

The CPH and CPTEG monomers and their corresponding prepolymers were synthesized as previously described\textsuperscript{37-39}. Polymers and copolymers of CPH, CPTEG and SA were synthesized using melt condensation as described elsewhere\textsuperscript{34, 37}. Polymer purity and molecular weight were determined using $^1$H NMR (Varian VXR300) and gel permeation chromatography (Waters GPC, Milford, MA) was used to confirm molecular weight. The purity and molecular weight of the 50:50 CPTEG:CPH and 20:80 CPH:SA copolymers used herein were consistent with previously published data\textsuperscript{37, 40}. The copolymer compositions were determined by 1H NMR and were consistent with the co-monomer compositions (i.e., 18:82 for 20:80 CPH:SA and 47:53 for 50:50 CPTEG:CPH). The number-average molecular weights of the 50:50 CPTEG:CPH and 20:80 CPH:SA copolymers were 5,800 Da with a polydispersity index (PDI) of 1.5 and 21,000 Da with a PDI of 1.4, respectively.
6.3.3 Nanoparticle fabrication

PspA-loaded polyanhydride nanoparticles were formulated using an anti-solvent, solid/oil/oil nanoprecipitation method as previously described\textsuperscript{41-43}. Briefly, lyophilized PspA (1\% w/w) and polymer (20 mg/mL) were co-dissolved in methylene chloride. The solution was sonicated at an output of 40 Hz with a VibraCell ultrasonic probe (Sonics & Materials, Inc., Newton, CT) to ensure a homogenized mixture. The resulting solution was rapidly added to pentane at a 1 to 250 (v/v) ratio of methylene chloride to pentane at room temperature for the CPH:SA formulation or at -40°C for the CPTEG:CPH copolymer due to the lower glass transition temperature of this polymer\textsuperscript{37}. Nanoparticles were collected using vacuum filtration. The nanoparticles were characterized using scanning electron microscopy (FEI Quanta SEM, Hillsboro, OR) and their size distribution was determined using ImageJ software (Version 1.44p, National Institutes of Health, Bethesda, MD). The resulting size distributions were 677 ± 254 nm for the 20:80 CPH:SA nanoparticles and 243 ± 84 nm for the 50:50 CPTEG:CPH nanoparticles.

6.3.4 Preparation of recombinant protein

The N-terminal region of a recombinant PspA (UAB055, PspA/Rx1 AA1 to 303, clade 2 PspA of the PspA family 1) was produced by Dr. David McPherson (University of Alabama at Birmingham) as described previously\textsuperscript{12}. Protein was dialyzed using a 10,000 MW cut-off dialysis cassette (Thermo Fisher Scientific, Rockford, IL) against sterile, nanopure water and frozen at -80°C. Protein was then lyophilized at -40°C under vacuum overnight and stored at -80°C until further use. For the \textit{in vivo} vaccination and biological functionality assays, endotoxin was removed from the protein using endotoxin removal beads (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer’s instructions prior to dialysis and lyophilization. The final endotoxin content of the protein prior to use was determined to be less than 1.9 EU/mg as
determined by a limulus amebocyte lysate (LAL) chromogenic endotoxin quantification kit (Thermo Fisher Scientific).

6.3.5 Culture and storage of bacterial strains

Two *E. coli* strains were used in this work, wild type MG1655 and LPS-deficient NR688. Prior to the start of experiments, LB broth was inoculated with approximately one colony of *E. coli* and allowed to grow overnight. Fresh LB broth was inoculated and incubated until an OD$_{600}$ of approximately 0.7 was obtained. Sterile glycerol (15% v/v) was added to aliquots of the bacterial culture and 1 mL aliquots were frozen and stored at -80°C.

6.3.6 Protein release and quantification

The *In vitro* release of PspA protein from polyanhydride nanoparticles was carried out as previously described$^{28, 30, 35, 37, 44}$. Briefly, 25 mg of nanoparticles loaded with 1% (w/w) PspA were weighed into 1.5 mL microcentrifuge tubes and suspended in 600 µL of 0.05 M phosphate buffered saline (PBS, pH 7.2). Microcentrifuge tubes containing the suspensions were incubated at 37°C under constant agitation. At each time point, suspensions were removed and centrifuged at 14,000 rcf for 20 min. The supernatant was removed and replaced with 450 µL of fresh PBS and the microcentrifuge tubes returned to incubation. Supernatant samples were stored at 4°C until analyzed. Supernatant samples were centrifuged at 10,000 rcf for 10 min before protein quantification to ensure removal of any remaining polymer particles. Protein released from polyanhydride nanoparticles was quantified using a micro bicinchoninic acid assay (Thermo Fisher Scientific) and was compared to the total protein used. The colorimetric changes were measured using a SpectraMax M3 microplate reader (Molecular Devices, Sunnyvale, CA) at an absorbance of 570 nm. All the samples were tested in triplicate. Encapsulation efficiency was determined by degrading PspA-loaded particles in a solution of 17 mM NaOH. In order to
determine the pH of the particle microenvironment over time. 25 µg of 20:80 CPH:SA and 50:50 CPTEG:CPH nanoparticles were incubated in 600 µL of 0.05 M phosphate buffered saline (PBS, pH 7.2). At each time point samples were centrifuged and the supernatant was removed for analysis. Solution pH was measured using an Orion Ross pH electrode (Thermo Fisher Scientific).

6.3.7 Analysis of released PspA by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

The primary structure of the released PspA was studied using SDS-PAGE under reducing conditions. After centrifugation, the protein concentration was adjusted to 100 µg/mL with PBS. Once concentrations of all the samples were standardized, 15 µL of solution was combined with 15 µL of Laemmli sample buffer (Bio-Rad, Hercules, CA) with 5% (v/v) β-mercaptoethanol (Bio-Rad). The resultant mixture was heated at 96°C for 10 min and allowed to return to room temperature. Samples were loaded into the wells of a 12% acrylamide gel and subjected to 150 V constant voltage for 60 min. Approximate molecular size of protein bands were estimated using pre-stained protein standards (Bio-Rad, Hercules, CA). Gels were incubated in fixative solution (50% water, 40% ethanol, 10% acetic acid) for 3 h at 4°C and stained with Flamingo Gel Stain (Bio-Rad) at 4°C overnight before scanning. Gels were scanned with a Typhoon 9400 (GE Healthcare Piscataway, NJ) and analyzed using ImageQuant TL 8.1 software (GE Healthcare).

6.3.8 Secondary structure of released PspA

Circular dichroism was used to analyze the secondary structure of PspA released from polyanhydride nanoparticles. Aliquots of released PspA (100 µg/mL), collected after incubating for two hours in PBS at 37°C, were analyzed on a Jasco J-710 spectropolarimeter (Jasco, Inc., Easton, MD) using a 1 mm cuvette at far-UV wavelengths from 190-260 nm. Values shown are
the average of two readings of at least two experimental replicates. In order to obtain a spectra of degraded protein, PspA at a concentration of 100 µg/mL was incubated in nanopure water adjusted to a pH of 2 with 12.1 N HCl (Thermo Fisher Scientific) overnight. Protein was then collected and secondary structure was analyzed as described above.

6.3.9 Antigenicity of released protein

The ability of anti-PspA antibody to bind to PspA released from polyanhydride nanoparticles (i.e., antigenicity) was determined using an enzyme-linked immunosorbent assay (ELISA) as previously described28, 29. PspA released from polyanhydride nanoparticles was collected, adjusted to a concentration of 0.5 µg/mL, used to coat a high-binding chemistry 96-well plate (Cole-Parmer, Vernon Hills, IL), and incubated overnight at 4°C. The PspA solution was removed and PBS with 1% fish gelatin was added and incubated for 2 h at room temperature. Next, the blocking buffer was removed and the plates were washed three times with PBS containing 0.5% Tween 20 (PBS-T). Anti-PspA monoclonal antibody clone number 22003 (QED Biosciences, San Diego, CA) was added at a concentration of 1 µg/mL and the plates were covered and incubated overnight at 4°C. Plates were washed thrice with PBS-T before the addition of alkaline phosphatase-conjugated goat anti-mouse IgG (heavy and light chain) (Jackson ImmunoResearch, West Grove, PA) at a 0.1 µg/mL, which was incubated for 2 h at room temperature before developing. For the development of the ELISA, alkaline phosphatase substrate was added at 1 mg/mL substrate buffer (50 mM sodium carbonate 2 mM magnesium chloride buffer (pH 9.3)). After a 15 min incubation at room temperature, the colorimetric changes were measured at an absorbance of 405 nm using a SpectraMax M3 microplate reader. The data is presented as relative antigenicity, which is herein defined as the optical density (OD) ratio between the experimental replicate and the native protein.
6.3.10 Functional assay for released PspA

*E. coli* strains NR688 (LPS-deficient) and wild type MG1655 (used as an experimental control), were cultured on Luria-Bertani (L-B) agar plates at 37°C overnight. Approximately one colony was removed from the overnight plate and used to inoculate broth. It was necessary to remove iron from the experimental system in order to prevent binding to ALF, which forms iron-rich hemolactoferrin. To accomplish this, both iron-free media and the iron-chelating agent, deferoxamine, were tested, and it was found that the addition of 1 mg/mL of deferoxamine successfully removed iron. The inoculated culture was grown at 37°C to an OD$_{600}$ of approximately 0.5. Cultures were diluted 1:20 into fresh broth and incubated until an OD$_{600}$ of approximately 0.1 was obtained. Bacteria were plated into the wells of a 96-well microtiter plate at a final concentration of 1 million cells per well, as estimated by a SmartSpec 3000 (Bio-Rad). ALF was dissolved in the appropriate medium at 3 mg/mL and added to the plated bacteria. After incubation for 3 h at 37°C, 10 µL of the solution was removed and added to 90 µL of the appropriate broth. Six 1:10 dilutions were prepared before plating in triplicate on L-B agar plates. The plates were incubated at 37°C overnight and colony forming units (CFUs) were counted.

The same procedure was followed to test the abrogation of killing by ALF through the addition of recombinant PspA. Briefly, *E. coli* was grown on LB agar overnight, a colony was inoculated into 5 mL LB broth and incubated at 37°C until the culture reached its exponential growth phase. The culture was diluted 1:20 into fresh broth and grown to its early log growth phase before inoculating at $10^6$ cells/well of a microtiter plate. ALF was dissolved in medium at a concentration of 3 mg/mL followed by the addition of native PspA or PspA collected after release from nanoparticles, which was mixed and allowed to incubate for approximately 5 min.
before addition to the bacteria. The microtiter plate was incubated at 37˚C for 3 h before enumerating viable bacteria as described above. Although the nanoparticles were removed prior to the addition of released PspA, it was verified that the presence of nanoparticles did not interfere with the experimental system. Additionally, it was confirmed that the acidic microenvironment produced by the nanoparticle degradation products did not interfere with the growth of *E. coli*.

CFUs were determined by counting colonies grown on LB agar plates after overnight incubation. The log CFU killed was calculated by subtracting the experimental values from control *E. coli* cultures grown under identical conditions. Data is presented as log CFU of killed *E. coli*.

6.3.11 Immunization studies

CBA/CaHN-Btk-<sup>xid</sup>/J (CBA/N) mice purchased from Jackson Laboratory (Bar Harbor, ME) were used for the immunization studies. Mice were immunized subcutaneously with 20 to 25 µg reconstituted PspA and 0 to 5 µg PspA encapsulated into 500 µg of polyanhydride nanoparticles and/or formulated with MPLA (10 µg, derived from *Salmonella enterica* serotype Minnesota Re 595 (Sigma Aldrich)), a traditional adjuvant approved for human use. The treatment groups were: 20 µg PspA delivered solubly with 5 µg PspA encapsulated into 500 µg of 50:50 CPTEG:CPH nanoparticles, 20 µg PspA delivered solubly with 5 µg PspA encapsulated into 500 µg of 20:80 CPH:SA nanoparticles, 25 µg PspA delivered solubly with 10 µg MPLA, and a saline control. The volume of the inoculum was 100 µL in each case. At least six animals were used in each experimental group. Blood was collected from animals via the saphenous vein before immunization and at 2, 4, and 7 weeks post-immunization. Serum was
collected after centrifugation (10,000 rcf for 10 min) of the blood samples and stored at 4°C until use.

6.3.12 Anti-PspA serum antibody titers

Antibody titers were determined using an ELISA as described above. Plates were coated with PspA at a concentration of 0.5 µg/mL and incubated overnight at 4°C. The blocking buffer used for these studies was 0.05 M PBS-T supplemented with 2% gelatin (Becton Dickinson, Franklin Lakes, NJ). PspA coated microtiter plates were incubated for 2 h at room temperature before washing thrice with PBS-T. Serum from immunized mice was added to the first well at a 1:200 dilution in PBS with 1% (v/v) heat-inactivated goat serum and serially diluted two-fold. After an overnight incubation at 4°C, plates were washed three times with PBS-T, and then alkaline phosphatase-conjugated goat anti-mouse IgG (heavy and light chain) (Jackson ImmunoResearch) was added at a concentration of 1 µg/mL followed by incubation for 2 h at room temperature. Plates were washed four times with PBS-T 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 colormetric development. The plates were read after 30 minutes at 405 nm. All the samples were tested in technical replicates of 2. Titer is herein defined as the serum dilution that produced an optical density value twice that of the saline group.

6.3.13 Antibody avidity

MagPlex-C microspheres (magbeads) were activated and coupled to PspA (20 µg/mL in PBS) as described by the manufacturer. Following the conjugation step, the PspA-magbeads were washed with PBS-T and stored in PBS with 1% bovine serum albumin and 0.05% sodium azide and covered to block overnight at 4°C. Serum samples were diluted in PBS-T and 50 µL
was added to each well of a 96 well black, clear bottom microtiter plate along with 50 µL of vigorously vortexed magbeads (120,000/mL) conjugated with PspA suspended in blocking buffer. Plates were immediately covered and placed on a plate shaker for 1 h at room temperature. Plates were washed three times with the blocking buffer using a BioPlex Pro II plate washer and 100 µL of either PBS-T or 6 M urea in PBS (chaotropois agent) were added to the appropriate wells. Plates were covered and put on a shaker for 15 min, and then washed three more times. Biotin-conjugated goat anti-mouse IgG (eBioscience, San Diego, CA) was diluted 1:200 and 50 µL was added to each well, and plates were covered and shaken at room temperature for 1 h. Plates were washed three times with wash buffer, 50 µL of streptavidin-phycoerythrin (PE) diluted 1:20 in PBS-T was added to each well, and incubated for 30 min. Plates were washed three times and filled with 100 µL of blocking buffer per well, and the mean fluorescent intensity (MFI) was measured using a BioPlex 200. To calculate the relative avidity of a serum sample having a demonstrable antibody response to the target antigen, the MFI obtained for the sample well treated with 6 M urea was divided by the MFI obtained for the corresponding sample well incubated with only PBS-T. This value was multiplied by 100 to obtain a relative percent avidity for each serum sample.

6.3.14 Calculations and statistics

Log CFU killed were calculated by subtracting the log of the experimental group CFU/mL concentration from the log CFU/mL of bacteria alone after incubation. All error bars represent standard error of the mean (S.E.M). MiniTab statistical package (MiniTab, Inc., State College, PA) was used to perform statistical analysis. A two sample t-test was used and statistical significance was characterized by p < 0.05.
6.4 Results and Discussion

Several proteins, including F1-V, *Bacillus anthracis* protective antigen (PA), bovine serum albumin (BSA), and ovalbumin have been shown to be stably released from polyanhydride particles\(^{28, 29, 30, 36}\). Each of these proteins has different mechanisms of instability and/or degradation. Herein, the lessons learned from these previous studies were applied to determine the optimal polyanhydride nanoparticle formulations for stabilization and sustained delivery of functional PspA for the development of a vaccine against *S. pneumoniae*.

6.4.1 Polyanhydride nanoparticles provide for the release of stable and functional PspA

PspA was encapsulated into and released from 50:50 CPTEG:CPH and 20:80 CPH:SA nanoparticles to study the release kinetics of the protein from different polyanhydride chemistries. These chemistries were chosen based on the previous success of 50:50 CPTEG:CPH-based nanovaccine formulations in inducing protective immunity\(^{32}\) and the ability of 20:80 CPH:SA to stabilize proteins for extended periods at elevated temperatures, obviating the need for cold storage\(^{29}\). Additionally, 20:80 CPH:SA nanoparticles are readily internalized by antigen presenting cells, which could lead to enhanced internalization of PspA by these immune cells\(^{41}\). The particles were fabricated using a solid/oil/oil double emulsion method with encapsulation efficiencies of 62 and 68% for 50:50 CPTEG:CPH and 20:80 CPH:SA, respectively. PspA-loaded nanoparticles were characterized using SEM and the photomicrographs are shown in Figure 1. The size and shape of the nanoparticles were consistent with previous work using polyanhydride nanoparticles, with smooth, roughly spherical surface morphology and an average diameter of 677 ± 254 nm for the 20:80 CPH:SA nanoparticles and 243 ± 84 nm for the 50:50 CPTEG:CPH particles\(^{41, 42}\). Particles were stored at -20°C until use and thoroughly sonicated before administration. Figure 2 shows the release of
PspA from polyanhydride nanoparticles over 30 days. Protein release from the 20:80 CPH:SA and 50:50 CPTEG:CPH chemistries showed an initial burst of 36% and 45%, respectively, followed by a sustained release, with smaller amounts of protein released from the particles over a period of time. The near-zero order release profile (after the initial burst) can be attributed to the surface erosion characteristics of polyanhydrides, in contrast to the bulk erosion exhibited by PLGA and other biodegradable polymers. The burst release observed is due to the relatively higher amount of surface area of the particle at initial time points and because of the propensity of the payload to migrate towards the surface of the particle during solvent evaporation. This burst release provides sufficient antigen initially to prime the immune response. These data are consistent with previous work on protein release kinetics from polyanhydride particles.

6.4.2 Preservation of PspA primary and secondary structure upon release from polyanhydride nanoparticles

Following the release of PspA from the nanoparticles, the supernatants were collected and the primary and secondary structures were analyzed using SDS-PAGE and circular dichroism (CD), respectively. Figure 3 shows that the primary structure of PspA remained intact after encapsulation and release based on the presence of a single band just under 50 kDa and the absence of smaller bands in all the lanes of the gel. Additionally, the lack of any larger bands indicated that the polyanhydride nanoparticle formulations did not cause any PspA aggregation.

It is known that the N-terminal portion of the recombinant PspA used in this work adopts a coiled coil, α-helical secondary structure. Figure 4 shows CD spectra for PspA released from the different polyanhydride nanoparticle formulations. The data showed that the α-helical secondary structure of the protein was maintained after release from the nanoparticles, as
evidence by the presence of characteristic minima at 208 nm and 222 nm, which are known to be associated with an α-helical structure\(^{49}\). Additionally, the observed secondary structure is consistent with previously published work on PspA\(^{48}\). Additionally, we present a spectrum of PspA that has been degraded with acid (Figure 4). It is clear from this experiment that acidity compromises the secondary structure of PspA, thereby denaturing it.

It is significant that both polyanhydride chemistries tested preserved the structure of PspA upon release. The option to choose from multiple chemistries provides the ability to tailor both the protein release profile and the host immune response. Differences in adjuvant chemistry, especially with respect to hydrophobicity and degradation kinetics, have been shown to significantly influence the character of the resulting immune response. For example, the 50:50 CPTEG:CPH and 20:80 CPH:SA nanoparticle formulations differentially affect cytokine secretion\(^{27}\). These two polymer chemistries also have different in vivo distribution and inflammation profiles, which can be exploited to further modulate the desired immune response\(^{50}\). Moreover, 20:80 CPH:SA nanoparticles have excellent thermal properties and can preserve protein antigenicity for up to one year at room temperature\(^{29}\). This feature could be exploited to eliminate the requirement for maintaining a cold chain during vaccine storage and delivery\(^{51}\).

6.4.3 Antigenicity of released PspA is retained upon release from polyanhydride nanoparticles

The results depicted in Figure 5 show that the antigenicity of PspA is maintained after release from the polyanhydride nanoparticles. The antigenicity was determined using an ELISA with a PspA-specific monoclonal antibody; this assay measured the ability of PspA released from polyanhydride nanoparticles be recognized by an anti-PspA-specific antibody. The relative antigenicity of the PspA released from nanoparticles was preserved for both polyanhydride
chemistries tested. These results indicated that PspA may not be as susceptible to hydrophobicity-induced conformational changes that have been observed with other proteins. However, it was observed that incubating PspA in acidic solutions, pH 5 and below, led to protein degradation (data not shown). Many biodegradable polymers, including polyanhydrides and polyesters such as PLGA, have acidic degradation products and, as demonstrated for PspA, incubation in acidic solution can lead to protein degradation. The amphiphilic 50:50 CPTEG:CHP has been shown to have local microenvironments of approximately pH 6, as compared to more acidic chemistries such as 20:80 CPH:SA and PLGA, which have been shown have local microenvironments of pH < 4. Supplemental Figure 1 shows the change in the pH of the release buffer in which the polyanhydride nanoparticles are eroding over time. These data show that degradation of 50:50 CPTEG:CPH nanoparticles results in a small decrease in pH, with a stable pH of 6.9 maintained for the duration of the study. In contrast, the degradation of the 20:80 CPH:SA nanoparticles resulted in a more pronounced pH decline to pH~5.2. Given this information, it is expected that PspA released from 50:50 CPTEG:CHP nanoparticles would maintain antigenicity, while PspA released from 20:80 CPH:SA nanoparticles would degrade from acidic effects. However, in the current experiments, PspA released from both 50:50 CPTEG:CHP and 20:80 CPH:SA nanoparticles maintained 90% relative antigenicity. Unlike bulk-eroding polymers such as PLGA, polyanhydrides degrade through surface erosion, which may explain why the PspA was well preserved. This erosion characteristic protects the encapsulated protein from the acidic microenvironment by preventing water penetration into the bulk. It is also important to note that during these in vitro release studies, released protein remained under these acidic conditions for 24 h before the supernatant was removed for testing and replaced with fresh PBS. However, when administered in vivo, polyanhydride nanoparticle
vaccine formulations disseminate quickly, which would prevent the local build-up of acidic degradation products. The preservation of 90% relative antigenicity of PspA upon release from both the 50:50 CPTEG:CPH and 20:80 CPH:SA nanoparticles under these harsh in vitro conditions indicates that these formulations may be promising delivery vehicles for in vivo administration.

6.4.4 Polyanhydride nanoparticles preserve the biological functionality of released PspA

In order to test the biological functionality of PspA released from polyanhydride nanoparticles, an E. coli killing assay was developed. This assay takes advantage of the bactericidal nature of ALF, which is found in the human mucosa in two forms, the iron-deficient ALF and the iron-rich hemolactoferrin. The role of PspA in pneumococcal infection is to bind to ALF and prevent its bactericidal and bacteriostatic effects. In the current studies, two E. coli strains were tested, the wild type MG1655 and the LPS-deficient NR688. Due to the absence of LPS, the NR688 strain proved to be more susceptible to killing by ALF.

Figure 6 shows that PspA released from both polyanhydride nanoparticle formulations retained its biological functionality using native PspA as a control. The log CFU of E. coli killed by ALF in the nanoparticle groups was statistically different from that of the untreated ALF group but not statistically different from the native PspA control. This indicates that the PspA protein released from polyanhydride nanoparticles is able to perform its biological function as well as the PspA control.

6.4.5 Vaccination with a single dose of PspA-containing polyanhydride nanoparticles provides a sustained immune response

CBA/N mice were vaccinated subcutaneously with polyanhydride nanoparticle-based and MPLA-based single dose vaccination regimens. Each animal was given a total of 25
μg of PspA. For the MPLA group, 10 μg of MPLA was used as an adjuvant. For the nanoparticle groups, 20 μg of PspA was delivered as soluble protein along with 5 μg of PspA encapsulated into 500 μg of polyanhydride nanoparticles of the specified chemistry. Based on the results from the in vitro studies described above, both nanoparticle formulations were tested. All vaccinated mice, regardless of chemistry (except for the saline formulation), developed anti-PspA antibody titers between 8,000 and 20,000 by seven weeks post-immunization (Figure 7a). These titers are consistent with those reported to be protective against lethal challenge with *S. pneumoniae*\(^{15}\). The mice receiving the 50:50 CPTEG:CPH nanoparticle vaccine formulation maintained their titer for at least seven weeks at titers above 10,000. The mice administered the 20:80 CPH:SA nanoparticle formulation and the MPLA formulation tended to have a decline in titer between weeks four and seven, although not statistically significant. In other work, the controlled release provided by nanoparticle-based vaccine formulations, specifically the 50:50 CPTEG:CPH chemistry, has sustained titer 23 weeks post-immunization and provided long-lasting immunity in a single dose\(^{32}\). The prolonged presence of protein as a consequence of the persistence of the nanoparticles may explain the effectiveness of the soluble plus encapsulated protein combination in the nanoparticle-based vaccine formulations.

The sustained release of protein is important as the continuous release of small quantities of antigen may be significant for the induction of a long-lasting immunity characterized by a high titer, high avidity antibody response. This effect may also have important implications in the development of a single dose PspA vaccine, limiting the need for repeat administrations. Many vaccine regimens typically require more than one immunization, including the current *S. pneumoniae* \(^7\) and 23-valent conjugate vaccines, which require three primary doses in infants and a booster dose at age two\(^{52}\). The single dose administration of particles based on
biodegradable polymers such as polyanhydrides has demonstrated the ability to provide antibody titers consistent with those induced by multiple doses of traditional adjuvants. Continuous release of antigen would mimic a replicating pathogenic infectious agent and generates a robust, long-lasting immune response as the continual exposure of B cells to antigen induces a strong memory response. Additionally, the initial burst of released antigen observed with both the 20:80 CPH:SA and 50:50 CPTEG:CPH nanoparticle formulations may provide sufficient antigen to successfully prime the immune response and generate T cell help. This step is critical because T cell interactions with B cells lead to development of germinal centers and generation of high affinity antibodies.

To assess the quality of the antibody response, avidity was measured. High avidity is indicative of high strength of binding antibody, which is important for a sustained and effective humoral immune response. The data in Figure 7b shows the relative avidity of the serum anti-PspA IgG antibody response at seven weeks post-immunization. Both of the polyanhydride chemistries tested demonstrated similar relative avidities, with the 50:50 CPTEG:CPH nanoparticle formulation resulting in a slightly higher relative avidity, though not significantly so, over the 20:80 CPH:SA. Additionally, the variability of the avidity of the anti-PspA antibody between mice was smaller with the nanoparticle formulations compared to that of the MPLA-immunized animals. The induction of highly avid antibodies by polyanhydride nanoparticle formulations is consistent with the sustained release demonstrated by these particles in vitro. The avidity indices observed in this work were similar to those generated against capsular polysaccharide 6B through a triple-immunization of an 11-valent pneumococcal conjugate vaccine adjuvanted with alum. In that study, it was demonstrated that the addition of alum to the vaccine did not provide a benefit with respect to avidity. Those observations, paired with our
present findings, highlight an important contrast between the use of alum and polyanhydride nanoparticle-based vaccine platforms in terms of the ability to increase antibody avidity\textsuperscript{57}.

This current work demonstrates the importance of rationally designing adjuvants and/or delivery vehicles for vaccine optimization. The encapsulation of PspA into polyanhydride nanoparticles provided a number of benefits over using off-the-shelf adjuvants such as MPLA, including sustained release, long-lasting immune responses, and higher avidity antibodies, as demonstrated previously with other protein antigens\textsuperscript{32, 53}. Because PspA was found to be structurally stable, antigenic, and biologically functional upon release from two separate polyanhydride nanoparticle chemistries, there exists the ability to rationally select nanoparticle formulations to protect vaccine antigens while tailoring the immune response to a phenotype that is most effective against \textit{S. pneumoniae}. The current studies lay the groundwork for the design of more robust and versatile vaccines against pneumonia that have the potential to provide significant benefits over current vaccination regimens.

6.5 Conclusions

The primary and secondary structure, antigenicity, and biological functionality of PspA protein were preserved during encapsulation and release from polyanhydride nanoparticles. This result is significant because the release of intact, functional protein increases the probability of preserving important, neutralizing epitopes and facilitates the development of an effective immune response. Herein, we demonstrate not only the release of functionally intact protein, but also demonstrate how a PspA vaccine based on polyanhydride nanoparticles can be used to generate a robust humoral immune response. PspA-containing polyanhydride nanoparticle vaccine formulations induced stable, high titers and avid antigen-specific antibody. These studies provide a critical foundation for the future design of robust and versatile vaccines against
streptococcal pneumonia that address both colonization and infection in a more patient-friendly manner.

6.6 Acknowledgments

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Figure 6.6. PspA released from nanoparticles retained biological functionality as indicated by the ability to reduce the bactericidal activity of ALF. Separate cultures of *E. coli* K-12 NR688 were incubated with bacteria culture medium containing ALF alone, ALF plus control PspA, or ALF plus PspA released from either 50:50 CPTEG:CPH or 20:80 CPH:SA as described in Materials and Methods. Data are presented as the mean ± SEM of two independent experiments with at
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Figure 6.7. CBA/N mice immunized with polyanhydride nanoparticle formulations produced high titer, high avidity anti-PspA antibodies. a. Anti-PspA IgG(H+L) antibody titer measured by ELISA at two, four, and seven weeks after vaccination. b. Antibody avidity indices measured seven weeks after vaccination. Error bars represent the mean ± the SEM.
Supplemental Figure 6.1. pH of supernatant from release samples evaluated as a function of time. In this experiment, 25 µg of 20:80 CPH:SA and 50:50 CPTEG:CPH nanoparticles were incubated in 600 µL of 0.05 M phosphate buffered saline (PBS, pH 7.2). At each time point samples were centrifuged and the supernatant was removed for analysis. Solution pH was measured using an Orion Ross pH electrode (Thermo Fisher Scientific). It was observed that degradation of 50:50 CPTEG:CPH nanoparticles resulted in a small decrease in pH to 6.9 whereas 20:80 CPH:SA nanoparticle degradation resulted in a more pronounced pH decrease to 5.2.
Chapter 7: RAPIDLY PROTECTIVE COMBINATION VACCINE AGAINST STREPTOCOCCUS PNEUMONIAE

Shannon L. Haughney¹, Janice King², David Briles²,³, Thomas W. Dubensky Jr⁴, Michael J. Wannemuehler⁵, and Balaji Narasimhan¹*  

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¹Department of Chemical and Biological Engineering, Iowa State University, Ames, IA, 50011  
²Department of Microbiology and ³Pediatrics, University of Alabama Birmingham, Birmingham, AL 35233  
⁴Aduro BioTech, Inc., Berkeley, CA 94710  
⁵Department of Veterinary and Preventive Medicine, Iowa State University, Ames, IA, 50011  

*To whom all correspondence may be addressed*
7.1 Abstract

Since its first isolation in the 1880s, *Streptococcus pneumoniae* has been found to be endemic globally in all areas that have been tested. Given the number of serotypes of the polysaccharide capsule of *S. pneumoniae*, a universal vaccine based on the capsule polysaccharides has not yet been developed. In the search for a universal vaccine many bacterial virulence factors have been investigated for use as protective antigens in vaccine formulations. The most well-known and well-studied *S. pneumoniae* subunit for potential vaccines is pneumococcal surface protein A (PspA). Polyanhydride nanoparticles have previously been reported to stabilize PspA and provide sustained release of the antigen. Herein we report the design of a novel combination nanovaccine that brings together innate immunity inducing cyclic dinucleotides (CDNs) and polyanhydride nanoparticles to elicit a protective immune response within two weeks of a single-dose vaccination.

7.2 Introduction

Since its first isolation in the 1880s, *Streptococcus pneumoniae* has been found to be endemic globally in all areas that have been tested. Considered to be part of the commensal bacteria flora of the human nasopharynx, with rates of colonization peaking in those of 1-2 years of age and declining thereafter, the bacteria may colonize the upper respiratory tract, specifically the nasopharynx, asymptotically for anywhere from several to upwards of 30 weeks depending on the immunogenicity of the serotype. The use of antibiotics has greatly improved the morbidity and mortality associated with *S. pneumoniae*, but with the increase in prevalence of antibiotic-resistant strains of *S. pneumoniae*, vaccination against this pathogen remains one of the most important strategies in combating pneumococcal disease. However, pneumonia vaccination programs have not been prioritized in the same way as influenza vaccine
programs. The increasing prevalence of antibiotic resistant *S. pneumoniae* strains and the lack of widespread pneumococcal vaccinations has led to renewed public health awareness about this pathogen.

Given the number of serotypes of the polysaccharide capsule of *S. pneumoniae*, a universal vaccine based on the capsule polysaccharides has not yet been developed. Polysaccharide-based vaccines have been utilized including Pneumovax 23 (Merck) and Pnu-Immune 23 (Lederle Laboratories). The 23-valent polysaccharide vaccine against *S. pneumoniae* has been shown to protect against the serotypes included in the vaccine, which are 1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F, and 33F. These vaccines tend to induce antibody-based immunity against the serotypes included in the vaccines, with a limitation being the T cell-independent nature of the immune response generated. Polysaccharide-conjugate vaccines use a carrier protein for the delivery of the purified polysaccharide capsule, eliciting a T cell-dependent immune response. However the limited number of serotypes (7-13) in this vaccine can lead to an increase in the prevalence of strains not included in the vaccine, from 5% to 49% of invasive pneumococcal disease (IPD) cases, known as serotype substitution.

In the search for a universal vaccine many bacterial virulence factors have been investigated for use as protective antigens in vaccine formulations, including pneumococcal surface protein A (PspA), PsaA, pilus components, and pneumolysin. The most well-known and well-studied *S. pneumoniae* subunit for potential vaccines is PspA. This choline-binding surface protein is an important virulence factor in *S. pneumoniae* infections and is therefore present on all medically relevant strains of the bacterium. PspA plays two different roles in invasive infection and nasopharyngeal carriage. In invasive systemic infections with *S. pneumoniae*, PspA
prevents the deposition of complement, an immune protein that assists antibodies and phagocytic cells\textsuperscript{9,10}. PspA also plays a role in carriage by binding the bactericidal glycoprotein, apolactoferrin\textsuperscript{11}. Vaccination with PspA has been shown to be protective against a lethal challenge with \textit{S. pneumoniae} making it a promising candidate for use in an improved vaccine\textsuperscript{12–14}. In addition, PspA can be economically synthesized as a recombinant protein and the protein-based vaccine avoids the loss of antigenicity in at-risk populations observed with the polysaccharide-based vaccine\textsuperscript{15}.

We have previously demonstrated the encapsulation and sustained release of stable, antigenic PspA from two polyanhydride chemistries based on sebacic acid (SA), 1,6-bis-(\textit{p}-carboxyphenoxy)hexane (CPH) and 1,8-bis-(\textit{p}-carboxyphenoxy)-3,6-dioxaoctane (CPTEG)\textsuperscript{16}. Herein we demonstrate the development of a protective subunit vaccine based on PspA using polyanhydride nanoparticles as a vaccine adjuvant/delivery vehicle. With the need to induce protective immunity at early time points, we combined the polyanhydride nanovaccines with cyclic dinucleotides (CDNs), a bacterial second messenger based on DNA nucleotides, that have been shown to stimulate the innate immune system through interactions with the \textit{ST}imulator of \textit{IN}terferon Gene (STING) pathway\textsuperscript{17,18}. Additionally we demonstrate the potential to formulate an effective PspA nanovaccine in which all the antigen is encapsulated with the nanoparticles. This eliminates the need for a soluble bolus of protein as previously included in polyanhydride nanovaccine formulations\textsuperscript{16,19}. When all of the antigen is encapsulated within the polyanhydride nanoparticles without a soluble component, there are potential implications for increased shelf-life and stability of the vaccine outside of the cold chain\textsuperscript{20}.
7.3 Materials and Methods

7.3.1 Materials

The following chemicals were used in the synthesis of polyanhydride copolymers and nanoparticles. 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). 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). 4-p-fluorobenzonitrile was obtained from Apollo Scientific (Cheshire, UK). Deuterated chloroform and dimethyl sulfoxide for \(^1\)H NMR analysis were purchased from Cambridge Isotope Laboratories (Andover, MA).

7.3.2 Polymer synthesis

Copolymer synthesis based on the monomers, 1,6-bis(p-carboxyphenoxy)hexane (CPH) and 1,8-bis(p-carboxyphenoxy)-3,6-dioxaoctane (CPTEG) was performed as described previously\(^{21,22}\). A 50:50 molar composition of CPTEG and CPH was synthesized using melt condensation polymerization\(^{22}\). The copolymer molecular weight was confirmed using \(^1\)H NMR analysis. The molecular weight of the 50:50 CPTEG:CPH copolymer used in these studies ranged from 4,790 g/mol – 6,160 g/mol.

7.3.3 Nanoparticle synthesis

50:50 CPTEG:CPH nanoparticles were synthesized using solid/oil/oil nanoprecipitation, as previously described\(^{21}\). PspA protein was dialyzed against water to remove salts before undergoing lyophilization overnight at -40°C. The resultant, dry PspA protein was encapsulated
into 50:50 CPTEG:CPH nanoparticles. The protein-loaded particles were characterized for size and morphology by scanning electron microscopy (SEM, FEI Quanta SEM, Hillsboro, OR).

7.3.4 Cyclic dinucleotides

Cyclic dinucleotides were synthesized by Aduro BioTech. Canonical rr-diGMP CDNs were synthesized from commercially available DMT-rA(Bz)-βCE-TBDMS-phosphoramidite\textsuperscript{12} using a phosphoramidite dimerization method followed by a H-phosphonate cyclization method\textsuperscript{13}.

7.3.5 Streptococcus pneumoniae

Infectious doses of \textit{S. pneumoniae} were prepared. 50 mL of Todd Hewitt + Yeast broth was inoculated with A66.1 \textit{S. pneumoniae}. Cultures were grown to log growth phase, which was indicated by an optical density of ~0.45. Cultures were then cooled on ice followed by the addition of 5 mL of 80\% glycerol. Frozen aliquots of the suspension were made and kept at -80°C until use.

7.3.6 Recombinant PspA protein

The N-terminal region of a recombinant PspA (UAB055, PspA/Rx1 AA1 to 303, clade 2 PspA of the PspA family 1) was produced by Dr. David McPherson (University of Alabama at Birmingham). Protein was dialyzed using a 10,000 MW cut-off dialysis cassette (Thermo Fisher Scientific, Rockford, IL) against sterile, nanopure water and frozen at -80°C. Protein was then lyophilized at -40°C under vacuum overnight and stored at -80°C until further use. Endotoxin was removed from the protein using endotoxin removal beads (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer’s instructions prior to dialysis and lyophilization. The final endotoxin content of the protein prior to use was determined to be less
than 3.7 EU/mg as determined by a limulus amebocyte lysate (LAL) chromogenic endotoxin quantification kit (Thermo Fisher Scientific).

7.3.7 Mice

Four to five week old male CBA/CaHN-Btk^{xid/J} (CBA/N) mice were purchased from Jackson Laboratory (Bar Harbor, ME). All studies were conducted upon receiving approval from the Iowa State University Institutional Animal Care and Use Committee.

7.3.8 Vaccination and challenge

CBA/N mice were used for the vaccination studies. Animals were vaccinated subcutaneously at the nape of the neck. All formulations were prepared in 100 µL sterile phosphate buffered saline (PBS). The vaccine formulations used are shown in Table 1. All animals received a total of 5-25 µg of PspA. Sterile PBS was used for the control group. Five to eight animals were used per group. Animals were challenged at 14 or 24 days after challenge with 2500 CFU of A66.1 \textit{S. pneumoniae} in a total volume of 100 µL through injection with a 26 gauge needle into the mouse tail vein.

7.3.8 Anti-PspA enzyme-linked immunosorbent assay

In order to determine anti-PspA antibody titers, enzyme-linked immunosorbent assays were performed, as previously described\textsuperscript{16}. Briefly, high-binding surface chemistry 96-well plates were coated with 100 µL of 0.5 µg/mL PspA suspended in PBS. Plates were incubated overnight at 4°C. The protein solution was removed and 300 µL of blocking buffer (2.5% skim milk dissolved on PBS with 0.05% Tween (PBS-T)). Plates were incubated with blocking buffer for 2 hours at room temperature. After incubation, plates were washed three times with PBS-T and serum was added to PBS-T containing 1% goat serum beginning at a 1:200 dilution, and serially diluted 3-fold 12 times. After the addition of serum, plates were incubated overnight at
4°C. Samples were washed three times with PBS-T, and alkaline phosphatase-conjugated goat anti-mouse IgG (heavy and light chain) (Jackson ImmunoResearch) was added at a concentration of 1 µg/mL followed by incubation for 2 hours at room temperature. Another three washes were performed with PBS-T, followed by the addition of 1 mg/mL alkaline phosphatase substrate (Fisher Scientific, Pittsburgh, PA) in 50 mM sodium carbonate 2 mM magnesium chloride buffer at pH 9.3. Plates were developed for 30 minutes before reading the optical density at 405 nm.

7.4 Results and Discussion

7.4.1 Soluble plus encapsulated nanovaccine formulation provides protection against lethal challenge with *S. pneumoniae*

We have previously demonstrated the encapsulation and sustained release of stable, antigenic pneumococcal surface protein A (PspA), a surface protein of *S. pneumoniae*, from two polyanhydride chemistries based on sebacic acid (SA), 1,6-bis-(p-carboxyphenoxy)hexane (CPH) and 1,8-bis-(p-carboxyphenoxy)-3,6-dioxaoctane (CPTEG). PspA is a choline-binding protein found on the surface of all pneumococcal strains and has been shown to be able to elicit protective immunity against *S. pneumoniae*.

In the current studies, mice were vaccinated with 25 µg PspA delivered alone or with 20 µg PspA and 5 µg PspA encapsulated into either 50:50 CPTEG:CPH nanoparticles or 20:80 CPH:SA nanoparticles (Figure 7.1). The immunization regimens are summarized in Table 7.1. Serum antibody titers were measured at 7 and 17 days after immunization and animals were challenged with 2500 CFU of *S. pneumoniae* 24 days post-vaccination (Figure 7.2). The data showed that adding CDNs to the nanovaccine formulations boosted the antibody response. Animals immunized with the 50:50 CPTEG:CPH nanovaccine formulation and two types of CDN formulations demonstrated 100% protection against challenge, while those receiving the
20:80 CPH:SA nanovaccine formulation alone (i.e., no CDNs) demonstrated 75% protection against challenge, and mice that received 25 µg PspA alone elicited only 25% protection against challenge (Figure 7.3).

7.4.2 100% antigen-encapsulated nanovaccine formulations provide protection against lethal challenge with *S. pneumoniae*

Next, animals mice were vaccinated with 5 µg PspA encapsulated at 10% (w/w) into 50:50 CPTEG:CPH nanoparticles (with no soluble antigen). The immunization regimens are summarized in Table 7.2. Animals were challenged with 2500 CFU of *S. pneumoniae* 14 days post-vaccination with the survival results summarized in Table 7.3. All the animals immunized with adjuvanted vaccine formulations demonstrated 100% survival against challenge, while the animals vaccinated with PspA alone had a survival rate of 87.5%. These results show a marked increase in the response to vaccination with the soluble PspA at a 5X reduced dose when compared to the previous study. These leads to the opportunity to further reduce the amount of protein needed, which has important implications for dose sparing with the combination nanovaccine formulations.

7.4.3 Dose sparing effects on 100% antigen-encapsulated nanovaccine formulation for enhanced protection against *S. pneumoniae* with reduced antigen requirements

With successful proof of concept studies, summarized above, indicating that mice vaccinated with 5 µg PspA encapsulated into 50:50 CPTEG:CPH nanoparticles (with no soluble antigen) were 100% protected against lethal challenge with *S. pneumoniae*, we next investigated whether the dose of PspA could be further reduced while maintaining high levels of vaccine efficacy. The total amount of PspA used in experimental vaccine formulations was reduced to a total dose of 1 µg. A formulation in which 100% of the antigen was encapsulated (i.e., no soluble
antigen) was investigated with 1 µg of PspA encapsulated at 5% loading into 20 µg of 50:50 CPTEG:CPH nanoparticles, with 30 µg of blank (i.e., no antigen) 50:50 CPTEG:CPH nanoparticles added to be consistent across groups, all of which contained 50 µg of nanoparticles. Additionally, a soluble + encapsulated formulation was included with 0.5 µg PspA encapsulated at 1% loading into 50 µg of 50:50 CPTEG:CPH nanoparticles, with 0.5 µg of PspA delivered as soluble protein. Finally, 1 µg PspA was delivered alone and 1 µg PspA with Alum was used as a positive control group. The experimental groups are summarized in Table 7.4.

Upon experimental challenge with 2500 CFU of *S. pneumoniae* it was found that the polyanhydride nanoparticles were able to provide greater than 80% protection in all of the nano-formulations studied with the reduced dose of PspA as shown in Figure 7.4. In contrast, the protection induced by the soluble 1 µg protein alone drops significantly, with only one animal out of eight surviving challenge. This clearly shows the adjuvant effect of the polyanhydride nanovaccine formulations. Antibody titer data, as shown in Figure 7.5, reflects these results. All of the nanovaccine formulations exhibit enhanced antibody titer over PspA alone and PspA adjuvanted with alum. Future studies will focus on the effect of shelf-life to demonstrate the benefit of the all-encapsulated nanovaccine formulation in comparison to the soluble + encapsulated formulation, which appear to be demonstrating an adjuvant effect on the protein.

7.5 Conclusions

Herein we demonstrate the development of a protective subunit vaccine based on PspA using a combination nanovaccine formulation based on polyanhydride nanoparticles and CDNs as vaccine adjuvants. The use of the CDNs obviated the need for soluble protein, resulting in a formulation that was protective upon lethal challenge only 14 days post vaccination. The use of
this formulation may have implications for increased shelf-life and stability outside of the cold-chain. We have also demonstrated the dose-sparing capabilities of the polyanhydride nanovaccine formulations, eliciting enhanced protection with just 1 µg of PspA. Additionally, in this work, we demonstrated protection both at early and late time points post-vaccination, providing foundational data for the development of a robust pneumonia nanovaccine.

7.6 References


7.7 List of Figures

Figure 7.1 Scanning electron microscopy images of 1% PspA-loaded 20:80 CPH:SA (left) and 50:50 CPTEG:CPH (right) nanoparticles. Scale bars: 4 µm (left) and 1 µm (right).

Figure 7.2 Anti-PspA antibody titers after subcutaneous vaccination of CBA/N mice with 25 µg PspA-based formulations (PspA alone, PspA adjuvanted with Alum, and PspA encapsulated within two different types of polyanhydride nanoparticles) measured at 7 and 17 days after immunization.
Figure 7.3. Survival data for CBA/N mice immunized with the PspA-based formulations in Table 7.2. The animals were challenged with 2500 CFU of A66.1 S. pneumoniae 24 days post immunization.

Figure 7.4. Survival data for CBA/N mice immunized with the PspA-based formulations in Table 7.4. The animals were challenged with 2500 CFU of A66.1 S. pneumoniae 30 days post immunization.
Figure 7.5. Anti-PspA antibody titers after subcutaneous vaccination of CBA/N mice with 1 µg PspA-based formulations (PspA alone, PspA adjuvanted with Alum, and PspA with three different polyanhydride nanoparticle formulations).

7.8 List of Tables

Table 7.1. Experimental groups of CBA/N mice immunized with 25 µg PspA in polyanhydride nanovaccine formulations or with Alum.

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Soluble PspA (µg)</th>
<th>Encapsulated PspA (µg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>50:50 CPTEG:CPH</td>
<td>20</td>
<td>5</td>
</tr>
<tr>
<td>20:80 CPH:SA</td>
<td>20</td>
<td>5</td>
</tr>
<tr>
<td>PspA + Alum</td>
<td>25</td>
<td>0</td>
</tr>
<tr>
<td>PspA Alone</td>
<td>25</td>
<td>0</td>
</tr>
</tbody>
</table>
Table 7.2. Experimental groups of CBA/N mice immunized with 5 µg PspA in polyanhydride nanoparticles with and without CDNs or with Alum.

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Soluble PspA (µg)</th>
<th>Encapsulated PspA (µg)</th>
<th>CDN (µg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>50:50 CPTEG:CPH</td>
<td>0</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>50:50 CPTEG:CPH + CDN</td>
<td>0</td>
<td>5</td>
<td>25</td>
</tr>
<tr>
<td>PspA + Alum</td>
<td>5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>PspA Alone</td>
<td>5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>PspA + CDN</td>
<td>5</td>
<td>0</td>
<td>25</td>
</tr>
</tbody>
</table>

Table 7.3. Survival data for CBA/N mice immunized with the formulations showed in Table 7.2. The animals were challenged with 2500 CFU of A66.1 S. pneumoniae 14 days post immunization.

<table>
<thead>
<tr>
<th>Group</th>
<th>Survival @ D14</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 µg PspA</td>
<td>7/8 (87.5%)</td>
</tr>
<tr>
<td>5 µg PspA + Alum</td>
<td>8/8 (100%)</td>
</tr>
<tr>
<td>5 µg PspA + 25 µg CDN</td>
<td>8/8 (100%)</td>
</tr>
<tr>
<td>5 µg PspA in 50 µg 50:50 CPTEG:CPH</td>
<td>8/8 (100%)</td>
</tr>
<tr>
<td>5 µg PspA in 50 µg 50:50 CPTEG:CPH + 25 µg CDN</td>
<td>8/8 (100%)</td>
</tr>
<tr>
<td>Saline</td>
<td>1/8 (12.5%)</td>
</tr>
</tbody>
</table>
Table 7.4. Experimental groups of CBA/N mice immunized with 1 µg PspA in polyanhydride nanoparticles or with Alum.

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Soluble PspA (µg)</th>
<th>Encapsulated PspA (µg)</th>
<th>Blank 50:50 CPTEG:CPH Nanoparticles (µg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Encapsulated PspA</td>
<td>0</td>
<td>1</td>
<td>30</td>
</tr>
<tr>
<td>Soluble + Encapsulated</td>
<td>0.5</td>
<td>0.5</td>
<td>0</td>
</tr>
<tr>
<td>Soluble PspA</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>PspA + Alum</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>
CHAPTER 8: RAPIDLY PROTECTIVE COMBINATION NANOVACCINE FORMULATIONS AGAINST YERSINIA PESTIS

A paper to be submitted to the International Journal of Nanomedicine

Shannon L. Haughney¹, Paul Lueth², Danielle Wagner², Thomas W. Dubensky Jr³, Bryan Bellaire², Michael J. Wannemuehler², Balaji Narasimhan¹*

¹Department of Chemical and Biological Engineering, Iowa State University, Ames, IA, 50011

²Department of Veterinary and Preventative Medicine, Iowa State University, Ames, IA, 50011

³Aduro BioTech, Inc., Berkeley, CA 94710

*To whom all correspondence may be addressed
8.1 Abstract

Infectious diseases pose a significant threat to the world population. In the case of re-emerging pathogens, and those that have been classified as potential bioterrorism agents, the need exists for a vaccine platform which can confer protective immunity in a short amount of time and also maintain a highly protective immune response for longer periods. Polyanhydride nanoparticles have previously been reported to generate a long-lasting protective immune response against pneumonic plague. Herein we report that combining novel innate immunity inducing cyclic dinucleotides (CDNs) with the polyanhydride nanovaccine platform elicited a protective immune response within days of a single-dose vaccination. We also confirmed the longevity of the immune response by demonstrating protection with this combination nanovaccine formulation up to six weeks after immunization.

8.2 Introduction

The use of biological agents as bioterrorism weapons remains a considerable threat due to their relative ease of manufacture and the high mortality rates induced by many select pathogens\(^1\). One pathogen that is considered to have the potential to be used as a biological weapon is *Yersinia pestis*, the causative agent of plague-related diseases, due to its ability to spread through aerosolized droplets and the ability to easily introduce antibiotic resistance\(^2\). Despite this, and the fact that plague is still endemic in many regions globally, no commercial vaccine against *Y. pestis* is available\(^2,3\). There are many experimental vaccines in development including live attenuated vaccines and a number of vaccines based on *Y. pestis* subunits, especially F1, LcrV, and F1-LcrV (F1-V) fusion proteins\(^2\).

A F1-V subunit vaccine based on polyanhydride nanoparticles has recently been demonstrated to provide protective immunity against lethal challenge with pneumonic plague.
The polyanhydride nanovaccine formulation provided 100% protection against challenge with CO92 *Y. pestis*, with both soluble protein alone and protein adjuvanted with MPLA failing to elicit a protective immune response. These results suggest the potential for an effective, easily synthesized vaccine against *Y. pestis*.

When designing a vaccine against a potential bioterrorism agent, post-exposure prophylaxis (PEP) is an important consideration, which has received much attention for potential bioterrorism agents such as *Bacillus anthracis*, the causative agent of anthrax, and *Filoviridae* viruses such as Ebola.

While polyanhydride nanovaccines have demonstrated protective immunity against lethal challenge with *Y. pestis*, the earliest time point after administration that has been tested is six weeks. In order to elicit protective immunity at early time points after administration, a vaccine formulation must effectively engage innate immune mechanisms as well as develop a potent adaptive immune response. Cyclic dinucleotides (CDNs), a bacterial second messenger based on DNA nucleotides have been shown to stimulate the innate immune system through interactions with STING. Herein, it is hypothesized that using these molecules in combination with polyanhydride nanoparticles will rapidly elicit an immune response after immunization.

While the short-term induction of protective immunity is important in the event of a bioterrorism attack, long-term immunity may be just as crucial for those who may be vaccinated against select agents as a preventative measure. In this work, we describe the design and performance of a protective combination nanovaccine formulation based on polyanhydride nanoparticles and novel CDNs that can fully protect upon challenge 10 days after administration,
without compromising the generation of long-term immunity, as evidenced by protective immune responses when challenged up to six weeks after immunization.

8.3 Materials and Methods

8.3.1 Materials

The following chemicals were used in the synthesis of polyanhydride polymers and the fabrication of nanoparticles. 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). 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). 4-p-fluorobenzonitrile was obtained from Apollo Scientific (Cheshire, UK). Deuterated chloroform and dimethyl sulfoxide for $^1$H NMR analysis were purchased Cambridge Isotope Laboratories (Andover, MA). F1-V fusion protein was obtained from BEI Resources (Manassas, VA).

8.3.2 Polymer synthesis

Synthesis of 1,6-bis(p-carboxyphenoxy)hexane (CPH) and 1,8-bis(p-carboxyphenoxy)-3,6-dioxaoctane (CPTEG) was performed as described$^9,10$. A 50:50 molar copolymer of CPTEG and CPH was synthesized through melt condensation polymerization, as described elsewhere$^{10,11}$. Molecular weight was confirmed using $^1$H NMR.

8.3.3 Nanoparticle synthesis

50:50 CPTEG:CPH nanoparticles were synthesized using solid/oil/oil nanoprecipitation, as previously described$^4,9$. F1-V protein was dialyzed against water to remove salts before
undergoing lyophilization overnight at -40°C. The resultant dry F1-V protein (2.5 µg) was encapsulated into 50:50 CPTEG:CPH nanoparticles (500 µg). The protein-loaded particles were characterized for size and morphology by scanning electron microscopy (SEM, FEI Quanta SEM, Hillsboro, OR).

8.3.4 Cyclic dinucleotides

Cyclic dinucleotides were synthesized by Aduro BioTech. Canonical rr-diGMP CDNs were synthesized from commercially available DMT-rA(Bz)-βCE-TBDMS-Phosphoramidite using a phosphoramidite dimerization method followed by a H-phosphonate cyclization method.

8.3.5 Yersinia pestis

Infectious doses of *Y. pestis* CO92 were prepared by scraping bacteria cultured overnight on Brain Heart Infusion (BHI) agar at 37°C. A bacterial suspension was made in PBS (pH 7.4) and cell density was adjusted to ~ 1 x 10^9 CFU/mL according to OD_{600} spectrophotometric readings. Frozen aliquots of the suspension were prepared and kept at -80°C.

8.3.6 Mice

Five to eight-week old female C57BL/6 were purchased from Harlan Laboratories (Haslett, MI). All studies were conducted in accordance with the Iowa State University Institutional Animal Care and Use Committee.

8.3.7 Vaccination and challenge

Animals were vaccinated subcutaneously at the nape of the neck. All formulations were prepared in 100 µL sterile phosphate buffered saline (PBS). Sterile PBS was used as the control
group. Five to eight animals were used per group with data representing between one and two independent experiments, as noted.

Enumeration of viable CFU was performed by thawing aliquots and plating serial dilutions of *Y. pestis* on BHI medium and incubating at 37°C. Viability of bacteria within the intranasal inoculum was confirmed in this manner to contain ~880 CFU within 20 µL PBS placed on the nares of anesthetized mice. Health of the infected mice was monitored several times daily for signs and symptoms of distress. Moribund mice were euthanized by CO₂ overdose. Animals found deceased and euthanized mice were recorded as having died on that day. Animals were challenged at either 14 or 42 days post-immunization. Mice were monitored for 14 days for clinical signs of infection and surviving mice were euthanized by CO₂ overdose.

8.3.8 Anti-F1-V enzyme-linked immunosorbent assay

In order to determine anti-F1-V antibody titers, enzyme-linked immunosorbent assays were performed, as previously described⁴,¹⁴,¹⁵. Briefly, high-binding surface chemistry 96-well plates were coated with 100 µL of 0.5 µg/mL F1-V suspended in PBS. Plates were incubated overnight at 4°C. The protein solution was removed and 300 µL of blocking buffer (2.5% skim milk dissolved on PBS with 0.05% Tween (PBS-T)). Plates were incubated with blocking buffer for 2 hours at room temperature. After incubation, plates were washed three times with PBS-T and serum was added to PBS-T containing 1% goat serum beginning at a 1:200 dilution, and serially diluted 3-fold 12 times. After the addition of serum, plates were incubated overnight at 4°C. Samples were washed three times with PBS-T, and alkaline phosphatase-conjugated goat anti-mouse IgG (Jackson ImmunoResearch) was added at a concentration of 1 µg/mL followed by incubation for 2 hours at room temperature. Another three washes were performed with PBS-T, followed by the addition of 1 mg/mL alkaline phosphatase substrate (Fisher Scientific,
Pittsburgh, PA) in 50 mM sodium carbonate 2 mM magnesium chloride buffer at pH 9.3. Plates were developed for 30 minutes before reading the optical density at 405 nm.

8.4 Results

8.4.1 Immunization nanovaccine formulations elicits higher antibody titers and provides enhanced protection over F1-V alone when challenged at 42 days post-administration

Protective immunity of vaccine formulations at extended time points was investigated through challenge with a lethal dose of *Y. pestis* at six weeks post-immunization. Animals were immunized with a total of 5 µg F1-V administered as soluble protein, encapsulated into 100 µg of 50:50 CPTEG:CPH polyanhydride nanoparticles, or 2.5 µg delivered as soluble protein along with 2.5 µg encapsulated into 500 µg of 50:50 CPTEG:CPH nanoparticles. Antibody titers were measured at 14 and 35 days after vaccine administration, as shown in Figure 8.1a. A statistically significant increase in the antibody response was observed at day 14 for the soluble plus encapsulated nanovaccine formulation as compared to the soluble protein alone and the nanovaccine formulation in which 100% of the antigen was encapsulated within the nanoparticles (i.e., no soluble antigen). By day 35 after administration, the statistical difference between the soluble protein and the soluble plus encapsulated vaccine formulation had waned, though the soluble plus encapsulated nanovaccine formulation remained significantly higher than the 100% antigen-encapsulated formulation. This may be explained by the lack of enough protein present initially to stimulate a robust B cell response to the vaccine administration for the 100% antigen-encapsulated formulation. This hypothesis is supported by the induction of weak antibody titers compared to the soluble plus encapsulated nanovaccine formulation and even the un-adjuvanted protein delivered solubly.
These observations are reflected in the survival data when the vaccinated animals were challenged 42 days after immunization, as shown in Figure 8.1b. The soluble plus encapsulated vaccine formulation provided 100% protection against lethal challenge with 880 CFU of CO92 \textit{Y. pestis}. In contrast, both soluble protein and the 100% antigen-encapsulated nanovaccine demonstrated poor protection, with all animals succumbing to challenge, though it may be interesting to note that the 100% antigen-encapsulated formulation resulted in a delayed onset of clinical signs of infection and death, hinting at a possibly weak immune response to challenge.

8.4.2 The addition of CDNs to nanovaccine formulations stimulates the innate immune responses eliciting high titers at early time points and shifts the response from IgG1-dominated to balanced (i.e., IgG1/IgG2) immune response

The addition of CDNs to the each of the vaccine formulations tested above stimulated innate immune responses and elicited high titers at early time points when compared to the formulations without the CDNs, as shown in Figure 8.2a. The addition of the CDN adjuvant to the soluble plus encapsulated vaccine formulation and the addition of CDNs to the soluble F1-V provided a marked increase because CDNs are known to stimulate innate immunity through the STING pathway, the observed results align with the expected response upon the inclusion of the CDNs in the nanovaccine regimens. It is interesting to note that, while higher, the antibody titers elicited by the 100% antigen-encapsulated formulation with CDNs, do not appear to indicate the same innate immune stimulation observed with the other groups. Again, this may be due to the lack of sufficient antigen present initially to stimulate a robust B cell response to immunization. Due to this lack of antigen, the adaptive immune response does not benefit from the recruitment of innate immune cells by the CDNs, as is suggested by the soluble plus encapsulated nanovaccine and the soluble F1-V group. It is also interesting to note that in addition to
improving the antibody response and survival of vaccinated animals, the addition of CDNs shifted response from an IgG1-dominated to a balanced (i.e., IgG1/IgG2) immune response, as shown in Figure 8.3.

Consistent with these observations the survival data when the vaccinated animals were challenged 42 days after immunization indicate the benefit of the addition of the CDNs to the vaccine regimens, as shown in Figure 8.2b. As shown in the previous figures, the soluble plus encapsulated vaccine formulation provided 100% protection against lethal challenge with 880 CFU of CO92 Y. pestis. Similarly the addition of CDNs did not hinder the efficacy of the formulation, with the soluble plus encapsulated vaccine with CDN formulation also providing 100% protection. In contrast, both soluble protein and the 100% antigen-encapsulated nanovaccine demonstrated little response, with all animals succumbing to challenge, without CDNs. The addition of CDNs to the soluble F1-V vaccine improved the response, with 80% of animals surviving challenge. Not surprisingly, based on the antibody titer results, the addition of CDNs did not improve survival of animals vaccinated with the all-encapsulated F1-V, with all of the animals succumbing to challenge.

8.4.3 An increase in the dose of F1-V along with the use of a hydrophobic polyanhydride nanoparticle chemistry increases efficacy of 100% antigen-encapsulated nanovaccine formulations at late time points

Based on our previous work, the chemistry of the nanoparticle adjuvant plays a critical role in the evolution of the immune response\textsuperscript{14}. The more hydrophobic the chemistry, the more danger signals are elicited by the presence of the polymer, leading to a more rapid response to immunization. Based on these observations, a more hydrophobic adjuvant (i.e., 20:80 CPTEG:CPH) was utilized in order to induce a protective response at earlier time points after
vaccination. Additionally, though the soluble plus encapsulated vaccine formulation provided protection against challenge, the use of soluble protein in a vaccine formulation may reduce shelf-life and need cold storage conditions in comparison to a 100% antigen-encapsulated nanovaccine formulation, which may be stored at room temperature for longer periods of time. Encapsulation into polyanhydride nanoparticles has been shown to prolong the shelf-life of fragile proteins and eliminate the need for cold storage\textsuperscript{16}. With this goal in mind, the dose of F1-V was increased to 10 µg in 100 µg of 20:80 CPTEG:CPH nanoparticles, which also increases the loading percentage from 5% to 10%. Increased loading has been shown to lead to a greater initial burst of antigen from the particles, resulting in higher a higher antibody titer response, possibly due to the protein needed initially to stimulate a robust B cell response\textsuperscript{17}.

Similar to the previous experiment, the inclusion of CDNs in the nanovaccine formulations increased the antibody response. In contrast to the data obtained from the 5 µg dose of F1-V delivered in the all-encapsulated formulation, the 10 µg dose elicited measurable antibody titers, which were increased by the addition of CDNs to the vaccine formulation, as shown in Figure 8.4. Additionally, these titers were maintained, and even increased, until at least 63 days post administration. By 63 days post vaccination, the mice that received 100% antigen-encapsulated formulation with CDNs also showed increased antibody titers, similar to the titers in animals that received the soluble plus encapsulated formulation. This observation indicates that, though less antigen may be available initially due to encapsulation, the continuous release of protein from the polyanhydride nanoparticles may lead to a sustained immune response and the accumulation of antibody in vaccinated animals. It has previously been shown that the persistent presence of antigen may lead to enhanced differentiation of B cells into long-lived plasma cells\textsuperscript{18–20}. 
8.4.4 Immunization with CDN + nanoparticle formulations enhances protection as compared to soluble F1-V when challenged at 14 days after immunization

Finally, based on the promising results from the 10 µg dose and the use of 20:80 CPTEG:CPH nanoparticle adjuvants, challenge studies at early time points were investigated. Additionally, an increased dose of F1-V of 20 µg in 200 µg of 20:80 CPTEG:CPH nanoparticles was tested, keeping the loading percentage the same (i.e., 10%). The results from this study are summarized in Table 1. While no vaccine formulation tested offered 100% protection, the 20 µg in 200 µg of 20:80 CPTEG:CPH nanoparticles with and without CDNs offered enhanced protection over 20 µg F1-V delivered alone.

8.5 Discussion

The plague causing pathogen, *Yersinia pestis*, remains a concern from the standpoints of antibiotic resistance and potential use as a biological weapon. Despite this, there is currently no commercially available vaccine in the United States and Western Europe. This work builds on previous work from our laboratories on the use of polyanhydride nanoparticles as a vaccine platform for *Y. pestis*, in which the use of a nanovaccine formulation with soluble antigen plus nanoparticle-encapsulated antigen was shown to be protective. In this work the titers induced by the soluble plus encapsulated nanovaccine formulation were significantly higher than the titers induced by the 100% antigen-encapsulated formulation. This may be due to the lack of sufficient protein present initially to stimulate a robust B cell response to the vaccine administration. This is supported by the reduced antibody titers compared to the soluble plus encapsulated nanovaccine formulation and even the un-adjuvanted protein delivered solubly (Figure 8.1a).

The use of cyclic dinucleotides (CDNs), which are viral and bacterial nucleic acids that regulate gene expression, is combination with the nanovaccine, was also investigated in this
work. These molecules are secreted by intracellular bacteria into the cytosol and are recognized by the innate immune system as PAMPs. This stimulates innate immunity and induces the release Th1-skewing pro-inflammatory cytokines and chemokines. This innate immune stimulating capability of the CDNs was utilized by combining them with the nanovaccine formulation to induce immune responses at earlier time points. In each case, the benefit of the combination nanovaccine is clear, as evidenced by the increased antibody titers, particularly within 14 days after immunization (Figure 8.2a.).

Additionally, the effect of increasing the antigenic dose was investigated in the attempt to design a protective, 100% antigen-encapsulated vaccine formulation, which is likely to exhibit enhanced shelf-life for extended periods of time. The elimination of the soluble portion of the nanovaccine formulation will have implications in the shelf-life, storage, and, possibly, administration of the final vaccine formulation, as a dry powder vaccine may allow for inhaled or intranasal delivery, thereby enhancing patient compliance\textsuperscript{16,21,22}. At later time points, the elimination of the soluble portion of the nanovaccine appears to not hinder the immune response in the presence of the CDNs. This provides promising evidence that a 100% antigen-encapsulated nanovaccine with extended shelf life at elevated temperatures will be protective when challenged at sufficiently long times (i.e., 6 weeks or later) post-vaccination.

Another consideration for a successful \textit{Y. pestis} vaccine however, is the induction of early immunity, with implications for use as a PEP therapy for those exposed to \textit{Y. pestis}, as well as for rapid vaccination in the event of a mass exposure event. In order to test the efficacy of the 100% antigen-encapsulated nanovaccine formulations, animals were challenged 14 days after vaccine administration, particularly with the use of a nanoparticle formulation that in our previous work showed rapid induction of antibody responses. An increased dose of F1-V of 20
µg in 200 µg of 20:80 CPTEG:CPH nanoparticles was tested in these experiments. While no vaccine formulation tested offered 100% protection, the 20 µg in 200 µg of 20:80 CPTEG:CPH nanoparticles, both with and without CDNs, offered enhanced protection over 20 µg of F1-V delivered alone (Table 1). These experiments are promising first steps in the design of a safe and versatile single dose combination nanovaccine with extended storage shelf life at elevated temperature that can provide protective immunity upon both pre- and post-exposure to Y. pestis.

8.6 Conclusions

Herein we demonstrate the development of a single dose combination nanovaccine formulation against Y. pestis with the goal of eliciting protective immunity in the first 14 days after administration and also generating long-lasting immunity at up to 6 weeks after vaccination. These studies have promising implications for use as a PEP therapy for those exposed to Y. pestis, as well as for rapid vaccination in the event of a mass exposure event.

8.7 References


8.8 List of Figures

![Graph A](image1.png)  
![Graph B](image2.png)

Figure 8.1. Anti-F1-V titers induced by 5 µg F1-V encapsulated into 100 µg of 50:50 CPTEG:CPH nanoparticles, 2.5 µg F1-V encapsulated into 500 µg of 50:50 CPTEG:CPH nanoparticles, and 5 µg soluble F1-V 14 and 35 days post a single intranasal immunization. The 50:50 CPTEG:CPH nanovaccine formulation provided 100% protection against lethal challenge with *Y. pestis* CO92 (880 CFU), while both the 100% antigen-encapsulated and soluble F1-V
formulations failed to provide any protection. Error bars represent standard error of the mean. Letters represent statistical significance between treatment groups at a given time point.

Figure 8.2. The addition of CDNs to nanovaccine formulations stimulates innate immune responses by eliciting high titers at early time points. Error bars represent standard error of the mean. Protection data indicates that both the soluble plus encapsulated nanovaccine formulation with and without CDN provided 100% protection against lethal challenge. Additionally, the inclusion of CDNs in the vaccine formulation enhanced the protection by soluble protein to 80% survival. Responses to the all-encapsulated formulations with and without CDN remained low, with no survivors.
Figure 8.3. The addition of CDNs shifts the immune response from an IgG1-dominated to a balanced (i.e., IgG1/IgG2) response. Optical density of serum samples of vaccinated mice was measured seven days post-vaccination and plated at a 1:1000 serum dilution. Error bars represent standard error of the mean.
Figure 8.4. The use of hydrophobic 20:80 CPTEG:CPH nanoparticles with an increased dose (20 µg) and loading percentage (10%) rapidly induced robust antibody titers. The total antibody titer of vaccinated animals was measured at days 14, 35 and 63 post-administration. Error bars represent standard error of the mean.
8.9 List of Tables

Table 8.1. Survival results for animals challenged with 1200 CFU of CO92 *Y. pestis* 14 days after a single intranasal immunization.

<table>
<thead>
<tr>
<th>Group</th>
<th>Survival @ D7</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 µg F1-V</td>
<td>2/8 25%</td>
</tr>
<tr>
<td>20 µg F1-V</td>
<td>1/8 12.5%</td>
</tr>
<tr>
<td>10 µg F1-V + 25 µg CDN</td>
<td>3/8 37.5%</td>
</tr>
<tr>
<td>20 µg F1-V + 25 µg CDN</td>
<td>5/8 62.5%</td>
</tr>
<tr>
<td>10 µg F1-V in 100 µg 20:80 CPTEG:CPH</td>
<td>3/8 37.5%</td>
</tr>
<tr>
<td>20 µg F1-V in 200 µg 20:80 CPTEG:CPH</td>
<td>5/8 62.5%</td>
</tr>
<tr>
<td>10 µg F1-V in 100 µg 20:80 CPTEG:CPH + 25 µg CDN</td>
<td>3/8 37.5%</td>
</tr>
<tr>
<td>20 µg F1-V in 200 µg 20:80 CPTEG:CPH + 25 µg CDN</td>
<td>4/7 57%</td>
</tr>
<tr>
<td>Saline</td>
<td>3/8 37.5%</td>
</tr>
</tbody>
</table>
CHAPTER 9: CONCLUSIONS AND FUTURE WORK

9.1 Conclusions

With increasing threats of biological pathogens such as *Yersinia pestis* being used as weapons in bioterror attacks and widespread overuse of antibiotics leading to multi-drug-resistant strains of bacteria, vaccination remains an important tool in the global fight against infectious diseases. Acute lower respiratory infections such as pneumonia are the leading cause of death among infectious diseases, and many microbes can be responsible for these deadly lower respiratory tract infections. In addition to bacterial pneumonia strains such as *Staphylococcus aureus*, *Psuedomonys aeruginosa*, and *Streptococcus pneumoniae*, viral infections such as influenza and respiratory syncytial virus as well as some fungi also contribute to the disease burden. However, community-acquired pneumonia remains the leading cause of lower respiratory infections, with *S. pneumoniae* being the most common causative agent. With this backdrop, the overall goal of this thesis is to design efficacious vaccine platforms against bacterial pathogens such as *S. pneumoniae* and *Y. pestis*, which offer improvements over commercially available vaccine regimens.

We first investigated the mechanism of protection associated with polyanhydride nanovaccines (Chapters 4 and 5). The F1-V fusion protein has been demonstrated previously to provide protection against *Y. pestis* challenge when delivered in the context of 50:50 CPTEG:CPH polyanhydride nanoparticles\(^1,2\). Herein, we investigated how F1-V encapsulated in 50:50 CPTEG:CPH nanoparticles interacted with immune cells at early time points after administration, and how these interactions compared with soluble protein alone and soluble protein delivered with MPLA. We demonstrated that polyanhydride nanoparticles bridged the
gap between the inert persistence of soluble protein alone and the rapid, inflammation-induced clearance observed with F1-V-MPLA formulations. The particles prolonged the presence of antigen in the lungs and induced a relatively mild inflammatory response compared to MPLA, while still inducing a long-lasting protective immune response. Finally, we assessed the long-term immune response induced by polyanhydride nanovaccines of different chemistries to determine the effect of particle persistence and polymer chemistry on the immune response. We determined that while long-term persistence seemed to have little effect on the immune response to F1-V, polymer chemistry affected the antigen release kinetics and the development and phenotype of the immune response to the nanovaccine. This work has important implications for vaccine design against different pathogens, which may require different phenotypes of immune responses.

Next, we demonstrated the value of polyanhydride nanoparticles in delivering and adjuvanting pneumococcal surface protein A (PspA), a protective antigen against S. pneumoniae (Chapter 6). PspA was encapsulated into polyanhydride nanoparticles of two different chemistries and released for one month. The released protein was found to retain its structure, antigenicity, and biological functionality upon release. Additionally we demonstrated the development of a protective subunit vaccine based on PspA using polyanhydride nanoparticles as a vaccine adjuvant (Chapter 7). Through the incorporation of cyclic dinucleotides in the nanovaccine formulation, we demonstrated the potential to formulate an effective PspA nanovaccine with 100% of the antigen encapsulated within the nanoparticles, which may have implications for increased shelf-life and stability outside of the cold-chain. Additionally, we designed combination nanovaccine formulations that demonstrated protection against lethal challenge at early time points after vaccination.
Finally, we investigated the development of a single dose combination nanovaccine formulation against *Y. pestis* with the goal of eliciting protective immunity in the first 14 days after administration and also generating long-lasting immunity at up to 6 weeks after vaccination (Chapter 8). These studies have promising implications for use as a post-exposure prophylaxis therapy for those exposed to *Y. pestis*, as well as for rapid vaccination in the event of a mass exposure event.

9.2 Future work

In addition to optimizing the nanovaccine formulations for bacterial infections that have been developed in this thesis, the nanoparticle platform can also be exploited to deliver antibiotics in a potentially simultaneous format with the vaccines. The particles offer advantages in terms of dose sparing (thus addressing antibiotic resistance), safety, and enhanced patient compliance. Some ongoing and future work in this area is described below.

9.2.1 Polyanhydride nanoparticle platform for the delivery of antibiotics against *Streptococcus pneumoniae*

In addition to designing polyanhydride nanovaccines for post-exposure prophylaxis against emerging diseases and potential bioterrorism threats, polyanhydride nanoparticles can be used for post-exposure treatment through the delivery of antibiotics against *S. pneumoniae* infection. Polyanhydride nanoparticles have previously been shown to be an effective delivery platform for antibiotics\(^3\). Encapsulation of antibiotics into particles resulted in an increase in the local concentration of drug and prevented rapid clearance of the antibiotic after administration. Additionally, particle persistence and sustained release of antibiotic (or antimicrobial agent) may obviate the need for a multiple-dose course of treatment. Together, these studies lay the groundwork for a polyanhydride nanoparticle-based combination treatment against *S.*
pneumoniae that addresses both pre-exposure (i.e., prophylaxis) as well as post-exposure (i.e., therapeutics).

9.2.2 Polyanhydride nanoparticle platform for the delivery of antibiotics against *Brucella abortus* and *Brucella melitensis*

Brucellosis, caused by the etiologic agents *Brucella abortus* and *Brucella melitensis*, which are gram-negative intracellular pathogens of humans and animals, is a chronic infection that infects both humans and animals\(^4\). In humans brucellosis is known as Maltese fever and Mediterranean fever and persists within resident monocytes in the spleen, liver and lymphatic system\(^4,5\). Brucella infections require dual antibiotic therapy of doxycycline twice daily and rifampicin once daily for 3-6 weeks due to difficulty of targeting antimicrobials into host cells at high enough concentrations to be therapeutic without inducing toxicity in host cells and tissues\(^6\).

There are a number of obstacles in reaching intracellular Brucella including limited cellular uptake by the host cell’s eukaryotic membrane and the presence of intracellular membranes that sequester the pathogen’s intracellular compartments, further protecting the bacteria from antibiotic exposure, even after cellular internalization\(^7\). Additionally, antibiotics can become inactivated through exposure to the acidic lysosomal environment after uptake. For example gentamycin requires a 64-fold higher dose to reach the minimum inhibitory concentration (MIC) when exposed to a pH 5 solution\(^7\).

Polyanhydride nanoparticles have been demonstrated to be readily internalized by antigen presenting cells, primarily through actin-mediated uptake\(^3\). In our initial studies, we demonstrated *in vitro* that nanoparticles were effectively internalized by monocytes, were able to enhance delivery of antibiotic to the cells, and were able to kill virulent Brucella persisting within monocytes as shown in Figure 9.1. *Brucella abortus* infection was established in a THP-1
cell human monocyte cell line for 24 hours followed by the delivery of equivalent amounts of
doxycycline either soluble or encapsulated into four different chemistries of polyanhydride
This was followed by bacterial enumeration as shown in Figure 9.1. The most readily
internalized chemistries, 20:80 CPH:SA and 20:80 CPTEG:CPH, demonstrated up to a four log
reduction in intracellular bacterial concentration.

The animals receiving nanoparticle-encapsulated antibiotic treatment also exhibited an
improved safety profile in comparison with antibiotic alone with reduced liver toxicity based on
alanine and aspartate transaminase concentrations. The liver enzymes alanine (ALT) and
aspartate (AST) transaminases were elevated upon delivery of one mg of soluble doxycycline. In
contrast, encapsulation of one mg of doxycycline into 20:80 CPH:SA nanoparticles reduced liver
toxicity and the liver enzymes ALT and AST remained in the normal range, as shown in Figure
9.2.

The nanoparticle-based treatment regimens also provided dose sparing effects, which in
addition to the lack of adverse side effects, will reduce antibiotic resistance and lead to enhanced
patient compliance. Weekly does of 0.5 mg doxycycline encapsulated into 20:80 CPH:SA and
20:80 CPTEG:CPH nanoparticles were shown to be as effective as 0.5 mg of soluble
doxycycline given daily for a period of three weeks, with the animals treated with the
polyanhydride nanoparticle group receiving a seven-fold reduction in the amount of doxycycline
over the length of study, as shown in Figure 9.3. Both the 10.5 mg total regimen of soluble
doxycycline and the 1.5 mg regimen of doxycycline encapsulated into polyanhydride
nanoparticles demonstrated significantly lower colonization compared to the untreated control in
the spleen (Figure 9.3A) and liver (Figure 9.3B). No significance was observed between the two
chemistries of polyanhydride nanoparticles used, indicating that both performed equally in reducing Brucella colonization. This work sets the stage for optimizing polyanhydride-based nanoparticle systems for antibiotic delivery.

9.2.3 Polyanhydride nanoparticle platform for the delivery of antiparasitic agents to filarial worms, \textit{Brugia malayi}

Lymphatic filarial diseases, transmitted by mosquitos in endemic regions, represent a significant global burden. The parasites are endemic in over 80 countries worldwide, particularly India and sub-Saharan Africa\textsuperscript{8,9}, and infecting up to 120 million individuals. This parasitic infection can cause diseases such as lymphedema, hydrocele, and elephantiasis.\textsuperscript{9} Traditional therapies utilize antifilarial drugs such as ivermectin, diethylcarbamazine, and albendazole. Many of these drugs have unwanted side effects and the use of up to four drugs over several months of continuous treatment can affect patient compliance. The use of amphiphilic polyanhydride nanoparticles for drug delivery may significantly improve the standard of treatment of filarial diseases by lowering the drug dose, shortening the killing time, and enhancing patient compliance. Antimicrobial treatments such as doxycycline have been added to antifilarial regimens to target Wolbachia, which are intracellular bacteria that exist in a symbiotic relationship with adult worms\textsuperscript{10}. Anti-Wolbachia drugs have been shown to reduce pathogenicity and reproductive capacity of adult filarial worms\textsuperscript{11}. Herein we demonstrate enhanced antifilarial activity upon co-delivery of doxycycline and the antiparasitic, ivermectin, using polyanhydride nanoparticles.

The unique chemistry of polyanhydride nanoparticles may be applied to address many of the challenges associated with mass drug administration against lymphatic filariasis. Drug-loaded nanoparticles were synthesized as described previously\textsuperscript{12} and their surface morphology
was found to be consistent with previous work as shown by the scanning electron photomicrographs in Figure 9.4\textsuperscript{13–15}. Additionally, particle size was also found to be consistent with previous publications\textsuperscript{16,17}. The average size for the 20:80 CPH:SA nanoparticles was found to be \(494 \pm 195\) nm, and the average size for the 20:80 CPTEG:CPH nanoparticles was slightly smaller at \(218 \pm 56\) nm. The surface erosion profile of the polyanhydride nanoparticles provided sustained release of drug over an extended time.\textsuperscript{13,18,19} The sustained release of doxycycline and ivermectin from the two polyanhydride chemistries is shown in Figure 9.5. A larger initial burst of the doxycycline was observed from the 20:80 CPH:SA nanoparticles, consistent with previous work\textsuperscript{12,13,17}. Additionally, a distinct profile was observed for the release of ivermectin from both polyanhydride chemistries, characterized by a very low burst, followed by a zero-order release over the course of the experiment. This may indicate a preferential partitioning of the ivermectin to the slower-degrading CPH-rich regions of both polymer chemistries.\textsuperscript{20} Effectiveness of the polyanhydride nanoparticles when directly compared to the standard soluble treatment was demonstrated by the overall percent survival of \textit{B. malayi} worms over the duration of the study period with a single treatment of the previously described groups (Table 9.1). The results clearly show the effectiveness of the nanoparticle-administered groups compared to the soluble group. This work sets the stage for testing the efficacy of polyanhydride nanoparticle-based delivery systems in animal models.

9.3 References


9.4 List of Figures

Figure 9.1. Intracellular concentration of *Brucella abortus* is reduced when an equivalent amount of doxycycline (10 µg/mL) is delivered together with 20:80 CPH:SA and 20:80 CPTEG:CPH nanoparticles as compared to soluble doxycycline.
Figure 9.2. Liver enzymes alanine (ALT) (A) and aspartate (AST) (B) transaminases are elevated upon delivery of one mg of soluble doxycycline while encapsulation of equivalent amount of doxycycline into 20:80 CPH:SA nanoparticles reduced liver toxicity.

Figure 9.3. Weekly doses of 0.5 mg doxycycline encapsulated into polyanhydride nanoparticles are as effective as 0.5 mg of soluble doxycycline given daily to mice for a period of three weeks, with the mice treated with the polyanhydride nanoparticle group receiving a seven-fold reduction in the amount of doxycycline over the length of study. * represents statistical significance (p < 0.05) in CFU colonization compared to the untreated control in the spleen (A) and liver (B). No significance was observed between the nanoparticle and soluble treatment groups.
Figure 9.4 Polyanhydride nanoparticles of 20:80 CPH:SA (A) and 20:80 CPTEG:CPH (B) were synthesized with 5% (w/w) of doxycycline and ivermectin. Particle size was analyzed using ImageJ software.

Figure 9.5. Cumulative release profile of doxycycline and ivermectin from 20:80 CPH:SA and 20:80 CPTEG:CPH nanoparticles. Cumulative mass of drug released was determined using high performance liquid chromatography (HPLC).
Table 9.1 Effects of soluble and 20:80 CPH:SA nanoparticle-encapsulated drugs on the survival of adult *B. malayi* worms *in vitro*.

<table>
<thead>
<tr>
<th>Formulation</th>
<th># Treated</th>
<th>% Death</th>
</tr>
</thead>
<tbody>
<tr>
<td>195 µM Soluble IVM/Dox</td>
<td>11</td>
<td>64%</td>
</tr>
<tr>
<td>195 µM Nano-IVM/Dox</td>
<td>13</td>
<td>100%</td>
</tr>
<tr>
<td>49 µM Soluble IVM/Dox</td>
<td>5</td>
<td>40%</td>
</tr>
<tr>
<td>49 µM Nano-IVM/Dox</td>
<td>5</td>
<td>100%</td>
</tr>
<tr>
<td>10 µM Soluble IVM/Dox</td>
<td>7</td>
<td>43%</td>
</tr>
<tr>
<td>10 µM Nano-IVM/Dox</td>
<td>4</td>
<td>100%</td>
</tr>
<tr>
<td>5 µM Soluble IVM/Dox</td>
<td>7</td>
<td>14%</td>
</tr>
<tr>
<td>5 µM Nano-IVM/Dox</td>
<td>7</td>
<td>100%</td>
</tr>
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</table>