Engineering amphiphillic polyanhydride particle platform for targeted drug and vaccine delivery

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Engineering amphiphillic polyanhydride particle platform for targeted drug and vaccine delivery

by

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

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Abstract

There is an urgent need to design novel and safe drug and vaccine delivery methods for efficacious prophylactic and therapeutic treatments to protect humans from new and re-emerging infectious diseases. Ideally, new regimens would provide protection from disease following a single immunization and to achieve this, the use of complementary strategies to stimulate both the innate and adaptive immune systems is required. This work has focused on the design of targeted drug and vaccine delivery platforms based on amphiphilic polyanhydride particles that can result in the generation of robust immune responses. A transdisciplinary approach that combines concepts from biomaterials, nanotechnology, carbohydrate and protein chemistry, molecular biology, immunology and computational analysis has been applied to rationally design and engineer novel vaccine platforms that encompass passive and active targeting strategies. *Yersinia pestis*, the causative agent of bubonic and pneumonic plague, is the disease of interest in this project; however, the platforms developed can be applied to different diseases.

First, the intrinsic properties of the polyanhydride systems utilized in these studies were tailored to achieve the stability of proteins with the potential to be used as antigens and/or therapeutic agents. Amphiphillic polyanhydride chemistries showed the most optimal properties to preserve structural integrity and functionality of different proteins. The surface chemistry of polyanhydride particles was also identified as an important attribute that directs the adjuvant properties of these carriers. In this context, this work
revealed changes in particle uptake and activation phenotype of antigen presenting cells as a consequence of chemistry-dependent surface adsorption of serum proteins.

Besides the passive targeting strategy based on the appropriate selection of polymer chemistry, active targeting strategies were also designed to improve and direct the adjuvant properties of polyanhydride particles. In this work, two approaches were taken: adjuvant and antigen modifications. Carbohydrate-functionalization of polyanhydride nanoparticles results in the development of “pathogen-like” particles with the ability to induce and enhance APC activation by specific interactions with the mannose receptor. Finally, antigenic modification with αGal residues in combination with polyanhydride particles as vaccine delivery vehicles results in the production of high antibody titers with high avidity and with the ability to recognize protective epitopes in vivo.

In summary, the studies described in this thesis demonstrate the promise of amphiphilic polyanhydride particles as drug delivery devices and vaccine adjuvants. This work provides key insights into the rational design of targeted platforms to enhance the induction of antigen-specific immune responses and to facilitate the design of protective targeted therapeutic treatments and vaccines.
CHAPTER 1

Introduction

1.1 Introduction

Novel diagnostic technologies and screening methods as well as the effective therapeutic agents have diminished mortality for several re-emerging infectious diseases. Even with the impressive advances in medical care witnessed over the past century, infectious diseases remain the second leading cause of death worldwide\(^1\)\(^2\). Many of these deaths occur in infants and young children from diseases that could be prevented by vaccination. On the other hand, the design of efficacious prophylactic and therapeutic treatments for emerging diseases, especially those that are considered by the U.S. Department of Defense as diseases with bioterrorism potential (i.e., plague, anthrax, etc), is also necessary\(^3\)\(^4\).

The 2006 PhRMA report “Biotechnology Medicines in Development” identifies 418 new biotechnology protein-based medicines (i.e., monoclonal antibodies, hormones, vaccine antigens) for more than 100 diseases, including infectious diseases, cancer, autoimmune diseases, HIV and related conditions, which are in human clinical trials or under review by the Food and Drug Administration\(^5\). However, the therapeutic potential of peptide and protein drugs, as well as their clinical application, is often hampered by a number of obstacles to their successful delivery\(^5\)\(^-\)\(^9\). Some of the obstacles in the delivery of these protein-based therapeutics is to deal with the physical and chemical instabilities of protein pharmaceuticals\(^6\). Chemical instability results when a new chemical structure
is formed as a result of bond formation because the reactivity of side chains or disruption of the primary sequence\textsuperscript{9}. Physical instability refers to changes in the structural level of proteins that can lead to denaturation, adsorption, and precipitation\textsuperscript{6,10}. The chemical and physical stability of proteins can be compromised by environmental factors such as pH, temperature, high pressure, organic solvents, detergents, agitation, etc\textsuperscript{6,8,10,11}. And most of these factors are present in common manufacturing process as well as during storage, transportation and administration of these therapeutic formulations. The fragile three-dimensional structure of protein therapeutics must be maintained for biological activity and avoid compromising the protein immunogenicity\textsuperscript{12,13}.

Another obstacle in the delivery of protein therapeutics is that being highly vulnerable molecules, proteins usually present short in vivo half-lives either at the site of administration or in distal location, on their way to the site of pharmacological action\textsuperscript{14}. The ability of large molecules such as pharmaceutical proteins to transport across biological barriers (i.e., epithelial, blood brain barrier, transmucosal) is low due to their low absorption\textsuperscript{15-17}. For these reasons, the effectiveness of potential peptides and proteins drugs is dependent on a frequent administration regimen, which compromises the patient comfort and makes these regimens expensive. A clear example is the only anthrax vaccine licensed by the FDA, BioThrax\textsuperscript{®} that requires a series of five doses administered at 0 and 4 weeks and 6, 12, and 18 months to convey protection\textsuperscript{18,19}. Therefore, the development of single dose therapeutics (e.g., vaccines) represents a much-needed solution to dispense with the requirements of multiple doses and, at the same time, to improve the efficacy of these treatments.
The need to design novel drug and vaccine delivery methods to overcome these challenges is urgent and biodegradable polymer-based carriers have emerged as an effective solution. These carriers can be fabricated into different devices geometries (i.e., films\textsuperscript{20,21}, implant coatings\textsuperscript{22,23}, drug eluting stents\textsuperscript{24,25}, microparticles or nanoparticles\textsuperscript{12,26-39}) to best fit the desired application of disease treatment; however, particles-like devices (i.e., microparticles and nanoparticles) offer several advantages over other geometries such as localized drug delivery, and administration by injectable or non-injectable (i.e., inhalable) routes for systemic drug delivery. Controlled release of a variety of therapeutic and prophylactic treatments has been achieved over the years by using polymer particles. Controlled polymeric drug delivery has the potential to reduce the quantity and frequency of doses while maintaining appropriate systemic or local drug concentrations better than conventional delivery techniques (Figure 1.1).

![Figure 1.1 Conventional vs. controlled release of therapeutic drugs.](image)

Various polymer chemistries have been applied in the drug and vaccine delivery area; however polyester microparticles, especially those composed of lactic and glycolic
acids (e.g. poly(lactide-co-glycolide), PLGA), have been the most extensively studied and used as delivery devices for biomedical applications\textsuperscript{26,27,32,34,37}. These devices have shown to provide a sustained release for different proteins and their degradation products are easily absorbed by neighboring cells\textsuperscript{29,40}. However, these degradation products can create an acidic microenvironment that has shown to affect the stability of proteins\textsuperscript{33}. In order to avoid these problems, researches have turned their attention to a class of surface eroding polymers: polyanhydrides\textsuperscript{41,42}. A special family of polyanhydrides based on copolymers of sebacic acid (SA), 1,6-bis(\(\rho\)-carboxyphenoxy)hexane (CPH), and 1,8-bis(\(\rho\)-carboxyphenoxy)-3,6-dioxaoctane (CPTEG) (\textbf{Figure 1.2}) have shown potential to be used as drug and vaccine carriers.\textsuperscript{35,43} The degradation mechanism of these polymer systems allows for controlled release kinetics of proteins that can vary from days to moths by choosing the appropriate polyanhydride chemistry\textsuperscript{30,44,45}. In addition, polyanhydride degradation products are non-cytotoxic\textsuperscript{46,47} and do not result in a severe pH decrease upon dissolution and therefore provide the adequate environment for protein stability\textsuperscript{44,45}. Finally, previous in vivo experiments performed by Dr. Narasimhan’s research group have shown the ability of CPH:SA and CPTEG:CPH copolymers to deliver vaccine antigens (i.e., tetanus toxoid and F1-V for \textit{Y. pestis}, respectively) resulting in an enhanced and modulated immune response in mouse models\textsuperscript{36,48}. All these characteristics make polyanhydrides promising candidates to be used as vaccine adjuvants.
Figure 1.2 Chemical structures of (from top to bottom): sebacic acid (SA), 1,6-bis(p-carboxyphenoxy)hexane (CPH), and 1,8-bis(p-carboxyphenoxy)-3,6-dioxaoctane (CPTEG).

Even with the advances showed in the area of drug and vaccine delivery, several challenges such as rapid clearance from circulation, directing delivery to specific immune systems and immunomodulation, still remain as unsolved in order to improve the efficacy of these delivery systems. In order to overcome these, complementary strategies to stimulate both the innate and adaptive immune systems are required. Development of targeting strategies to overcome physical barriers and/or direct drug and vaccine carriers to specific target cells and tissues of immunological importance is a growing area of research\textsuperscript{49}. Targeting strategies can be recognized as two main types: passive and active targeting and both types can be achieved by engineering the
drug/antigens to increase their immunogenicity and/or the particle-based carriers to enhance their adjuvant properties\textsuperscript{50,51}. Passive targeting or physical targeting involves the preparation of an antigen and/or drug/vaccine carrier that can avoid the elimination due to body defense mechanisms like metabolism, excretion and opsonization followed by phagocytosis\textsuperscript{51}. Some of the variables that can be tailor in order to achieve passive targeting are the size and shape of the device and the molecular weight and chemistry of the materials that will dictate the surface charge and hydrophobicity of the device. On the other hand, active targeting involves attaching molecules like antibodies, proteins, peptides, carbohydrates or any other ligand to the drug/antigen or the drug/vaccine delivery system, which allow it to meet and complex with the immune target that has the specific receptor for the attachment\textsuperscript{49,51}. In the case of vaccines, it is imperative that the immune response be primed to the delivered antigen. In order for effective priming to be accomplished, antigen must be supplied to antigen presenting cells (APCs). If these APCs are properly activated and present the correct antigenic epitopes, an adaptive immune response can be mounted that will build long-term immunological memory\textsuperscript{52,53}. By targeting receptors (i.e., Toll-like receptors (TLRs), and C-type lectin receptors (CLRs)) present on the surface of DCs, it is possible to enhance and modulate the generated immune response\textsuperscript{54,56}.

With all this in mind, the overall goal of this research is to design innovative platforms for targeted vaccine delivery based on polyanhydride particles that can result in efficacious immune responses. A graphical summary of the approach taken to rationally design these targeted platforms is presented in Figure 1.3.
Figure 1.3 Pictorial summary of the rational approach developed in this thesis to design targeted platforms.

1.2 References


CHAPTER 2

Literature Review

2.1 Summary

Micro and nanoparticles have great potential to be used as delivery vehicles. Different natural and synthetic polymers have been used to fabricate particulate carriers for delivery of drugs, proteins, and vaccines. Some of these polymers exhibit adjuvant properties that can be explored for the design of effective vaccines. Section 2.2 overviews the use of micro and nanoparticles as drug delivery vehicles, exploring the different biomaterial chemistries and their fabrication and characterization methods. In Section 2.3 the immune system and its interaction with vaccine adjuvants is discussed, with special emphasis on the adjuvant properties of polymeric particles (i.e., polyesters and polyanhydrides). Section 2.4 discusses current and potential strategies for the development of novel vaccine platforms based on targeted systems. Finally, Section 2.5 provides an overview of Yersinia pestis, the causative agent of plague.
2.2 Micro and Nanoparticles as Drug Delivery Vehicles

2.2.1 Introduction

Even during the 20th century, drug discovery frequently resulted from empiricism (e.g., the accidental discovery of penicillin). As technology advanced, particularly after 1970, methods of drug and vaccine production became more sophisticated and rational. In parallel with the advent of modern pharmaceutical technology and the explosive ascent of biotechnology, the cellular and molecular basis for the action of many drugs is now understood. New technology and clearer biological insights have led to new classes of therapeutics and prophylactic agents. Examples of these new agents that are commercially available include: Recombivax HB® (Merck & Co., Inc), a hepatitis B subunit vaccine, Orthoclone OKT® (Ortho Biotech), a monoclonal antibody against CD3 used in the prevention of organ rejection, and Avonex® (Biogen, Inc), which uses interferon beta-1a as an agent to treat multiple sclerosis. This wealth of new technology and "ammunition" in the war against disease has incited novel strategies for drug and vaccine administration.

An increasing number of drugs are based on biomolecules such as peptides, proteins, oligonucleotides, and DNA. The manufacture of therapeutic proteins represented the first true industrial application of recombinant DNA technology. Approximately 36% of the new biopharmaceutical products currently approved within the EU are protein-based. Actually, a large fraction of biotechnology R&D is focused on the creation of vaccines for different kinds of diseases and many of these future vaccines will be based on peptide or protein subunits. One of the most challenging tasks in the development of protein pharmaceuticals is to deal with the physical and the
chemical instabilities of the protein. These molecules often show low bioavailability and display a need for protection against enzymatic or acid-catalyzed breakdown in the human body. The fragile three-dimensional structure of proteins must be maintained for biological activity and this could be lost due to physical as well as chemical instability.

Drug delivery systems are an alternative to improve the bioavailability of drugs while at the same time providing the necessary protection of drug molecules. Successful efforts to produce pharmaceutical formulations that would prolong the action of the therapeutic agents minimizing the frequency between dosing go back to the late 1940s and early 1950s with the introduction of the first commercial product, known as Spansules®. This product consisted of small drug spheres coated with a soluble coating and by varying the coating thickness, capsule dissolution times could be varied, thus prolonging the action of one dose of the orally administrated therapeutic agent. Such formulations are now known as “controlled-delivery” products.

Following a single dose administration of a therapeutic agent, the blood plasma level rapidly rises and then exponentially decays as the drug is metabolized and/or eliminated from the body. The purpose behind developing systems that release drugs in a controlled manner is to achieve more effective therapies while eliminating the potential for both underdosing in which the drug is not therapeutically effective and overdosing in which the drug produces undesirable side effects (Figure 2.1). It is desirable to achieve a therapeutic index that is between the toxic and the minimum effective levels, for longer periods of time (i.e., “zero order” release). Other advantages of using controlled delivery systems can include the need for fewer administrations, optimal use of the drug
in question, and increased patient compliance—all of these aspects can lead to a reduction of costs for the pharmaceutical industry. While these advantages can be significant, the potential disadvantages cannot be ignored. These include the possible toxicity or non-biocompatibility of the materials used, undesirable degradation products, any surgery required to implant or remove the system, and the possibility of patient discomfort from the delivery device.  

**Figure 2.1** Drug level verses time profile showing differences between zero order, controlled release, slow first order sustained release and conventional release. Reprinted and modified from 2.

In order to design effective delivery systems, it is necessary to design materials that are biocompatible and that can provide an adequate microenvironment to maintain the biological activity of a drug and provide a sustained drug release profile. Polymers, especially biodegradable polymers, have been extensively used in the biomedical arena in the form of sutures, wound covering materials, and artificial skin. Polymeric drug
delivery systems have been considered for many applications to supplement the standard means of medical therapeutics. In the 1960’s, researchers discovered alternative uses for these biomaterials in applications for drug delivery. Recent advances have shown that polymeric devices are useful for high molecular weight drugs and for those drugs that should be delivered in minute quantities with zero-order kinetics. Different types of “polymer therapeutics” have been used in order to improve drug functionality, for example polymer-protein conjugates, polymer-drug conjugated or complexes, and polymer micelles with drugs covalently or physically incorporated in the core. The most common polymer-protein conjugate system is PEG-protein conjugates. In this kind of system, therapeutic proteins are conjugated with poly(ethylene glycol) (PEG) by a process known as PEGylation that was first described by Davis, Aubuchowski and colleagues. There are multiple PEGylated protein drugs that are commercially available or in clinical trials, for example Adagen® is composed of PEG-adenosine deaminase and used for SCID syndrome, and PEGASYS® marketed in 2002 is composed of PEG-α-interferon 2a and is used to treat hepatitis C. On the other hand, block copolymers constructed from hydrophilic and hydrophobic segments are known to assemble in an aqueous environment into polymeric micelles. Because of their core-shell structure, polymeric micelles have excellent potential as drug delivery devices. Kataoka, Okano and coworkers have published extensively on polymer micelles, and have designed many interesting block copolymers for use in polymeric micelles. Polymeric micelles have principally been used as drug carriers in tumor targeting applications. Pluronics and poly(ethylene glycol) are the main used polymers for micelles formation.
Even with the advantages provided by the “therapeutic polymers” described above, biodegradable polymeric drug carriers have been extensively studied. Such systems combine the advantage of long-term zero order drug release with biodegradability. In this type of controlled delivery system, the drug is not chemically attached to the polymer, and it is simply encased within the polymer matrix. Because there are no chemical interactions between the drug and the polymer, the drug remains in a biological active form, and can exert its effect upon the body as soon as it is released from the polymeric matrix. Numerous polymers have been evaluated for these types of drug delivery systems, and they can be classified into three basic types. These are water-soluble polymers, which are typically used for short-term delivery, non-biodegradable polymers, and biodegradable polymers, which require some chemical reaction or alteration in the body before they become water-soluble.

The decision to use a suitable polymer from these three general classes depends upon the route of administration, the type of drug used, the amount of drug to be delivered, the duration of release required, and the specific site of delivery (i.e., targeted delivery). The same criteria that are used to select the appropriate polymer in the design of drug delivery systems are applicable in determining the required form of the delivery device. Many drug delivery devices have been fabricated as implants (i.e., wafers, films, multilayered disks). The disadvantage of this type of device is that usually the implantation requires surgery and there is the possibility of patient discomfort from the delivery device. These issues can be obviated by the development of injectable devices, mainly micro and nanoparticles – which can also serve as vaccine delivery devices. In recent years, biodegradable polymeric particles have attracted considerable attention as
potential drug delivery devices in view of their applications in controlled release of drugs, their ability to target particular organs/tissues, as carriers of DNA in gene therapy, and for their ability to deliver proteins, peptides and genes through a specific route of administration \(^{28-31}\).

This section provides an overview of biodegradable polymeric micro and nanoparticles and their applications as drug delivery carriers. First, the most common biodegradable polymers used for particle fabrication are introduced and discussed in context of their chemical properties and applications in drug delivery. This is followed by a summary of the main fabrication methods used for micro and nanoparticle preparation. Finally, the biological consequences of some important properties (i.e., size, surface properties) of micro and nanoparticles are discussed.

### 2.2.2 Biodegradable Polymers for Drug Delivery

#### 2.2.2.1 Naturally occurring polymers

Polymers are commonly found in nature. Naturally occurring polymers can be classified into three types: lipid-based (e.g., liposomes), proteins (e.g., collagen, gelatin, fibrin, and albumin), and polysaccharides (e.g., chitosan, hyaluronic acid, cellulose, and alginate (Figure 2.2)) \(^{32}\). Generally, the degradation of these polymers is driven by hydrolytic or enzymatic cleavage of their main chain.
Liposomes are one of the most well-known drug delivery carriers employed in the treatment of different diseases\textsuperscript{33}. Liposomes can be constructed from biodegradable and nontoxic constituents (i.e., double lipid bilayer) and are able to non-covalently encapsulate molecules (i.e., chemotherapeutics agents, hemoglobin, imaging agents, drugs, and genetic material) within their 100-200 nm diameter interior (Figure 2.3)\textsuperscript{7, 33-36}. The factors that must be taken into account when preparing liposome formulations include the size, the surface charge\textsuperscript{37} and the membrane fluidity\textsuperscript{38}. There are many liposomes for the treatment of malignancies that are already approved, awaiting approval or in clinical trials. For example, doxorubicin liposomes have shown significant activity against AIDS-related Kaposi’s sarcoma, breast and ovarian cancers in different clinical trials\textsuperscript{39-41}. However, developmental work on liposomes has been limited due to
inherent problems such as low encapsulation efficiency, rapid leakage of water-soluble drug in the presence of blood components, and poor storage stability. 

Figure 2.3 Basic liposome structure.

In another example of naturally occurring polymers, green plants have the ability to take glucose and make long chains containing many glucose units. These long chains are known as cellulose. Cellulose has been routinely used in medical and pharmaceutical applications for many years (e.g., in pharmaceutical excipients or cellulose wadding for surgical use), and is a benign material that is well tolerated by the body. For example, cellulose nanocrystals of suitable sizes can be used as carriers in drug delivery, and have many reactive functional groups on their surface to which drugs or targeting molecules could be attached.

Other polysaccharides like chitosan and hyaluronic acid have shown promise as drug delivery vehicles. There are specific enzymes for chitosan (papain) and hyaluronic acid (hyaluronidase) degradation. In drug delivery they have been used as hydrogels,
microparticles, as stealth or Trojan coatings or co-polymerized with other polymers of interest 12, 46-48.

Sodium alginate and gelatin have been used to prepare carrier matrices because they are naturally occurring, biodegradable, biocompatible, and hydrophilic polymers that are suitable for the entrapment of water soluble drugs 49-51. Alginate has been utilized as a scaffold for skeletal muscle tissue engineering due to its biocompatibility and ability to form gels with a gentle gelation process 52.

The use of collagen as a biomaterial has focused on the aspects of cellular growth or delivery of proteins capable of stimulating cellular response. The most successful and stimulating applications are shields in ophthalmology, injectable dispersions for local tumor treatment, sponges carrying antibiotics and minipellets loaded with protein drugs 12, 53-55.

It is clear that the biocompatibility offered by naturally occurring polymers is an important characteristic that is difficult to find in synthetic polymers. However, there are significant limitations (i.e., difficulty for chemical modification, the low drug concentration that these systems can deliver) that must be addressed to improve their function.

Next, some synthetic polymer systems for drug delivery applications are discussed.

2.2.2.2 Poly(ortho esters) and Poly(phosphoesters)

The first poly(ortho ester) was described in the 1970s in a series of patents 56-59 and represent the first example of a new synthetic polymer specifically designed for controlled drug delivery. These polymers contain hydrophobic units linked together along the polymer chain by functional groups very susceptible to hydrolysis. Such
polymers are bioerodible and the rate of hydrolytic degradation at the surface of the polymer is much faster than the rate of water permeation into the polymer matrix. However, these polymers contain an orthoester linkage which is more susceptible to hydrolysis under low pH conditions than basic conditions. Four families of poly(ortho esters) have been developed by Alza Corporation and by Dr. Heller and colleagues at the Stanford Research Institute. Figure 2.4(a) show the structure of poly(ortho esters). Control of the degradation rate of these polymers has been achieved by incorporating acidic or basic excipients into the polymer matrix. The last family of poly(ortho esters), developed by Advanced Polymer Systems, a short segment of lactic or glycolic acid in the polymer backbone. This segment acts as a latent acid catalyst and eliminates the need to add acidic excipients to the polymer to control erosion. The two most commonly used polyorthoester-based devices are implants and microspheres. Drug delivery applications for these polymers include the delivery of cancer chemotherapeutics, periodontal therapeutics, anti-inflammatory agents, intraocular therapeutics, anesthetics, and DNA.

The general chemical structure of poly(phosphoesters) is shown in Figure 2.4(b). In the past, interest in phosphorus-containing polymers centered on their flame-retardant properties. However, it was their hydrolytic instability that renewed interest in such polymers for drug delivery applications. Because the ultimate hydrolysis products are phosphate ions, alcohol, and a diol, the polymer has the potential to be non-toxic. While these polymers are being investigated for delivery of a number of different therapeutics, most research has focused on the delivery of cancer and gene therapies. These
two types of polymers may receive more attention in the future especially for optimization of drug delivery carriers.

Figure 2.4 (a) Structure of poly(ortho esters), and (b) general structure of poly(phosphoesters).

2.2.2.3 Poly(alkylcyanoacrylates)

Poly(alkylcyanoacrylates) (PACAs), shown in Figure 2.5, were not employed as polymers until the early 1980s. However, the corresponding monomers, alkylcyanoacrylates, have been used since at least 1966 because of their excellent adhesive properties resulting from the bonds of high strength they are able to form with most polar substrates, including living tissues and skin. Therefore, the monomers have been extensively as tissue adhesive for the closure of skin wounds, as surgical glue, and as embolitic material for endovascular surgery. More recently, PACAs have been used as nanoparticulate drug delivery carriers. This area of research has gained increasing interest in therapeutics, especially for cancer treatments, which generally involve highly toxic molecules in contact with healthy tissue. Other molecules of interest, including poorly stable compounds such as peptides...
and nucleic acids, have been combined with PACA nanoparticles for targeting purposes.  

Today, PACA nanoparticles are considered of the most promising polymer colloidal drug delivery system and are already in clinical development for cancer therapy.  

![Chemical structure of alkylcyanoacrylates](image)

**Figure 2.5** Chemical structure of alkylcyanoacrylates.

### 2.2.2.4 Polyethers

The two most common polyethers are poly(ethylene glycol) (PEG) and poly(propylene glycol) (PPG) (Figure 2.6). These polymers are water-soluble and do not have to undergo any chemical degradation; they simply have to become hydrated, ionized, or protonated to lose their form and dissolve within the body. PEG has been used widely in the pharmaceutical industry and especially in the drug delivery area. PEGylation is the covalent coupling of PEG to another large macromolecule (i.e., drugs, proteins, peptides). When attached to various protein medications, PEG allows a slow clearance of the carried protein from the blood. This makes for a longer acting medicinal effect and reduces toxicity, and it allows longer dosing intervals. Examples include
PEG-interferon alpha, which is used to treat hepatitis C, and PEG-filgrastim (Neulasta), which is used to treat neutropenia.

![Chemical structures of (a) poly(ethylene glycol), and (b) poly(propylene glycol).](image)

**Figure 2.6** Chemical structures of (a) poly(ethylene glycol), and (b) poly(propylene glycol).

Researchers in the drug delivery area have taken advantage of the amphiphilic properties of PEG by incorporating it into the backbone of some of the most common polymers used for drug delivery (i.e., polyanhydrides, poly(cyanoacrylates)) because of their ability to alter the degradation rate of these polymers. As mentioned before, it has been demonstrated that the presence of PEG inhibits material interactions with blood components and therefore could increase the circulation time of a delivery device in the bloodstream. Taking advantage of this property, PEG has been attached by different methods to delivery devices (i.e., micro and nanoparticles) in order to increase their biocompatibility.

Another useful biomaterial based on polyethers is the block copolymer Pluronic® ((PEG)n-(PPG)m-(PEG)n). This copolymer has unique micelle forming properties, via self assembly in water, due to the hydrophilic PEG and hydrophobic PPG, enabling the
formation of nanometer sized particles. In summary, polyethers show promise in the design of efficacious drug delivery vehicles.

2.2.2.5 Polyesters

Poly(lactic acid) (PLA), poly(glycolic acid) (PGA), poly(ε-caprolactone) (PCL), and their copolymers (e.g., PLGA) (Figure 2.7) have been the most studied polyesters, especially in the drug delivery area. These polymers were originally developed as biodegradable sutures and were approved because they degrade to the natural metabolites L-lactic acid and glycolic acid. Several studies have been published describing these polymers, their synthesis, characterization, and fabrication into microcapsules, films, fibers, rods, microparticles, and nanoparticles for controlled release of drugs ranging from low molecular weight antibiotics and steroids to high molecular weight proteins such as vaccines and growth hormones. Although the majority of drug delivery systems developed with these polymers have been injected or implanted either subcutaneously or intramuscularly, there are studies showing their use in nasal and oral administration.

The erosion rate of PLGA copolymers can be controlled by varying the ratio of glycolic to lactic acid in the copolymer, and the higher the glycolic acid content, the faster the erosion rate. However, as the amount of glycolic acid in the copolymers is increased, crystalline domains of PGA may form, and solubility in toxicologically acceptable solvents decreases; thus the highest amount of PGA normally used is about 50 mol%. These copolymers have an average lifetime of about 2-4 weeks.
PCL is also an FDA-approved polyester for applications in drug delivery and biodegradable sutures. PCL is semi-crystalline, similar to PLA. However, PCL has a much slower degradation profile ranging from one to three years in vivo\textsuperscript{95, 104-107}. Due to this prolonged degradation rate it has been utilized in blends or copolymers with expedite the degradation rate \textsuperscript{107}.

![Chemical structures of polyesters](image)

**Figure 2.7** Chemical structures of the most commonly used polyesters and their copolymers.
Polyesters undergo a bulk erosion process, which can be described in four steps (Figure 2.8). First, water diffuses into the polymer and ester bond cleavage starts. Second, differentiation between the surface and interior begins, with a drastic decrease in molecular weight in the inner part of the matrix, where the acidic environment accelerates the degradation. Third, low-molecular-weight oligomers begin to diffuse through the thinning outer layer, and when the molecular weight of these oligomers is low enough to allow solubilization in the medium, weight loss begins. Finally, a polymer shell remains after the oligomers have solubilized and slow erosion of the shell takes place.

![Figure 2.8 Schematic representation of bulk erosion process.](image)

Such an erosion process can have a significant impact on the use of polyesters in drug delivery. When the polymer is used in orthopedic applications such as bone plates, the massive release of acidic products can exceed local tissue clearance capabilities, and it has been shown to produce serious inflammatory responses\(^ {108} \). In addition to accelerating degradation, the low internal pH can also have adverse effects on acid-sensitive incorporated agents, such as DNA, antigens, or some proteins. These limitations demonstrate significant hurdles for the use of polyesters in drug delivery.
2.2.2.6 Polyanhydrides

Figure 2.9 shows the general chemical structure of polyanhydrides. These materials were first prepared in 1909 and were subsequently investigated as potential textile fibers, but found unsuitable because of their hydrolytic instability. However, it is this hydrolytic instability that makes them excellent candidates for the design of drug delivery systems. Polyanhydrides are a class of versatile biomaterials and can be grouped into three primary classes: aliphatic, unsaturated and fatty-acid derived, and aromatic. Figure 2.10 shows the most common polyanhydrides used for drug delivery applications. The promising characteristics of polyanhydrides for biomedical applications rely on their surface erosion mechanism (Figure 2.11) that translates into well-controlled release kinetics, where the drug release rate coincides with the degradation rate of the polymer. In an aqueous environment, the macromolecules at the surface break into smaller chains before water penetrates into the device. Thus, the drug is released as the polymer degrades. Because aliphatic polyanhydrides hydrolyze rapidly while aromatic polyanhydrides hydrolyze slowly, good control over hydrolysis rate can be achieved by using copolymers of aliphatic and aromatic polyanhydrides. In this way, erosion rates from days to many months have been demonstrated. Another aspect that makes polyanhydrides excellent candidates for drug delivery carriers is their biocompatibility as they degrade into non-toxic, non-mutagenic degradation products capable of being metabolized intracellularly. Polyanhydride-based drug delivery devices (Gliadel®) have been approved by the U.S. Food and Drug Administration (FDA) for the treatment of brain tumors. This device is a polyanhydride wafer composed of sebacic acid (SA) and 1,3-bis(p-
carboxyphenoxy)propane (CPP) (CPP:SA copolymer in 20:80 molar ratio) loaded with the chemotherapeutic agent, carmustine, 1,3-bis(2-chloroethyl)-1-nitro-sourea (BCNU)\textsuperscript{110}.

![General structure of polyanhydrides.](image)

**Figure 2.9** General structure of polyanhydrides.

Sebacic acid is the most widely used aliphatic polyanhydride and it degrades within weeks. When copolymerized with the aromatic CPP, this aliphatic-aromatic system can be used for drug delivery. Sebacic acid can also be copolymerized with unsaturated polyanhydrides like poly(fumaric acid) (FA) and acetylenedicarboxylic acid (ACDA) increasing their bioavailability for the oral delivery of proteins\textsuperscript{110,111}. Another aromatic polyanhydride that has been widely used in drug delivery is 1,6-bis(p-carboxyphenoxy)hexane (CPH) and it has been copolymerized with SA in order to result in controlled degradation times\textsuperscript{110,119}. Several modifications of anhydride monomers have been carried out in order to obtain desired characteristics for particular applications. An example is the incorporation of triethylene glycol (TEG) into an aromatic monomer (CPH) in order to enhance the hydrophilicity of the monomer, resulting in faster degradation rate with a combination of bulk and surface erosion. The resulting polymer is poly(1,8-bis(p-carboxyphenoxy)-3,6-dioxaoctane) (CPTEG)\textsuperscript{120,121}. 
Figure 2.10 Typical polyanhydrides used for drug delivery applications.

Recent efforts have focused on the use of two polyanhydride-based copolymer systems (i.e., CPH:SA and CPTEG:CPH) as drug delivery carriers. Different device forms including tablets, films, microparticles and nanoparticles have been fabricated for a broad range of drug delivery applications including injectable microparticle delivery, inhalable/injectable nanoparticle delivery, multi-component implants, and tissue engineering constructs. The chemistry of the copolymer has been shown to play a significant role in the release kinetics, the ability to stabilize proteins, and the immunomodulatory capabilities of these polyanhydride systems.

Figure 2.11 Schematic representation of surface erosion process.
The release kinetics of both the CPH:SA and the CPTEG:CPH systems have been well studied \(^{114, 115, 125, 128, 130}\). By changing the ratio of the monomers during fabrication, the degradation rate of the copolymers can be extended from days to months. As the release profile of an encapsulated drug depends on the degradation rate of the copolymer (because of the surface erosion mechanism), the release kinetics can be tailored by choosing the appropriate copolymer composition. Increasing the CPH content results in slower release profiles, while the presence of CPTEG generates higher initial bursts and faster release.

One of the most important issues related to the rational design of drug delivery carriers is their ability to maintain the biological activity of the different payloads (e.g., peptides, recombinant proteins) that they have to deliver. In this sense, previous research has studied the effects of polyanhydride degradation products on several proteins. Determan et al. \(^{126}\) demonstrated the stability of tetanus toxoid (TT), ovalbumin (Ova), and lysozyme when incubated with anhydride (i.e., CPH and SA) monomers. The stability of Ova and bovine serum albumin (BSA) after encapsulation and release from CPH:SA microspheres was also shown by Determan et al. \(^{114}\). Torres et al. \(^{117}\) studied the stability of lysozyme and Ova in the presence of degradation products of the CPTEG:CPH system and found that the CPTEG:CPH system preserves the structural integrity of these two proteins.

These studies demonstrated the potential of polyanhydride carriers for controlled drug delivery and make them serious candidates for the design of efficacious drug delivery vehicles.
2.2.3 Polymeric Particle Fabrication

As previously described, in order to improve the efficiency of drug delivery and targeting, biodegradable polymers have been fabricated into different devices to be used for controlled delivery. Of the different devices forms, microparticles and nanoparticles have gained much attention, due to their tendency to accumulate in inflammation areas \(^{28, 81, 82, 84}\). A special interest has arisen in particulate systems for vaccine delivery because they facilitate injection. In this regard, nano and microparticles offer attractive properties.

Microspheres are, in a strict sense, spherically empty particles. In addition different terms are commonly used to refer to microparticles, for example microbeads or beads are used alternatively. The term “microparticle” refers to a particle with a diameter of 1-100 µm and it has become the most well studied device geometry for polymeric drug delivery. Nanoparticles were first developed around 1970. The term “nanoparticle” is used when the particle is <1 µm in size and they were initially devised for vaccines and anti-cancer drugs. Next, the most common fabrication techniques for polymeric micro and nanoparticles are briefly described.

2.2.3.1 Microparticles

Microparticles have been fabricated based on both natural occurring and synthetic polymers. The most common synthetic polymers used for microparticles fabrication are: polyesters (i.e., PLGA, PCL) \(^{5, 8, 66, 100, 102}\), poly(orthoesters) \(^{68}\), poly(alkylcyanoacrylates) \(^{35}\), poly(amino acids) \(^{7}\), polyacrylamides \(^{12}\), and polyanhydrides \(^{98, 115-117, 128}\).
Four general techniques have been used to fabricate microparticles: (i) hot-melting, (ii) emulsion solvent extraction, (iii) phase separation processes, and (iv) spray drying or atomizing.

The hot-melt technique involves heating the polymer and drug in a non-solvent above the melting point of the polymer. Particles are formed and dispersed by the use of mechanical stirring. However this method is not ideal for encapsulating temperature sensitive drugs, such as proteins.

In phase separation processes particle formation is triggered by addition of an anti-solvent to a solution. This type of fabrication method has been applied especially for naturally occurring polymers (i.e., chitosan).

Solvent extraction is the most common method for polymeric microparticles fabrication. Oil-in-water (O/W), and water-in-oil (W/O) emulsions, and water-oil-water (W/O/W), water-oil (W/O/O), and solid-oil-oil (S/O/O) double emulsions can be used. In the w/o/w method the drug (typically proteins) is dissolved in an aqueous phase and then emulsified with a larger volume of polymer dissolved in an organic solvent, typically methylene chloride. The inner emulsion is then added to a larger volume of water that contains a surfactant, usually PVA, and allowed to stir for several hours in order to extract the solvents. In the case of w/o/o or s/o/o the outer aqueous PVA phase is replaced with an immiscible organic, i.e. silicone oil.

In the spray-drying technique (Figure 2.12(a)), a solution stream breaks down into droplets while passing through a nozzle, followed by drying of the droplets prior to their collection. Benefits of spray drying include one step fabrication without the use of multiple solvents or secondary drying and easy scale-up for mass production. In the
case of spray-atomization (Figure 2.12(b)), the drug/polymer suspension passes through an atomizing nozzle. As the polymer/drug particles leave the nozzle they are collected in a bath of liquid nitrogen sitting on top of a frozen layer of non-solvent (e.g., ethanol)\(^{115, 117, 131}\). The liquid nitrogen/non-solvent bath is then stored at -80ºC for three days, allowing the non-solvent to thaw and the frozen microparticles to fall into it. As the microparticles reside in the non-solvent the organic solvent (e.g., methylene chloride) slowly diffuses out, leaving solid spheres to be collected by filtration. More recently, microspheres of controlled size have been fabricated using acoustic excitation to force single droplet emulsions to precipitate in a passing non-solvent stream\(^ {132}\).

![Figure 2.12](image)

**Figure 2.12** Schematic representation of (a) spray-drying, and (b) spray atomization methods for polymeric micro/nanoparticle fabrication.

### 2.2.3.2 Nanoparticles

Several methods have been developed for preparing nanoparticles and are optimized on the basis of their physicochemical properties (e.g., size and hydrophilicity)
with regard to their in vivo fate after parenteral administration. The selection of the appropriate method for preparing drug-loaded nanoparticles depends on the physicochemical properties of the polymer and the drug. On the other hand, the procedure and the formulation conditions will determine the inner structure of these polymeric colloidal systems.

Preparation methods of nanoparticles are broadly divided into two categories, those based on physicochemical properties such as phase separation and solvent evaporation, and those based on chemical reactions such as polymerization and polycondensation. The first route is normally been used to produce nanoparticles from hydrophobic or crosslinked water-insoluble hydrophilic polymers (i.e., PLGA, PCL, poly(alkylacrylates, and polyanhydrides). For the second route, emulsion polymerization and precipitation polymerization methods have been described.

Solvent precipitation techniques have been generally applied for hydrophobic polymers. Several techniques described in the literature are based on the mechanism of polymer precipitation. In a solvent extraction-evaporation technique, a hydrophobic polymer is dissolved in an organic solvent, such as chloroform, ethyl acetate, or methylene chloride and is emulsified in an aqueous phase containing a stabilizer (e.g., PVA). Just after formation of the nanoemulsion, the solvent diffuses to the external phase until saturation. The solvent molecules that reach the water-air interphase evaporate, which leads to continuous diffusion of the solvent molecules from the inner droplets of the emulsion to the external phase; simultaneously, the precipitation of the polymer leads to the formation of nanospheres.
In the solvent displacement or nanoprecipitation method, the organic solvent selected is completely dissolved in the external aqueous phase, obviating the need for evaporation or extraction for polymer precipitation. The organic solvent diffuses instantaneously to the external aqueous phase, followed by precipitation of the polymer and drug. The advantage of this method is that no surfactant is employed; however, the method is limited to drugs that are highly soluble in a polar solvent.

Recently, many studies have been published describing modified methods according to the specific properties of the polymer matrix. For example, PACA nanoparticles have been prepared by an emulsion polymerization method in which droplets of water-insoluble monomers are emulsified in an aqueous and acidic phase that contains a stabilizer. Another recent study used a modified water-oil-water double-emulsion/solvent evaporation technique that could be tuned to fabricate nanospheres using polyanhydrides as polymer matrix.

2.2.4 Particle Characterization

Microparticles and nanoparticles as colloidal carriers mainly depend on the particle size distribution, surface charge, and hydrophilicity. These physicochemical properties affect not only drug loading and release, but also the interaction of these particulate carriers with biological membranes.

2.2.4.1 Particle Size Analysis

Two techniques have been used to determine the particle size distribution of colloidal systems: Photon correlation spectroscopy (PCS) and electron microscopy.
including both scanning electron microscopy (SEM) and transmission electron microscopy (TEM). PCS is based on dynamic light scattering at constant temperature, and the method requires knowledge of the viscosity of the suspending fluid for an estimation of the average particle size and its distribution function \(^{135}\). Quasi-Elastic Light Scattering (QELS) also offers an accurate procedure for measuring the size distribution of nanoparticles.

SEM provides images of the particles to be measured and imaging software (e.g., ImageJ) can be used to determine size distribution. Images obtained with TEM can be used to determine the size of particles but also offers a more detailed analysis of shape and inner structure of the particles.

2.2.4.2 Surface Charge and Hydrophobicity

The surface charge of colloidal particles can be determined by measuring the particle velocity in an electric field. Laser light scattering techniques are fast enough to measure the surface charge with high resolution. The hydrophobicity of the nanoparticles can be determined by methods including the adsorption of hydrophobic fluorescent or radio labeled probes, two phase partitions, hydrophobic interaction chromatography, and contact angle measurements. Recently, XPS has been developed which offers the identification of chemical groups in a 5 Å-thick coat on the external surface of the nanospheres \(^{136}\).
2.2.5 Particles Properties: Consequences for their Biological Fate

As mentioned before, the *in vivo* behavior of microparticles and nanoparticles as drug delivery carriers depends on properties such as particle size and surface characteristics (i.e., surface charge and hydrophobicity). These properties can affect not only the particle interaction with the payload that needs to be released, but also the barriers that they will encounter in the biological system.

At various steps of the different fabrication methods described above, the payload (i.e., drug, protein, peptides, DNA) can be added and encapsulated *in situ* while the particles form. As described before, it is important to select the appropriate polymer matrix that possesses chemical properties that are compatible with the chemical characteristics of the drug. The release of encapsulated drug occurs via diffusion through aqueous channels or pores within the polymer matrix or during surface or bulk erosion of the carriers. Both the percent of encapsulation and the release profile depend not only on the chemistry of the polymer matrix but also the shape and the size of the delivery device. In the case of particulate systems, size plays an important role in the biological fate of the carrier. A successful particulate delivery system may have high loading capacity to reduce the quantity of the carrier required for administration. In this sense, microparticles will offer a high encapsulation capacity. In addition, microparticle size seems to be adequate for administration through peritoneal delivery route, but injections of microparticles can be sometimes difficult and painful leading to patient non-compliance while other delivery routes (i.e., intranasal) cannot be explored with microparticles because of their size.
The motivation to develop an alternative drug delivery system using nanoparticles instead of microparticles is based on several assumptions and observations. First, more administration routes can be explored for a more efficacious delivery. Second, the larger surface area to volume ratio of nanoparticles compared to microparticles should result in a faster polymer degradation and drug release. However, this effect might not be as pronounced as expected. Third, particles having a size below 100 nm can evade mechanical filtration from the blood stream and accumulate in liver, spleen and kidneys, resulting in longer blood circulation times. Fourth, nanoparticles are able to deliver payloads across a number of biological barriers, for example the blood-brain barrier (BBB), different types of mucosa and epithelia, and cell membranes for transfection applications. Finally, nanoparticles offer a large surface area that could be used for surface-modification for specific targeting (Section 2.4).

The interaction of particles with their biological environment and electrostatic interaction with biological compounds occur due to the charge on the surface, e.g., a negative charge promotes the adsorption of positively charged drug molecules such as proteins. Apart from the size and the surface charge of the particles, the surface hydrophobicity determines the amount of adsorbed blood components, mainly proteins (opsonins). These will also have an effect in the in vivo fate of these delivery vehicles. Binding of these opsonins on to the surface of particulate devices, called opsonization, acts as a bridge between particles and phagocytes.

A detailed characterization of the properties of micro or nanoparticle-based delivery carriers and their interactions with biological components is extremely important in the design of adequate and efficacious delivery carriers.
2.3 Vaccine Adjuvants

2.3.1 Introduction

One of the most successful medical interventions in the reduction of disease caused by infectious agents over the last 200 years is the use of vaccines\textsuperscript{137}. A good example of this is the worldwide eradicated of the human smallpox virus through vaccination\textsuperscript{138}. Programs that include a childhood vaccination schedule have been implemented in different countries to eradicate diseases such as polio\textsuperscript{139}. In addition to human benefits, vaccines have been used in veterinary medicine to control and eradicate diseases such as pseudorabies virus\textsuperscript{140}. Therefore, vaccination is one of the greatest medical achievements in the 20\textsuperscript{th} century.

Even with all the advancements and improvements in the efficacy and implementation of vaccines over the past several decades, infectious disease still remains the largest cause of death worldwide; many of these diseases could be prevented by efficacious vaccination\textsuperscript{140, 141}. This shows that there are many challenges that still remain unsolved to derive the full benefits of immunization programs. These obstacles include the low level of understanding of the immune system and all the biological events that a vaccine will initiate in order to generate an immune response, the development of single-dose vaccines, methods to increase the poor immunogenicity of immunogens, and the ability to rapidly develop vaccines against emerging pathogens.

Promising solutions include the development of new vaccine adjuvants or carries that enhance the effectiveness of vaccines. Although recent developments in synthetic and naturally derived adjuvants suggest that single-dose vaccines for many pathogens may be realized in the near future, no single adjuvant will be effective for all vaccine
applications. Therefore, it is important to design effective vaccine adjuvants which can be adjusted to different diseases. In order to do this, it is necessary to develop interdisciplinary science-based approaches.

The next sections report and discuss the most common vaccination techniques employed during the past years as well as new approaches based on the use of vaccine adjuvants. Special attention is given to polymeric carriers as the new generation of vaccine adjuvants.

2.3.2 Innate and Adaptive Immune Responses: An overview

When designing vaccine systems, it is essential to have a detailed understanding of the complex interplay between the cells of the innate and adaptive immune systems. Protection from diseases includes the activation of both the innate and adaptive immune systems working together as an integrated system. The innate immune system is described by Janeway and Mezhitov as "a non-specific first line of defense of the immune system" and involves a variety of cells including phagocytic cells (i.e., macrophages and dendritic cells (DCs)), NK cells and the complement system. The components of the innate system use pattern recognition receptors (PRRs) (e.g., Toll-like receptors (TLRs), mannose receptor (MR) on macrophages, and C-type lectin receptors) to recognize pathogen-associated-molecular-patterns (PAMPs) present on foreign invaders. The innate immune system has evolved two main functions: to react quickly to molecular patterns found in microbes, and to develop slowly, precisely targeted specific adaptive immune responses.
In contrast to the innate system, the adaptive system involves a more direct immune response in which antigen presenting cells (APCs) recognize specific antigen epitopes by specialized receptors. Then, APCs become responsible for the activation of killer T cells, helper T cells, and B cells that will provide an adequate immune response. The APCs responsible for connecting the innate system with the adaptive system are mainly DCs, which are present in blood and tissues in an “immature” state characterized by their phagocytic functions. After encountering pathogens or antigens, DCs migrate to lymph nodes (LN) where they “mature” increasing the surface expression of co-stimulatory molecules (i.e., CD80, CD40, and CD86) and starting the release of cytokines. Inside the DC, pathogens are degraded into small peptide fragments that will bind to Major Histocompatibility Complexes (MHC) to be displayed on the surface of the cell for T cell recognition. There are two main pathways of antigen presentation. In the first pathway, MCH I molecules present antigenic peptides to CD8+ T cells, also known as cytotoxic T lymphocytes (CTLs), which are responsible for killing infected cells directly (cell-mediated immunity (CMI)). In the second pathway, antigen is presented by MHC II molecules to CD4+ T cells (T helper cells) which are classified as Th1 and Th2. Th1-type immune responses are characterized by the production of cytokines, like interferon gamma (IFN-γ) and tumor necrosis factor beta (TNF-β), and generate a cell-mediated immunity. An immune response of the type Th2 is characterized by the secretion of a variety of interleukins, such as IL-4, IL-5, IL-10, and activation of B cells to produce antibodies (humoral response), specially IgG1. Figure 2.13 shows a cartoon representation for DC maturation and activation.
The induction of the appropriate immune response is necessary for a successful vaccination \cite{146}.

**Figure 2.13** Cartoon representation of dendritic cell maturation and activation and the resulting immune response pathways.
2.3.3 Current Vaccine Strategies

Vaccines have been designed to mimic the immune response induced by an active infection, but by avoiding the effects of disease. To be effective, a vaccine must contain some portion of the disease-causing agent and may include an immune-enhancer (e.g., adjuvant). Generally, vaccine regimens include an initial dose that initiates immune responses that involve DCs, followed by booster doses that not only induce the activation of effector cells but also memory cells\textsuperscript{138}. Memory cells provide a faster immune response of greater magnitude\textsuperscript{147}. In a general way, vaccines can be classified into three categories: live, killed, or subunit.

2.3.3.1 Live Vaccines

Relative to other vaccines, those that consist of live, attenuated organisms, induce the most potent and lasting immune response in the host. These vaccines generally require the fewest number of inoculations, require no adjuvants, confer lifelong immunity, and can be delivered through the same route as the natural infection\textsuperscript{148}. One of the advantages of these vaccines is that they retain many of the natural microbial compounds, enhancing immunity.

Attenuation, which is the reduction of the virulence capacity of the organism, is necessary so that these vaccines can be used in a safe way. There exist different strategies to attenuate organisms, like genetic changes to delete critical virulence attributes through repetitive passages (several 100 to 1,000 times) in a non-human host or in vitro. Some examples of this kind of vaccine are the Sabin oral polio vaccine and the Flu-mist\textsuperscript{149}.
However, modified live vaccination can cause the opposite effect because they can replicate in the host and generate an infection and reversion to the more virulent form. Another problem with this class of vaccines is that the live organism can spread and induce the disease in other non-vaccinated individuals\textsuperscript{149}. Even when live vaccines effectively induce both cellular and humoral immunity, they can cause severe reactions and their effectiveness also depends of their proper handling before administration\textsuperscript{150-152}.

\subsection*{2.3.3.2 Killed Vaccines}

To produce this kind of vaccine the whole organism is treated with either heat or chemicals, so that the organism is not able to replicate in the host but the cellular integrity of the pathogen is preserved. Killed vaccines can also induce a potent humoral immune response but without the risk of live vaccines because the organism cannot replicate and therefore, cannot cause infection, spread to other individuals or revert to a virulent form\textsuperscript{148}. Another advantage is that these types of vaccines possess a longer shelf life and are less sensitive to changes in storage conditions\textsuperscript{153}. Some of the limitations of killed vaccines are that they require multiple doses to achieve protective immunity and as with live vaccines, are associated with high incidence of side effects, such as high fever, severe pain, redness, and swelling\textsuperscript{154}.

\subsection*{2.3.3.3 Subunit or Recombinant Protein-Based Vaccines}

Subunit vaccines contain only a portion of the infection agent; some examples of these kinds of vaccines are those against diphtheria and tetanus toxoids\textsuperscript{148}. These
vaccines offer several advantages including the reduction or elimination of side effects. However, subunit vaccines lack strong immunogenicity and require multiple doses for protection. Usually to reverse the poor immunogenicity that subunit vaccines offer, they are delivered with an immunoenhancer (adjuvant)\(^{155}\). Subunit vaccines are easier to produce and safer than live vaccines, but require an efficacious adjuvant\(^ {154, 156}\).

### 2.3.4 Current Vaccine Adjuvants

#### 2.3.4.1 Ideal Adjuvant Properties

Adjuvants are functionally defined as components added to vaccine formulations that enhance the immunogenicity of antigens in vivo\(^ {137}\). The functions that an adjuvant performs are: provide an adequate environment for the antigen including an antigenic reservoir for slow release, facilitate targeting of the antigen to APCs, increase phagocytosis, and modulate and enhance the type of immune response induced by the antigen alone\(^ {157-160}\). They may also provide the danger signal that the immune system needs to respond in the case of an infection\(^ {138}\).

In order to provide an adequate environment for the immunogen, the antigen is entrapped in a non-dissolving or slowly dissolving substance; alums and their gel-like matrix are examples of adjuvants that create a slow-release environment\(^ {156}\). This adjuvant function can also be achieved by incorporating the antigen into biodegradable polymeric micro or nanoparticles. As the particles degrade, the antigen is released. Adjuvants have different ways of enhancing the immune response; by targeting the antigen to immune cells, enhancing phagocytosis, and/or activating APCs. There are some properties of the antigen carriers that accomplish this function. For example,
lipopolysaccharide (LPS), a bacterial-derived immunostimulant, interacts directly with the innate system via LPS receptors like CD14\textsuperscript{161}.

Immune modulation can be influenced by many factors including the route of antigen delivery, antigen dose, duration of antigen presentation, number of immunizations and their frequency and even the inclusion of co-stimulatory molecules with the antigen\textsuperscript{162}. Adjuvants can affect all these factors, and that is why it is important to use the correct adjuvant to generate the desire immune response\textsuperscript{162}.

Currently, adjuvants have been divided into two classes based on their dominant mechanisms of action: i) immune potentiators which activate innate immunity directly (e.g., cytokines) or through PRRs (e.g., bacterial components\textsuperscript{161}), and ii) delivery systems which may concentrate and display antigens in repetitive patterns, target vaccine antigens to APCs and help co-localize antigens and immune potentiators (e.g., polymeric micro and nanoparticles)\textsuperscript{137, 160}.

Thus, both immune potentiators and delivery systems can serve to augment antigen-specific immune response \textit{in vivo}. Even so, for each specific situation there will be a set of characteristics that would constitute an ideal vaccine adjuvant. Within those characteristics are the particular delivery route, the desired immune response, the pathogen, and the stage of the disease. For example, for subunit vaccines it is likely that a combination of delivery systems, immune potentiators, and isolated antigens will be required to elicit optimal immune responses.

The use of vaccine adjuvants should reduce the amount of antigen necessary to generate an immune response. On the other hand, a good vaccine adjuvant must stimulate the desired immune response without toxicity or the induction of excessive
inflammation. The production of an appropriate cytokine profile to the desired immune response is another characteristic of a good vaccine adjuvant.

Other factors such as stability over time and the ability to provide immunity with a single dose should be considered in the selection of an ideal adjuvant.

### 2.3.4.2 Current Adjuvants

Table 2.1 presents the different categories of adjuvants that are commercially available or in development. Most of the currently licensed adjuvants were developed using empirical methods, thus they are not optimal for many of the challenges in vaccination today.

<table>
<thead>
<tr>
<th><strong>Adjuvant category</strong></th>
<th><strong>Examples</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>Mineral salts</td>
<td>Aluminum and calcium salts^162, 168, 169</td>
</tr>
<tr>
<td>Emulsions and surfactant-based formulations</td>
<td>MF59, AS02, ISA-51 and ISA-720^172</td>
</tr>
<tr>
<td>Microbial derivatives</td>
<td>Monophosphoryl lipid A, CpG oligonucleotides, heat labile toxin from <em>Escherichia coli</em>, lipoproteins^173, 174</td>
</tr>
<tr>
<td>Cells and cytokines</td>
<td>Dendritic cells, IL-12 and GM-CSF^176</td>
</tr>
<tr>
<td>Particulate delivery vehicles</td>
<td>Micro and nanoparticles, immunostimulatory complexes; liposomes, virosomes, virus-like particles^127, 176</td>
</tr>
</tbody>
</table>

Alums are salts of aluminum hydroxide or aluminum phosphate and were the first compounds used as adjuvant in 1926. Alum-based vaccines are prepared by suspending the antigen in a phosphate buffered solution and allowing the antigen to
adsorb to the aluminum hydrogel. This type of adjuvant may not be ideal for antigens with low immunogenicity (i.e., small peptide vaccines), and its use induces IgE production which is associated with allergy. Other disadvantages of alums are their limited shelf lives due to the impossibility of lyophilizing them, and their non-suitability for oral or intranasal immunization.

Calcium phosphate has been used for many years as adjuvant in the DTP (diphtheria-tetanus-pertussis) vaccine. It is used in the delivery of allergens because it does not induce IgE production.

Freund’s complete (CFA) and incomplete (IFA) adjuvants are very potent systems and are comprised of a water-in-mineral oil emulsion with the emulsifier mannide monooleate. Recent studies have been shown that CFA induces a very strong immune response while IFA is thought to induce tolerance.

Introduced in Europe in 1997, MF59 is an oil-in-water microemulsion that has been shown to stimulate a strong Th2 biased immune response to a large number of antigens and may be more suitable for subunit vaccines than alum. MF59-based vaccines that have incorporated recombinant antigens induce high titer antibody responses and T cell proliferative responses.

Bacteria-based adjuvants have also been widely used. Lipopolysaccharide (LPS) is known to stimulate the production of cytokines and chemokines that control DC movement and maturation. But even with its potency to induce immune responses, LPS is used only as experimental adjuvant because of its toxicity. Monophosphoryl Lipid A (MPLA) can bind to Toll-Like Receptor 4 (TLR-4) initiating the synthesis of interleukin-12 (IL-12) and interferon-gamma (IFN-γ) which induce DC maturation, migration and
initiation of the T cell response\textsuperscript{174}. Bacterial toxins have a high degree of immunogenicity and immune enhancing capabilities as well as a high cellular receptor specificity. These characteristics have led researchers to study their potential as vaccine adjuvants. Pertussigen and Cholera enterotoxin are some examples of bacterial toxins that are currently under study\textsuperscript{175}.

Virosomes and virus-like particles are generated by inserting viral fusion proteins into pre-formed liposomes\textsuperscript{176}. These particles allow the induction of both humoral and cell mediated immunity because they deliver the antigen directly into the cytosol of the target cell by mimicking infectivity of native viruses\textsuperscript{176}. Vaccines for human influenza and human papiloma virus are based on these adjuvants\textsuperscript{137,176}.

2.3.5 Biodegradable Polymers as Vaccine Adjuvants

Biodegradable polymers have characteristics that make them promising for the development of single dose vaccine adjuvants\textsuperscript{177,178}. Some of the advantages of these compounds are: the ability to stabilize and sustain the release of antigens by a controlled mechanism over extended periods of time, which eliminates the need of booster doses, and their immunomodulatory properties that can be achieved with the appropriate polymer chemistry\textsuperscript{179}.

Polymeric particles can be administered by various routes (e.g., intramuscularly, subcutaneously, intranasal or orally), and they have been shown to induce immune responses in all these regimen\textsuperscript{174}. The biggest advantage of these materials is that because they are biodegradable, they do not have to be removed after administration because they degrade into compounds that are not toxic for the body\textsuperscript{180}. 
Currently there are two main classes of biodegradable polymers that have been studied for controlled release vaccines: polyesters \(^{102, 179-185}\) and polyanhydrides \(^{114, 117, 118, 127, 178, 186-191}\).

### 2.3.5.1 Polyesters

Lactic acid and glycolic acid (e.g. poly(lactide-co-glycolide), PLGA) have been utilized in biomedical applications and recently have been proven effective as vaccine delivery vehicles for the induction of protective immunity in laboratory animals \(^{179, 182, 183, 192, 193}\). Specifically, microparticles based on polyester chemistries have been widely studied and recently, nanoparticles are being used as vaccine adjuvants \(^{194, 195}\). PLGA has the benefit that its degradation products are naturally occurring metabolites and are absorbed by neighboring cells. However, these degradation products create an acidic environment that does not benefit the stability and immunogenicity of proteins \(^8\).

Antigen-loaded PLGA microparticles have been shown to enhance phagocytic uptake by APCs (i.e., DCs) \(^{184}\). These particles also enhance cytokine production and cell proliferation in \textit{in vitro} experiments \(^{181}\).

A large number of antigens (i.e., \textit{Yersinia pestis} antigens, ovalbumin, tetanus toxoid, malaria antigens, influenza antigens, cancer cell antigens) have been encapsulated in PLGA, PLA, or PGA variant microparticles and nanoparticles resulting in the induction of immune responses \(^{175, 177}\). Encapsulation of antigen in PLGA microspheres was shown to enhance antigen presentation via MHC class I, increasing the activation of T-cytotoxic cells \(^{102}\). However, most of these studies were conducted \textit{in vitro} and with the inclusion of known Th1 immune response activators such as MPLA.
Even when different studies have shown the ability of PLGA microparticles to induce and modulate immune response, there are others that have shown low immunomodulatory efficacy by these materials. These can be due by variations in experiment conditions such as dose of antigen, method of encapsulation, route of immunization and/or size of the particles\textsuperscript{118, 182}. For example, it is known that microparticles $\leq 10$ $\mu$m in diameter are readily phagocytosed by macrophages and DCs and would enhance antigen presentation\textsuperscript{127}. It is important to remark that all this extensive research done with PLGA has not shown induction of an immune response in humans; therefore there is still an amount of experimental research that need to be accomplished in order to determine the effectiveness of this vaccine delivery system.

2.3.5.2 Polyanhydrides

Polyanhydrides are surface erodible polymers that have been used as carriers for controlled drug delivery\textsuperscript{110, 127, 178, 187, 188, 191} and are approved by the FDA for biomedical applications. These biomaterials degrade into carboxylic acids that are non-cytotoxic\textsuperscript{112}. These polymers have shown a controlled release profile that ranges from days to months, depending on the copolymer composition and this is because of their surface erosion mechanism\textsuperscript{113, 114, 117, 128, 130}. Other features of polyanhydride delivery systems are: improved adjuvanticity\textsuperscript{129, 189}, antigen stabilization\textsuperscript{125, 126, 128}, and enhanced immune responses\textsuperscript{127, 178}.

**Figure 2.14** compares the adjuvant properties of polyesters and polyanhydrides. The main advantage of polyanhydrides over polyesters is that polyanhydrides offer protein stability after encapsulation, release, and storage from micro and nanoparticles.
The hydrophobicity and surface erosion properties of these biomaterials prevent water from penetrating to the interior of the particles increasing protein stability and providing a sustainable release profile.\textsuperscript{114,128} In addition, polyanhydride degradation products are less acidic than those of polyesters, which also contributes to the enhanced protein stability.\textsuperscript{117,125,126}

**Figure 2.14** Comparison between the adjuvant properties of polyesters and polyanhydrides.

Even though polyanhydrides have not been extensively studied as adjuvants, recent \textit{in vivo} studies with antigen-loaded microparticles based on the anhydride monomers sebacic acid (SA) and 1,6-bis\textit{(p-carboxyphenoxy)}hexane (CPH) demonstrated that tetanus toxoid (TT) maintains its immunogenicity and antigenicity following encapsulation.\textsuperscript{178} Furthermore, the type of immune response generated, Th1 vs. Th2, was evaluated by antibody isotype production, resulting in observations that suggest that different copolymer chemistries induce different immune responses. These studies indicate that polyanhydrides are promising adjuvants for single dose vaccines.
Current research has focused on the CPH:SA system as well as on the adjuvant properties of novel amphiphilic polyanhydride chemistries that include copolymers of CPH and 1,8-bis(p-carboxyphenoxy)-3,6-dioxaoctane (CPTEG) \(^{110, 120, 121}\). These studies included *in vitro* adjuvanticity experiments that have shown the ability of CPH:SA and CPTEG:CPH-based micro and nanoparticles to activate APCs (i.e., DCs). These studies showed an adjuvant chemistry effect on cell surface marker expression (i.e., MHCI, MHCII, DC40, CD86) and cytokine production (i.e., IL-6, IL-12, TNF-α) by murine DCs \(^{129, 189}\). A chemistry effect has been also observed in micro and nanoparticles phagocytosis experiments conducted with human monocyte \(^{196}\) cell lines and murine-derived DCs \(^{189}\). Finally, *in vivo* studies are ongoing to evaluate the efficacy of these polyanhydride systems as vaccine delivery vehicles for different antigens (i.e., *Yersinia pestis* antigens, anthrax antigens, ovalbumin, influenza antigens).

The design of improved adjuvants can lead to the solution of various problems related to effective vaccine design, and in this context, biodegradable polymer particles (i.e., polyanhydride-based) may play an important role, especially because of their ability to modulate the immune response. More *in vitro* and *in vivo* studies with these adjuvants are necessary to understand the role that chemistry and particle size play in the modulation of immune response and to design more efficacious vaccines.

### 2.4 Targeted Vaccines Strategies

#### 2.4.1 Introduction

The ultimate goal of vaccination is to stimulate a specific immune response and to induce a long lasting immunologic memory to protect against subsequent disease \(^{137, 197}\).
Vaccines are considered to be one the most successful medical interventions against infectious diseases (i.e., small pox, tetanus, diphtheria, malaria, hepatitis B, etc)\textsuperscript{198}. Even so, many significant obstacles in the design of effective vaccines remain. These include improving current suboptimal vaccines, developing new vaccines for disease for which vaccines have not yet produced, and responding rapidly to newly emerging pathogens\textsuperscript{199}.

In order to design efficacious vaccines many key elements are required. First, an antigen is required to which a memory immune response will be developed. An important aspect in the selection of adequate antigens for a specific pathogen is its presentation and this includes the preservation of its structural conformation (i.e., preservation of adequate epitopes) for induction of specific functional antibodies\textsuperscript{200-202}. It is well known that an effective adaptive immune response is necessary to fight pathogen infection and it is now known that the innate system plays a crucial role in the evolution of the adaptive immune response\textsuperscript{203}. Thus, inclusion of immune potentiators (i.e., vaccine adjuvants), which trigger early innate immune response to aid in the generation of robust and long-lasting adaptive immune responses, is crucial to vaccine effectiveness. Finally, delivery systems that target the vaccine to appropriate cells of the immune system will ensure optimal stimulation\textsuperscript{172}.

In the search for novel platforms for a more effective vaccine design, researchers have identified different immune targets that could be used to generate the require immune response. The selection of the appropriate target, as well as the selection of the target-agent (i.e., target strategies), will depend greatly upon the type of disease to confront. As an example, the effectiveness of cancer treatments will depend on their
ability to target and kill cancer cells while affecting as few healthy cells as possible. In other cases, the success of novel vaccine technologies will depend on an adequate antigen presentation by antigen presenting cells (APCs) (i.e., macrophages, dendritic cells, B cells) that can activate effector cells of the immune defense. It is crucial for the design of novel targeted vaccine platforms to have a clear understanding of the specific immune cell to target and the downstream activation mechanisms.

Targeted vaccines that can direct their action to a specific immune target represent a potential solution to overcome the remaining obstacles in the design of effective vaccines. Many strategies have been developed in the targeted vaccine area, and the most studied one involves the use of monoclonal antibodies that are recognized by specific receptors on immune cells directing antigens to the place of action. Monoclonal antibodies can be delivered together with the antigen or by a different administration route to target specific receptors on immune cells; however, a new approach focuses on the chemical attachment of macromolecules to antigens (i.e., monoclonal antibodies, synthetic peptides, carbohydrates).

As mentioned before, the inclusion of vaccine adjuvants can help in the stimulation of an immune response. Four major reasons have turned research efforts towards the development of new adjuvants. First, single dose vaccination is desirable, especially when considering the necessity to deliver health care to developing countries. Second, the use of many protein and peptide based antigens requires an adjuvant. Even when such antigens have shown to be more safe than traditional vaccination methods (i.e., live and killed vaccines), they are poorly immunogenic and need the inclusion of effective adjuvant. Third, a better understanding of innate immunity has
drawn attention to the mechanism(s) of adjuvanticity and guided new design approaches. Finally, it has been recognized that adjuvants (mimicking pathogens) can direct the nature of the immune response as well as its magnitude.

Polymeric micro and nanoparticles have shown to be effective as delivery carriers and have properties that make them ideal as vaccine adjuvants. Engineering these particle-based vehicles (i.e., controlled antigen delivery, size manipulation, and surface modification) can induce passive or active targeting, leading to an enhanced and specific immune response. The use of these novel biomaterial-based platforms has clear potential for the design of effective targeted vaccines. The next sections described the current options for targeted vaccines and show the potential of functionalized-polymer particles for the design of effective vaccines against a new range of diseases.

2.4.2 Immune System Targets

Currently, vaccines act mainly through the humoral arm of the immune system (i.e., antibody-mediated), but this does not seem to be sufficient to offer protection from many diseases. Many chronic diseases, including cancer, malaria, hepatitis, HIV, and tuberculosis are better defended against though cell mediated immunity, which indicates that a balanced immune response is desired.

In order to generate new strategies that can provide optimal immune responses, it is necessary to have a better understanding of the immune system components and their functions in the disease of interest. This knowledge will allow for the identification of immune targets and will guide the rational design of vaccines.
2.4.2.1 Cancer cells

Current cancer therapy involves intrusive processes including chemotherapy, and surgery to remove the tumors if possible, followed by more chemotherapy and radiation. Lately, a more important role has emerged via immunotherapy (i.e., cancer vaccines) as a treatment for cancer. The selection of the cancer treatment to apply to a specific cancer patient depends on the particular cancer cell to attack. In all cases, the effectiveness of the treatment is directly related to the ability of the treatment to target and eliminate cancer cells while reducing the number of healthy cells affected.

Research in cancer therapy has identified different properties of cancer cells that can be used to target treatments to the specific tumor site. The first step in order to target cancer cells is to engineer technologies that are able to avoid the reticuloendothelial system (RES) (i.e., liver and spleen) and ascertain transport to the solid tumor from the bloodstream. Cancer's own structure is also used to target treatment to specific cancer cells. In many cases, cancer cells overexpress particular antigens on their surfaces and these antigens become ideal targets or drug delivery, but it is important that the targets for a particular cancer cell type can be identified with confidence and that they are not expressed in significant quantities anywhere else in the body.

Angiogenesis is defined as a physiological process involving the growth of new blood vessels from pre-existing vessels and is a vital process for the development of a tumor mass. This process is the result of numerous interactions between regulators, mediators and stimulatory molecules. Many angiogenesis stimulatory molecules have been identified (e.g., vascular endothelial growth factor (VEGF), basic fibroblast growth factor, platelet-derived growth factor) in the body. However, there are also different angiogenesis...
inhibitors that can be used to regulate angiogenesis as a target strategy. Some examples of angiogenesis inhibitors are the interferon family, thrombospondin-1 and -2, and protein fragments such as angioatin and endostatin \(^{224, 225}\). Finally, targeting of the tumor vasculature can be also used as a strategy to target delivery to a wide range of tumor types \(^{224, 226}\).

Developing strategies to target these different properties of cancer cells can lead to adequate design of immunotherapy for cancer treatment.

2.4.2.2 Dendritic cells

Dendritic cells (DCs) have emerged as the most potent antigen-presenting cell because of their unique T cell stimulatory capacities \(^{176}\). In their immature state, DCs can efficiently take up antigens, and then process them through both MHC class I and II which are required for the activation of CD8\(^+\) cytotoxic T lymphocytes (CTL) and CD4\(^+\) T helper cells, respectively \(^{191}\). In order to activate T cells, DCs have to undergo maturation and migrate to lymphatic organs. Antigen recognition can initiate DC maturation and therefore induce T cell differentiation. Normally, the immune system’s response to a pathogen initiates because they share similar structures known as pathogen-associated molecular pattern (PAMPs), which enable their recognition \(^{227, 228}\). DCs express pattern recognition receptors (PRRs) that interact with PAMPs leading to DC activation and secretion of cytokines that can recruit other cells creating an inflammatory environment that consequently leads to an adaptive immune response \(^{137, 229}\). PRRs include Toll-like receptor (TLRs) as well as non-TLRs, retinoic acid-inducible gene I-like receptors and C-type lectin receptors (CLRs) \(^{227, 228}\).
So far the most studied PPRs have been TLRs and this is mainly because eleven such receptors have been identified in humans. TLR signaling plays an important role in determining the quality of the T cell response. For example, the use of lipopolysaccharide (LPS), which is a TLR4 ligand, leads to a preferential cell-mediated response (Th1). Table 2.2 shows some of the identified TLRs and their ligands. The role of TLRs in the initiation of innate immune response makes them potential targets for vaccines by using their ligands as vaccine adjuvants.

**Table 2.2** Toll-like receptors and their ligands.

<table>
<thead>
<tr>
<th>Toll-like receptors</th>
<th>Ligands</th>
</tr>
</thead>
<tbody>
<tr>
<td>TLR1</td>
<td>Bacterial lipoproteins from <em>Mycobacteri, Neisseria</em></td>
</tr>
<tr>
<td>TLR2</td>
<td>Lipoproteins, glycolipids, lipopolysaccharide</td>
</tr>
<tr>
<td>TLR3</td>
<td>Viral double-stranded RNA</td>
</tr>
<tr>
<td>TLR4</td>
<td>Bacterial lipopolysaccharide</td>
</tr>
<tr>
<td>TLR5</td>
<td>Bacterial flagellins</td>
</tr>
<tr>
<td>TLR6</td>
<td>Yeast zymosan particles, lipotechoic acid, lipopeptides from mycoplasma</td>
</tr>
<tr>
<td>TLR7</td>
<td>Single-stranded RNA, synthetic compounds such as loxoribine and bropirimine</td>
</tr>
<tr>
<td>TLR8</td>
<td>Single-stranded RNA</td>
</tr>
<tr>
<td>TLR9</td>
<td>CpG oligonucleotides</td>
</tr>
<tr>
<td>TLR10</td>
<td>Unknown</td>
</tr>
<tr>
<td>TLR11</td>
<td>Bacterial components from uropathogenic bacteria</td>
</tr>
</tbody>
</table>
C-type lectin receptors bind sugars (i.e., mannose, fucose, and glucan) in a calcium-dependent manner using highly conserved carbohydrate-recognition domains\textsuperscript{228,235}. CLR\textsuperscript{s} exist both as soluble and transmembrane receptors that function as PRR\textsuperscript{s}. Together, these CLR\textsuperscript{s} recognize most classes of human pathogens (i.e., viruses, fungi, and mycobacteria)\textsuperscript{197,227,235,236}. Recognition by CLR\textsuperscript{s} leads to the internalization of the pathogen, its degradation and subsequent antigen presentation\textsuperscript{237} and these properties are important for vaccine efficacy. However, it is believed that targeting of the signaling pathways of CLR\textsuperscript{s} can be more powerful to tailor the immune response. These signaling pathways are complex processes that depend on crosstalk with other PRR\textsuperscript{s} (i.e., TLR\textsuperscript{s}), the ligand (i.e., carbohydrate specific signaling pathway) and the DC subset\textsuperscript{235,238-240}. Some examples of CLR\textsuperscript{s} that are expressed by DC\textsuperscript{s} are the mannose receptor\textsuperscript{241-245}, DEC-205\textsuperscript{246-249}, DC-SIGN\textsuperscript{250-254}, Dectin-1\textsuperscript{205,228,235,255,256}, and Dectin-2\textsuperscript{238,257}. Table\textsuperscript{2.3} offers more complete information about the CLR\textsuperscript{s}, the cells that express these receptors, and their ligands. Even while research on CLR\textsuperscript{s} is relatively new and most of their ligands are not yet identified and their mechanism of action is unclear, they represent a promising opportunity to be used in the design of targeted vaccines.

Fc family receptors (i.e., FcγRs, FcεRs)\textsuperscript{258-260}, integrin receptors (i.e., CD11c)\textsuperscript{261} and the TNF-receptor superfamily (i.e., CD40)\textsuperscript{262,263} can be also used for DC targeting purposes. The diversity of DC targeting makes it one of the most recurrent strategies for the design of targeted vaccines.
2.4.3 Current Targeting Strategies

2.4.3.1 Antibody-Targeted Vaccines

The high binding and specificity of antibodies are ideal properties for delivering a payload to target cells. Monoclonal antibody (mAb)-based therapies have become an important part of the treatment of patients with cancer, autoimmune, and infectious diseases.

**Table 2.3** C-type lectin receptors and their ligands.

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Expression by cells</th>
<th>Ligands</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mannose Receptor (CD206)</td>
<td>Dendritic cells (DCs), Langerhans cells (LCs), monocytes, macrophages</td>
<td>Mannose, fucose and sulfate sugars</td>
<td>Phagocytosis and antigen presentation</td>
</tr>
<tr>
<td>DEC-205 (CD205)</td>
<td>DCs, LCs, thymic epithelial cells, NK cells, B cells and T cells</td>
<td>Unknown</td>
<td>Endocytosis and antigen presentation</td>
</tr>
<tr>
<td>DC-SIGN (CD209)</td>
<td>DCs, macrophages</td>
<td>HIV-1, SIV, mannann, ICAM-2, ICAM-3</td>
<td>T cell interaction, HIV-1 pathology, migration, antigen uptake</td>
</tr>
<tr>
<td>Dectin-1</td>
<td>DCs, LCs, monocytes, macrophages, neutrophils, B cells, subpopulation of T cells</td>
<td>β-Glucan</td>
<td>T cell interaction</td>
</tr>
<tr>
<td>Dectin-2</td>
<td>DCs, monocytes, macrophages, B cells and neutrophils</td>
<td>High mannose</td>
<td>Antigen uptake, induction of TNF and IL-6 production</td>
</tr>
</tbody>
</table>
In the case of cancer treatment, tumor-antigen specific mAbs are used to target chemotherapy drugs and/or tumor-growth inhibitors to cancer cells. There are many mAb-based cancer treatments that are approved by the FDA, for example Rituximab (Rituxan®) that consist of an anti-CD20 antibody to fight Hodgkin’s lymphoma. While cancer treatments try to kill cancer cells directly, cancer vaccines (like any other vaccine) look for the activation of the immune system to respond against the cancer cells.

Antibody-targeted vaccines (ATVs) exploit the same targeting properties of antibodies, but are directed to normal cells of the immune system. Mainly, mAbs are used to deliver a cargo of antigens to APC (i.e., dendritic cells) with the goal of inducing or enhancing immunity to the antigen. With biotechnology advances, fusion proteins between antibodies and antigens can be constructed and used as target agents to different DC receptors. MHC molecules and Fc receptors (FcR) were the first targets of antibody-targeted vaccines. Based on the knowledge that FcRs mediated recognition of opsonized antigens by APCs, Snider et al. targeted antigen specifically to FcRs for IgG or MHC molecules on APCs in vitro resulting in enhanced antigen presentation to T cells. Later, the same targets were evaluated in vivo corroborating a strong humoral response to the antigens targeted in the absence of adjuvants. Studies that apply mAbs to target CD11c, which is expressed on DCs, resulted in high antibody titer responses.

ATVs have also explored CLRAs as targets. For example, studies have demonstrated that targeting of tumor antigens to the mannose receptor (MMR) on monocyte-derived DCs using a human MMR-specific mAb allows for antigen-specific T cell proliferation.
Monoclonal antibodies specific to other CLRs (i.e., DC-SIGN, DEC-205, Dectin-1, and Dectin-2) have shown promising results in directing different antigens to DCs in in vitro and in vivo studies.

Even when the highest improvement in targeting strategies has been in the mAb area (i.e., ATVs), there are some disadvantages (e.g., high cost in mAb production) that have turned researches’ attention to the development of alternative approaches.

2.4.3.2 Cell-Based Targeting

Most of the efforts in DC targeting have been focused on ex vivo approaches. DCs were isolated for the first time ca. 1994. In ex vivo targeting, DCs are isolated from the blood of patients, then the cells are exposed, in vitro, to an antigen or other maturation stimuli (i.e. peptides, loaded with proteins or transfected with RNA encoding specific antigens), and, finally, re-injecting them into the patient. An example of DC-based therapy is in cancer immunotherapy. The first attempt to use cell-based targeting in cancer immunotherapy was to directly take tumor cells and conjugate them with antibodies that are specific for certain tumor antigens and in this way direct the antibody-based therapy to cancer cells and not to healthy cells. Other approaches have used DC cell-based therapy for the creation of cancer vaccines. Several clinical trials have evaluated the effect of DCs that have been transfected with tumor-derived RNA or even fused with tumor cells; these tumor-hybrids have been able to induce antigen-specific T cell responses. Researchers have explored the option of using alginate gels as DC delivery vehicles, and recent studies have shown the ability of these alginate-DC systems to accumulate in tumors areas.
Although encouraging results have been obtained with cell-based targeting, there are many medical, economic, and logistic complexities that make it difficult to apply this approach for many different diseases and especially when trying to get vaccination regimes to third world countries.

2.4.3.3 Modified Antigens as Target Agents

As described in Section 2.4.3.1 monoclonal antibodies have been used as targeting strategies, and they are usually delivered together or by different routes with the antigen of interest. However, other approaches are available including the chemical binding of mAbs to the antigen to form antibody-antigen complexes that can be delivered as target agents. The targets remain the same (i.e., cancer cells, dendritic cells receptors), but having antibodies and antigens as one complex allow for a more direct delivery of the antigen.

Antibody-antigen complexes have been used to target different antigens to FcRs, CLR (i.e., mannose receptor, DEC-205), and other receptors like CD40, and B7. Antibody-based modification can be performed in two ways: (i) by chemically attaching the antibody to the antigen (i.e., proteins and/or peptides), and (ii) by encoding the antigens with the desired mAbs and creating a DNA vaccine. The second approach has been widely used to target DC receptors. Chaplin et al. used a DNA vaccine encoding detoxified phospholipase D fused to the CTLA-4 gene (a B7 ligand); the DNA vaccine was tested in sheep and show to be partially effective against Corynebacterium pseudotuberculosis.
Not only have mAbs been linked to antigens for targeting purposes, synthetic peptides have been also used as target agents. In this approach, amino acids sequences are identified by immune cells and initiate an immune response. This targeting strategy has been especially applied to attack virus-based diseases (i.e., influenza).

Glycosylation is a natural enzymatic process that consists of the linking of saccharides to proteins, lipids, or other macromolecules. The majority of the proteins that are produced in the endoplasmic reticulum undergo glycosylation. These glycosylate proteins are part of the immune system in a soluble or membrane bound form and usually serve in the immune system to recognize sugar residues on the surface of pathogens. Taking advantage of this natural process, sugars (i.e., mannose, mannan, glucan) have been chemically linked to antigens to target some receptors on immune cells (i.e., C-type lectin receptors). In different Phase I clinical trials, patients with advanced carcinoma of breast, colon, and stomach were treated with mannann-conjugated MUC1 (tumor-associated antigen mucin 1). Results showed that most of the patients were able to develop an antigen-specific humoral response while some of them also developed a cell-mediated response.

Even when modified antigens have been successfully used as target agents in some clinical trials, they still have the disadvantage that protein-based antigens are generally poor immunogenic. Even when they can be targeted to specific cells by modifications (i.e., antibody, synthetic peptides and/or sugar attachment) their fragile structure can be affected during the delivery process (e.g., breaking linkages that keep the antigen and target molecule together) leading to a failed immune response.
2.4.4 Polymeric Particles as Target Agents

Polymeric particles (i.e., microparticles and nanoparticles) may overcome the weakness shown by other strategies in order to design effective targeted vaccines. Different degradable polymers have been used to fabricated particulate delivery systems, including polyesters (i.e., poly(lactic-co-glycolic acid) (PLGA)), polyorthoesters, poly(alkylcyanoacrylates), and polyanhydrides. Polymeric particles have shown to be effective as drug delivery vehicles but also offer suitable properties to be considered as vaccine adjuvants. The physical and chemical properties of polymeric particles (i.e., chemistry of the polymer matrix, surface charge, surface hydrophobicity, size) have to be carefully selected and triggered in order to design effective carriers for efficacious targeted vaccines. This section reviews the strategies used to design polymeric particles as target agents for vaccine design.

2.4.4.1 Passive Targeting

The main property that attracted the attention of researchers to degradable biomaterials (i.e., synthetic polymers) is their ability to delivery payloads in a controlled manner. Antigens (i.e., proteins, peptides, DNA) can be encapsulated and/or conjugated to polymeric particles allowing for their protection and leading to the delivery of intact antigens that can be presented and processed by APCs (i.e., DCs). The release of antigens from polymeric particles can be controlled by choosing the adequate polymer chemistry; for example, more hydrophobic chemistries offer long-time release profiles while the incorporation of hydrophilic motifs can accelerate the degradation of the polymer matrix and therefore the antigen release kinetics.
Tailoring the release kinetics by controlling the chemistry of the polymer matrix in particulate delivery systems has been used as an indirect approach (i.e., passive targeting) to target delivery of antigens to DCs because it allows for structurally intact delivery of the payload plus long term release simulating the effect of boosting doses in vaccination. Before an antigen can be processed and presented by DCs, the particulate delivery vehicle has to be internalized by DCs. The mechanism by which DCs uptake and internalize particles will determine the subsequent processing and presentation pathway (i.e., MHC class I, MHC class II, or cross-presentation pathways). The uptake of particles can be partially controlled by their properties (i.e., particle size and surface charge); particle size appears to be a key determinant for the uptake and internalization of particles. Particles having a size range between 0.5 to 10 µm (i.e., microparticles and large nanoparticles) are optimally taken up by phagocytosis. Both in vitro and in vivo studies have shown that microparticles (1-10 µm) and nanoparticles (200-600 nm) made of PLGA and polyanhydrides (i.e., CPH:SA and CPTEG:CPH) are efficiently taken up by DCs. On the other hand, macropinocytosis is the uptake mechanism that DCs use for small nanoparticles (<50 nm). Reddy et al. showed that ca. 25 nm pluronic-stabilized polypropylene sulfide (PPS) nanoparticles are able to be efficiently transported into lymphatic capillaries and their draining lymph nodes targeting DCs present in lymph nodes. Particle size is also an option to target a specific and desirable delivery route; for example, for mucosal delivery it is known that particles between 100 and 500 nm in size are preferred because they can pass through the nasal cavity into the lungs.
After internalization, polymeric particles should be able to deliver the payload for processing by DCs. In order to achieve antigen delivery into the cytoplasm (to allow for MHC I presentation) a group of pH-sensitive biomaterials have developed and tested\textsuperscript{326-329}. As an example, pH-responsive core-shell PDEAEMA nanoparticles have been developed that can achieve delivery of small molecules and proteins to the cytosol in DCs\textsuperscript{329}.

While the passive-targeting strategies described above allow for a more direct and controlled delivery of payloads, in many cases, they are not sufficient to strategically target DCs because other APCs (i.e., macrophages) can also uptake particulate vehicles, but they are not effective at activating T cells\textsuperscript{207}.

2.4.4.2 Active Targeting

Strategies to preferentially target biomaterials to DCs in vivo will improve their efficacy as vaccine adjuvants. As mentioned before, nanoparticles can migrate into the lymphatic system and accumulate in DC-rich lymph nodes\textsuperscript{208,324}. Other strategies have included the release of cytokines or chemokines that can attract DCs and initiate an immune response\textsuperscript{330,331}. A novel strategy has been to conjugate molecules (i.e., mAbs, peptides, sugar), known as active targets, to the particle surface that will interact with specific receptors on DCs. One of the first attempts in surface modification of polymeric particles was the attachment of poly(ethylene glycol) (PEG) chains to the surface of micro and nanoparticles\textsuperscript{92,93}. The reason behind the inclusion of PEG on particle surface is that the hydrophilic nature of this polymer reduces interactions between the polymer device and blood components (i.e., proteins) increasing its biocompatibility\textsuperscript{124}.
Figure 2.15 shows the possible uptake mechanisms of particulate devices by DCs and their relation with particle size was discussed in the previous section. There is a third mechanism (i.e., receptor-mediated endocytosis) that is also used for pathogen uptake and can be targeted for micro and nanoparticle uptake. Receptor-mediated endocytosis can be achieved by targeting specific receptors on DCs. As mentioned before, a variety of TLRs and CLR s and their functions have been identified \(^{137, 228, 235}\) (Tables 2.2 and 2.3) and many of them are involved in antigen uptake and presentation (i.e., receptor-mediated endocytosis, and DC maturation) and can, therefore, be used as targets to enhance and direct the resulted immune response. Some biomaterials used for vaccine delivery exhibit adjuvant properties that can help DC maturation. For example, PLGA \(^{332-335}\) and polyanhydride \(^{129, 189}\) micro and nanoparticles have shown to help DC maturation by the upregulation of maturation cell surface markers (i.e., CD40, CD86, CD80, MHC II). A second attempt to improve antigen uptake and presentation (including DC maturation) is the functionalization of the particle surface for targeting purposes.
Figure 2.15 DC-particle interactions for antigen uptake and processing: particle internalization through phagocytosis or micropinocytosis (size-dependent), particle internalization through receptor-mediated endocytosis (interaction of functionalized particles and pattern recognition receptors (e.g., C-type lectin receptors), conjugation of molecular danger signals to induce DC maturation (interaction of functionalized particles and pattern recognition receptors (e.g., Toll-like receptors). Particle uptake and internalization end in DC maturation and migration as well as antigen specific T cell activation.

The synthesis of functionalized polymeric micro and nanoparticles is a relatively new area and it has been mainly focused on the physical (i.e., adsorption) and chemical (i.e., chemical linking) of mAbs specific to DCs receptors. CLRs (i.e., DEC-205,
and the mannose receptor (MMR) have been targeted using functionalized particles that include anti-DEC-205 \(^{261}\) or anti-CD206 \(^{336-338, 341}\) on their surfaces. CLRs have carbohydrate recognition domains, and therefore, can be also target sugar residues. Copland et al. \(^{338}\) showed that DCs can internalize mannosylated liposomes at a higher degree than free antigen or neutral liposomes resulting in a more efficacy presentation to T cells \(^{338}\). Jiang et al. \(^{341}\) performed \textit{in vitro} and \textit{in vivo} studies that showed the potential of mannosylated chitosan microparticles to target the mannose receptor in alveolar macrophages, and these results are important for intranasal administration \(^{341}\). Sugar-based functional micro and nanoparticles have been rarely explored using synthetic polymers because of the difficulty of solid-phase coupling reactions involving sugars. In one example, polyesters and poly(alkylcyanoacrylate) (PACA) nanoparticles decorated with polysaccharides (i.e., dextran, and chitosan) were fabricated, but the targeting properties of these particles were not tested \(^{342}\).

Another novel and rarely applied strategy for the active targeting of immune cells is the chemical inclusion of synthetic peptides on the surface of micro and nanoparticles. Townsenda et al. \(^{343}\) showed the \textit{in vitro} efficacy of nanoparticles based on a novel PLGA-PEG-biotin polymer to which tetanus toxin C fragment (TTC) was surface-conjugated, to selectively target neuroblastoma cells \(^{343}\).

Functionalized particles based on polymeric biomaterials present an intriguing opportunity to improve the design of targeted vaccine platforms.
2.5 *Yersinia Pestis*: An Overview

2.5.1 Introduction and Facts about *Y. Pestis*

*Y. pestis* is the etiological agent of bubonic and pneumonic plagues to which an estimated 200 million human deaths are attributable. History has recorded three plague pandemics. Although some of the numerous references to plagues in ancient texts may be attributable to *Y. pestis*, the cycles of plague epidemics between 541 and 750 A.D. are called the first pandemic. The first plague, the Justinian plague, began in Egypt in 541 A.D. and quickly spread throughout Mediterranean Europe and the Middle East over the next 3 years. From the 8th to the 14th centuries, Europe appeared to have escaped most epidemic diseases, and it experienced a population increase. Plague spread from 1330 to 1346, probably from the steppes of central Asia along trade routes, and its introduction into Sicily in 1347 heralded the start of the second pandemic, which again encompassed the “known world.” The second pandemic is believed to have accelerated and in some instances directed great changes in economic, societal, political, religious, and medical systems and convictions. The third pandemic started in 1855 in the Chinese province of Yunnan; troop traffic from the war in that area caused rapid spread of the disease to the southern coast of China. It was during the last period of the third pandemic when the causative agent of the pandemic, *Y. pestis*, was identified by Alexander Yersin. By 1900, plague had spread throughout the world including the United States. The 1994 outbreak of two plague epidemics in western India was the latest demonstration that plague is not an eradicated disease. This disease, with reservoirs on nearly every major continent, exhibits an impressive ability to overcome mammalian host defenses.
After the last endemic events, considerable amount of research has been focused on the development of effective treatments to avoid the spread of *Y. pestis*. Even when antibiotics (e.g., Ciprofloxacin) can prevent mortality, they cannot avoid the spread of the bacteria and should be administered within 24 hours in order to be effective \(^{350}\).

Thus, new therapeutic and prophylactic strategies are necessary to correctly fight this disease. This becomes especially important given the bacteria’s potential as a bioterrorism agent. The ability of *Y. pestis* to be genetically modified to overcome vaccination may be used for the creation of bacteria-based terrorism weapons. Therefore, it is necessary to design adequate technology platforms (i.e., recombinant antigens, vaccine delivery vehicles) for the generation of effective and robust vaccines that allow for a quick reaction against possible plague spreads.

### 2.5.2 Bubonic, Systemic and Pneumonic Plague

Through the start of the third pandemic, transmission from urban rodents (especially rats) was the norm \(^{351, 352}\). In the United States, transmission to humans occurs primarily via the bites of fleas from infected rodents \(^{353, 354}\). Most human cases present three different forms: bubonic, systemic or pneumonic plague. Over the past 25 years, the majority of patients in the United States have had either bubonic or septicemic plague \(^{355}\). Case fatalities for untreated bubonic plague range from 40 to 60%, while untreated septicemic and pneumonic forms of the disease are invariably fatal \(^{355}\).

Bubonic plague is the classic form of the disease. Patients usually develop symptoms of fever, headache, chills, and swollen, extremely tender lymph nodes (buboes) within 2 to 6 days of contact with the organism either by flea bite or by
exposure of open wounds to infected materials\textsuperscript{346}. Bacteremia or secondary plague septicemia is frequently seen in patients with bubonic plague causing 50\% of the deaths.

Clinically, plague septicemia resembles septicemias caused by other gram-negative bacteria. The mortality rate for people with septicemic plague is fairly high, ranging from 30 to 50\%\textsuperscript{346}.

Primary pneumonic plague is a rare but deadly form of the disease that is spread via respiratory droplets through close contact (i.e., 2 to 5 ft) with an infected individual. It progresses rapidly from a febrile flu-like illness to an overwhelming pneumonia with coughing and the production of bloody sputum\textsuperscript{355, 356}. The incubation period for primary pneumonic plague is between 1 and 3 days. In general, patients who develop secondary plague pneumonia have a high fatality rate.

2.5.3 Vaccines against \textit{Y. Pestis}

As with many other diseases, the first plague vaccine consisted of the killed infection agent (i.e., \textit{Y. pestis})\textsuperscript{357} and it was shown to provide immunity to bubonic plague, but, as expected, it caused severe side effects that made this vaccine unsuitable for human use. Another disadvantage of the killed-vaccine is that it did not provide protection against pneumonic plague\textsuperscript{358, 359}.

In recent years, plague vaccine design, like that for many other diseases, has focused on the use of recombinant proteins such as F1 and V\textsuperscript{360-363}, which are found on the surface of the \textit{Y. pestis} bacterium. Previous work demonstrated that the full length F1-V fusion protein offers protection in several animal models\textsuperscript{345, 363, 364}. Powell \textit{et al.}\textsuperscript{345}
proposed a subunit vaccine in which the active component included an improved combination of the F1 capsular protein and V virulence antigen of *Y. pestis*. This antigen provided protection to wild-type mice and is now commercially available.\(^3\) Vaccination with F1-V monomer and its multimeric forms resulted in protection in mice after subcutaneous administration as shown by Goodin *et al.*\(^3\) In another study, it was shown that the same full length F1-V antigen provides protection in murine models, especially when different prime and boost routes are used; this study also highlights the importance of the inclusion of an appropriate adjuvant for immunization.\(^3\) Tripathi *et al.*\(^3\) developed a peptide-based vaccine, based upon the constructs between B and T cell epitopes of the F1 antigen of *Y. pestis*. In their work, a new F1-V construct, F1B2T1-V10, was developed and it consists of three F1 immunogenic epitopes (B1, B2 and T) and the V protein without the interleukin-10 inducing sequence (-V10). This recombinant antigen induced both cell-mediated and humoral immunity. The efficacy of these antigens could be enhanced by using vaccine adjuvants (i.e., polymer particles).

### 2.6 Conclusions

Micro and nanoparticles based on polymeric materials could be used as vaccine delivery vehicles. Both micro and nanoparticles offer different opportunities based on their interaction with the immune system that is triggered by physical and chemical properties like polymer chemistry, size, and surface charge. Different natural and synthetic polymers can be used for particle fabrication; however, the unique properties of polyanhydrides (i.e., surface erosion, amphiphilicity, controlled release kinetics) make them excellent candidates for vaccine adjuvants. A direct target of these novel delivery
carriers to immune cells (i.e., dendritic cells) responsible for the initiation of an immune response can be achieved by the functionalization of the surface of the particle. These strategies can be combined and optimized for the design of novel vaccine platforms to convey protection against current and emerging diseases.

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CHAPTER 3

Research Objectives

The overall goal of this research is to design innovative platforms for targeted vaccine delivery based on polyanhydride particles that can result in efficacious immune responses. In order to achieve this goal, a transdisciplinary approach was adopted by combining concepts from biomaterials, nanotechnology, carbohydrate and protein chemistry, molecular biology, immunology, and computational analysis. This rational pathway to the design of vaccine delivery platforms combines passive and active targeting strategies. The specific goals (SGs) of this research are:

**SG1:** Evaluation of stabilization and release mechanisms of protein antigens from polyanhydride particles.

**SG2:** Determination of serum protein adsorption patterns on polyanhydride particles and their effect on *in vitro* interactions with antigen presenting cells.

**SG3:** Design and characterization of novel targeted vaccine delivery vehicles based on carbohydrate-functionalized polyanhydride particles and mechanistic evaluation of their interactions with antigen presenting cells.

**SG4:** *In vivo* evaluation of immune responses induced by loading novel galactose-containing carbohydrate modified F1-V antigen within polyanhydride particles to rationally design a protective vaccine against *Y. pestis*. 
Passive strategies evaluated in this work involved the selection of appropriate polyanhydride chemistries for antigen stability, which is an important determinant of an effective immune response. To this end, a systematic assessment of antigen stability after release from polyanhydride particles was evaluated for several protein antigens including antigens candidates for *Yersinia pestis* (Chapter 4) and monoclonal antibodies with potential to be used in passive immunotherapy (Chapter 5). Additionally, serum protein adsorption patterns on polyanhydride particles were determined was evaluated (Chapter 6) and extended to study the effect of the chemistry-dependent adsorption of serum proteins on the uptake and activation of antigen presenting cells (Chapter 7).

The knowledge obtained by the experiments designed to understand the role of particle chemistry on the adjuvant properties of polyanhydride particles and their interactions with antigens and immune system components was translated to generate novel active targeting strategies based on chemical modification of both the antigens and the vaccine carriers. Novel approaches to target specific receptors on antigen presenting cells and/or specific uptake pathways were developed and evaluated. First, polyanhydride particles were functionalized with specific carbohydrates and their targeting capabilities were evaluated by activation studies of antigen presenting cells (Chapter 8). In this work, we also studied the mechanisms dictating the interaction of these novel materials with immune cells. A second active targeting approach was pursued by modifying a vaccine antigen (i.e., F1-V) attaching novel galactose-containing carbohydrate (αGal) epitopes in Chapter 9. This technology was combined with the use of amphiphilic polyanhydride particles as a delivery system and evaluated in murine models for its capacity to induce robust immune responses.
Taken together, these approaches provide key insights into the rational design of targeted platforms that will enhance the induction of antigen-specific immune responses and facilitate the design of protective vaccines and therapeutic treatments.
CHAPTER 4

Encapsulation into Amphiphilic Polyanhydride Microparticles Stabilizes *Yersinia pestis* Antigens

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4.1 Abstract

The design of biodegradable polymeric delivery systems based on polyanhydrides that would provide for improved structural integrity of *Yersinia pestis* antigens was the main goal of this study. Accordingly, the full length *Y. pestis* fusion protein (F1-V) or a recombinant *Y. pestis* fusion protein (F1\textsubscript{B2T1}-V10) were encapsulated and released from microparticles based on 1,6-bis(p-carboxyphenoxy)hexane (CPH) and sebacic acid (SA) copolymers and 1,8-bis(p-carboxyphenoxy)-3,6-dioxaoctane (CPTEG) and CPH copolymers fabricated by cryogenic atomization. An enzyme-linked immunosorbent assay was used to measure changes in the antigenicity of the released proteins. The recombinant F1\textsubscript{B2T1}-V10 was unstable upon release from the hydrophobic CPH:SA microparticles, but maintained its structure and antigenicity in the amphiphilic CPTEG:CPH system. The full length F1-V was stably released by both CPH:SA and CPTEG:CPH microparticles. In order to determine the effect of the anhydride monomers on the protein structure, changes in the primary, secondary, and tertiary structure, as well as the antigenicity of both *Y. pestis* antigens were measured after incubation in the presence of saturated solutions of SA, CPH, and CPTEG anhydride monomers. The results indicated that the amphiphilic environment provided by the CPTEG monomer was important to preserve the structure and antigenicity of both proteins. These studies offer an approach by which a thorough understanding of the mechanisms governing antigenic instability can be elucidated in order to optimize the *in vivo* performance of biodegradable delivery devices as protein carriers and/or vaccine adjuvants.
4.2 Introduction

According to the American Association of Pharmaceutical Researchers and Manufacturers, approximately 80% of the biopharmaceutical products in development are protein-based. Many current efforts in biotechnology are focused on the creation of vaccines based on peptide or protein subunits and the design of new and effective delivery vehicles is a key objective in this area. One of the most challenging tasks in the development of protein pharmaceuticals is to deal with the physical and the chemical instabilities of proteins; these changes can be induced by factors such as temperature and pH. The fragile three-dimensional structure of a protein must be maintained for biological activity and this could be lost due to physical as well as chemical instability. In order to avoid these problems, it is necessary to design vehicles that will minimize protein degradation, maximize its in vivo immunogenicity, and provide controlled release of the encapsulated protein.

It is well known that the mechanisms affecting protein stability are protein-specific; therefore, it is necessary to elucidate principles that will rationally select polymer formulations to stabilize the specific protein of interest. Preservation of protein structure is important in order to generate effective immune responses when recombinant or purified proteins are used in vaccines. Conformational stability of the immunogen is a key factor in maintaining the ability to induce T and B cell responses. Instability of antigenic epitopes often favors induction of Th2 and allergic immune responses because of the antigenic low dose resulting from the degradation. Thus, immunogens exposed to aqueous environments with a low pH may lose key elements of tertiary, secondary, or even primary structure that may affect the type of immune
response induced following vaccination. For the development of efficacious vaccines, it is desirable to use protein antigens containing multiple immunogenic epitopes as opposed to peptide antigens containing a single epitope \(^{12}\). This suggests that antigen stability is a critical factor in the development of subunit vaccines. Indeed, antigen stability influences the outcome of antigen processing and presentation by antigen presenting cells (APCs) that govern the outcome of the resultant immune response \(^{10, 11}\).

Polymers have been well-studied as matrices for drug delivery systems in the pharmaceutical industry \(^{13}\), and encapsulating proteins into polymer microparticles is one of the most commonly used methods for parenteral delivery \(^{14}\). The most extensively investigated biodegradable polymers are polyesters such as poly(glycolic acid), poly(lactic acid) and their copolymers. These polymers undergo bulk erosion which can result in aggregation of proteins \(^{14}\). In addition, this class of polymers creates an acidic pH microenvironment during degradation that may lead to chemical instability of proteins \(^{14-18}\).

Polyanhydrides are surface-erodible polymers and provide advantages that enhance protein stability and controlled release. However, these materials are hydrophobic which can lead to protein instability \(^{19, 20}\). A well-studied aliphatic-aromatic copolymer system for protein delivery is based on copolymers of sebacic acid (SA) and 1,3-bis(\(\rho\)-carboxyphenoxy)hexane (CPH) (Figure 4.1) \(^{19, 21}\). In order to prevent deleterious protein-polymer interactions, the ideal protein delivery device must be amphiphilic. One way to achieve this is by introducing oligomeric ethylene glycol units into the backbone of an aromatic polyanhydride (i.e., CPH) \(^{22, 23}\). In previous work, a novel amphiphilic polyanhydride system based on CPH and 1,8-bis(\(\rho\)-carboxyphenoxy)-3,6-dioxaoctane
(CPTEG) monomers (Figure 4.1) was shown to provide a more favorable environment for encapsulated proteins. Another advantage of these polyanhydride systems (i.e., CPH:SA and CPTEG:CPH) is that by changing the ratio of the monomers during fabrication, the degradation rate of the copolymers can be extended from days to months. Previous studies evaluated the effects of polyanhydride degradation products on several proteins. Determan et al. demonstrated the stability of tetanus toxoid (TT), ovalbumin (Ova), and lysozyme when incubated with anhydride (i.e., CPH and SA) monomers. The stability of Ova and bovine serum albumin (BSA) after encapsulation and release from CPH:SA microparticles was also shown by Determan et al. Torres et al. studied the stability of lysozyme and Ova in the presence of degradation products of the CPTEG:CPH system and found that the CPTEG:CPH system preserves the structural integrity of these two proteins.

**Figure 4.1** Chemical structures of (from top to bottom) poly(SA), poly(CPH), and poly(CPTEG).
The work outlined in this study demonstrated the preservation of the structure and antigenicity after release from polyanhydride microparticles of two candidates that may be used as antigens for *Yersinia pestis*. *Y. pestis* is the etiological agent of bubonic and pneumonic plagues to which an estimated 200 million human deaths are attributable. In recent years, plague vaccine design, like that for many other diseases, has focused on the use of recombinant proteins such as F1 and V, which are found on the surface of the *Y. pestis* bacterium. Previous work demonstrated that the full length F1-V fusion protein offers protection in several animal models. Powell et al. proposed a subunit vaccine in which the active component included an improved combination of the F1 capsular protein and V virulence antigen of *Y. pestis*. This antigen provided protection to wild-type mice and is now commercially available through BEI Resources. Vaccination with F1-V monomer and its multimeric forms resulted in protection in mice after subcutaneous administration as shown by Goodin et al. In another study, it was shown that the same full length F1-V antigen provides protection in murine models, especially when different prime and boost routes are used; this study also highlights the importance of the inclusion of an appropriate adjuvant for immunization. Tripathi et al. developed a peptide-based vaccine, based upon the constructs between B and T cell epitopes of the F1 antigen of *Y. pestis*. In their work, a new F1-V construct, F1B2T1-V10, was developed and it consists of three F1 immunogenic epitopes (B1, B2 and T) and the V protein without the interleukin-10 inducing sequence (-V10). This recombinant antigen induced both cell-mediated and humoral immunity. The efficacy of these antigens could be enhanced by using vaccine adjuvants (i.e., polyanhydride microparticles), but an important consideration is that the
adjuvants have to prolong the antigen release and stabilize them. This study compares the stabilization of F1B2T1-V10 and full length F1-V antigens using different polyanhydride microparticle adjuvants.

4.3 Materials and Methods

4.3.1 Materials

Chemicals needed for monomer synthesis and polymerization, sebacic acid (99%), p-carboxy benzoic acid (99+%), and 1-methyl-2-pyrrolidinone anhydrous (99+%), were purchased from Aldrich (Milwaukee, WI); 4-p-hydroxybenzoic acid, 1,6-dibromohexane, 1-methyl-2 pyrrolidone, and tri-ethylene glycol were purchased from Sigma Aldrich (St. Louis, MO); 4-p-fluorobenzonitrile was obtained from Apollo Scientific (Cheshire, UK); potassium carbonate, dimethyl formamide, toluene, sulfuric acid, acetic acid, acetonitrile, acetic anhydride, methylene chloride, and petroleum ether were purchased from Fisher Scientific (Fairlawn, NJ). F1-V fusion protein and monoclonal anti-Y. pestis LcrV protein were purchased from Biodefense and Emerging Infections Research Resources Repository (Manassas, VA). Polyclonal goat anti-LcrV antisera was obtained from Dr. John Clements from Tulane University, and donkey anti-goat IgG-alkaline phosphatase (AP) conjugated was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Goat anti-mouse IgG (H+L)-AP was purchased from Jackson ImmunoResearch (West Grove, PA). Phosphatase substrate was purchased from Aldrich (St. Louis, MO). Polyacrylamide 4-20% Tris-HCl pre-cast gradient gels, unstained protein standards, pre-stained broad range molecular weight standards, and Flamingo Gel Stain were purchased from BioRad Laboratories (Richmon, CA).
Bicinchoninic acid (BCA) and Micro BCA protein assay kits and Slide-A-Lyzer dialysis cassettes (10,000 MW cut-off membranes) were obtained from Pierce Biotechnology Inc. (Rockford, IL).

4.3.2 F1_{B2T1}-V10 Protein Construction and Purification

4.3.2.1 Plasmid Construction

To express the recombinant F1_{B2T1}-V10 protein, a gene construct was first synthesized (Integrated DNA Technologies, Coralville, IA) and then cloned into the expression vector pDEST17 (Invitrogen, Carlsbad, CA). The synthetic gene was designed to encode a product with an amino terminal region comprised of 3 distinct immune-stimulating domains/epitopes of the F1 capsular protein. Specifically, these regions comprised amino acids 32-50 and 53-76 of the full-length F1 antigen representing B cell epitopes, and amino acid residues 79-93, a T cell epitope. The fusion of these three F1 regions was designated as F1_{B2T1}. These epitopes were shown to elicit a humoral immune response and protect mice against Y. pestis challenge.

The carboxy-terminal region of the fusion construct (amino acids 96-390 of the recombinant antigen) represents a modified version of LcrV lacking a domain (residues 271-300 of Y. pestis LcrV) shown to trigger induction of interleukin 10 by host immune cells and was designated as V10.

To enable purification of F1_{B2T1}-V10, the synthetic gene also includes bases that encode an enterokinase cleavage site near the amino-terminus (amino acids 25-29) of the recombinant gene product. To maximize expression, the nucleotide sequence of the F1_{B2T1}-V10 coding region was also modified so the codon usage of the resulting
transcript matches that of highly expressed genes in *E. coli*. Finally, the synthetic gene also includes *attB1* (5'-end) and *attB2* (3'-end) sequences that enabled use of the Gateway cloning system (Invitrogen, Carlsbad, CA) for insertion into pDEST17, yielding pISM51. The final construct expresses a 390 amino acid product that includes an amino-terminal hexa-histidine epitope tag (amino acids 5-10) to simplify protein purification. F1* <sub>B2T1</sub>-V10 is expressed from pISM51 by the T7 promoter.

Purification of F1* <sub>B2T1</sub>-V10 protein was followed by western blot analysis using anti-V antigen antisera. Approximately 10 µg of unpurified induced or uninduced whole cell lysate and 200 ng of purified unmodified fusion F1-V protein were loaded on a 10% Criterion Tris-HCl gel (Bio-Rad), resolved by electrophoresis and blotted to nitrocellulose. The immunoblot was developed with anti-V antiserum (1:10,000) and alkaline phosphatase-labeled donkey anti-goat conjugate antiserum (1:50,000).

### 4.3.2.2 Protein Purification of F1* <sub>B2T1</sub>-V10

*E. coli* BL21 (*E. coli* B F− *dcm ompT hsdS(rB−, mB−) gal l(DE3)) containing plasmid pISM51 was grown overnight at 37°C in Luria-Bertani broth containing 100 µg/mL ampicillin, diluted 1:100 in the same media and then induced with 1 mM isopropyl β-D-thiogalactopyranoside for 4 h. Cell pellets were obtained by centrifugation (6,000 x g, 30 min) and washed once with phosphate buffered saline (0.05 M, pH 7.2) by centrifugation. The final cell pellet from a 2 L culture was resuspended in 50 mL of lysis buffer (20 mM Tris, 100 mM sodium chloride, 20 mM imidazole, 8 M urea, pH 8.0), passed through three cycles of freeze-thaw, and then cellular debris was removed by centrifugation at 12,000 x g for 10 min followed by filtration through a 0.45 µm filter.
A BioLogic Protein Purification system (Bio-Rad Laboratories, Hercules, CA) was used for recombinant F1\textsubscript{B2T1}-V10 protein purification. Fifty mL of induced cell lysate was passed through a 1.2 x 20 cm column packed with 5 mL of Proaffinity IMAC nickel chromatography resin (Bio-Rad). The column was then washed with 10 column volumes of binding buffer (50 mM sodium phosphate, 300 mM sodium chloride, 10 mM imidazole, 8 M urea, pH 8.0) until baseline was achieved. The protein was eluted with a linear gradient of 20 – 500 mM imidazole in binding buffer. Aliquots of 5 mL fractions were analyzed by western blot using anti-Yersinia V antigen or anti-6xHis monoclonal (Clontech Laboratories, Inc., Palo Alto, CA) with its appropriate conjugant [alkaline phosphatase labeled donkey anti-goat (Santa Cruz Biotechnology, Santa Cruz, CA) or alkaline phosphatase labeled goat anti-mouse Ig (H+L) (Southern Biotechnologies, Birmingham, AL).

Recombinant protein containing fractions were combined, and endotoxin was removed using ActiClean Etox (Sterogene Bioseparations, Inc., Carlsbad, CA). The combined fractions from one chromatography run were passed through 5 mL of resin and then dialyzed against water using 7000 Da MW cutoff dialysis tubing (Pierce Chemical Co., Rockford, IL). Endotoxin levels were determined using a chromogenic QCL-1000 Limulus Amebocyte Lysate assay (Lonza Group Ltd, Basel, Switzerland) and protein was considered “endotoxin-free” if contamination was below 50 ng of contaminants per mg of protein (as determined by a Bicinchoninic Assay [Thermo Scientific, Waltham, MA] against a BSA standard). If contaminants were found to be above this level, the protein solution was run over a Detoxi-Gel endotoxin removal column three times (Thermo Scientific) and the LAL assay was re-run. This procedure
was repeated until contamination fell below the designated acceptable level. The protein yield after endotoxin removal was 74%.

4.3.3 Polymer Synthesis and Characterization

Diacids of CPH and CPTEG were synthesized as described previously. SA and CPH prepolymers were synthesized by the methods described by Shen et al. and Conix et al. Subsequently, 20:80 CPH:SA and 50:50 CPTEG:CPH copolymers were synthesized by melt polycondensation as respectively described by Kipper et al. and Torres et al. H nuclear magnetic resonance (NMR) spectroscopy on a Varian VXR 300 MHz spectrometer (Varian Inc., Palo Alto, CA) was used to characterize the polymer structure. NMR spectra were consistent with previously published data and confirmed the synthesis of the desired copolymer compositions. In addition, polymer molecular weight was determined utilizing gel permeation chromatography (Waters HPLC System, Milford, MA using Varian Inc. GPC columns). The 20:80 CPH:SA copolymer had a M\(_w\) of 21000 g/mol and polydispersity index (PDI) of 2.1, while the M\(_w\) and PDI of the 50:50 CPTEG:CPH copolymer were 9000 g/mol and 1.8, respectively. These values are consistent with previous published data.

4.3.4 Microparticle Fabrication

Encapsulation of F1-V and F1\(_{B2T1}\)-V10 in 20:80 CPH:SA and 50:50 CPTEG:CPH microparticles was performed by cryogenic atomization using parameters specified by Torres et al. and Lopac et al. Briefly, 100 mg of each copolymer composition was dissolved in methylene chloride and 2 mg of lyophilized F1-V or F1\(_{B2T1}\)-V10 was
suspended in this solution using a Tissue-Tearor™ (10,000 rpm, 1 min). The solution was sprayed with a syringe pump through an 8700-1200 MS ultrasonic atomizing nozzle (SonoTek Corporation, Milton, NY) into 200 mL of frozen ethanol (with an excess of liquid nitrogen). The procedure was carried out at 4°C for the CPTEG:CPH composition and at room temperature for 20:80 CPH:SA. The resulting solution was stored at -80°C for three days. For CPTEG-based compositions, after the first 24 h, ~200 mL of cold ethanol was added to avoid aggregation; the solutions were stirred at 300 rpm for 15 min and placed back in the freezer at – 80°C. After this period, microparticles were collected by vacuum filtration and dried overnight at vacuum conditions. This procedure yields at least 70% recovery. The microparticle morphology was analyzed by scanning electron microscopy (SEM) (JEOL 840 A, JEOL Peabody, MA) using four replicates per sample. The particle size distribution was obtained from SEM images using ImageJ image analysis software (National Institutes of Health, Bethesda, MD). An average of 200 particles per image was analyzed.

4.3.5 Protein Release Studies

*In vitro* release kinetics of the two antigens were measured by suspending fifteen mg of the protein-loaded microparticles in 1 mL of saline phosphate buffer (0.1 M, pH 7.4) with 0.01% w/v of sodium azide, and incubated at 37°C and 100 rpm. Aliquots of 750 µL were taken at different time intervals and replaced with fresh buffer. The aliquots were stored at 4°C to measure protein concentration using a Micro BCA analysis at an absorbance of 570 nm and gel electrophoresis (SDS-PAGE) under non-reducing conditions. A SDS-PAGE quantification method was performed as previously described.
Equal amounts of the released proteins were mixed with non-reducing buffer (SDS (10% w/v), Tris-HCl (1M, pH 6.8), glycerol (3 mM), bromophenol blue (0.01% w/v)) at a 1:1 volume ratio. The samples were heated at 70°C for 10 min and cooled to room temperature, after which they were loaded onto 4-20% Tris-HCl pre-cast gels and run at 140 V and 4°C for 90 minutes. Five µL of unstained protein standard were used as reference to determine the molecular weight of the samples. The gels were incubated in fixative solution (40% ethanol, 10% acetic acid) at 4°C for 3 h. Next, they were stained with fluorescent flamingo gel stain overnight, and washed with a 0.01% Tween 20 solution to reduce non-specific fluorescence. A Typhoon 8600 (GE Healthcare, Piscataway, NJ) fluorescence scanner was used to obtain the images of the gels and fluorescence intensity was quantified by ImageQuantTL (GE Healthcare, Piscataway, NJ) using a standard curve with corresponding protein concentrations from 250 to 1 ng/mL. At least three replicates of each sample were analyzed. The cumulative release was normalized by the total protein encapsulated that was determined as described by Torres et al. 25

4.3.6 Protein Antigenicity after Release

F1-V and F1227-V10 Specific Enzyme-Linked Immunosorbent Assay (ELISA)

High protein binding 96-well Costar microtiter plates (Corning Life Sciences, Lowell, MA) were coated overnight with 100 µL of phosphate buffer saline (PBS) at a pH of 7.4 containing 5 µg/mL of released protein. Samples collected after release from 20:80 CPH:SA and 50:50 CPTEG:CPH were analyzed in triplicate. In some cases, Vivaspin 500 centrifugal concentrators (Sartorius Stedim Biotech, Aubagne, France) were used to
concentrate the released samples. PBS containing 0.05% Tween 20 (PBS-T) and 2% (w/v) gelatin as a non-specific blocking agent (BD Biosciences, San Jose, CA) was used to block plates for at least 2 h at room temperature. After the blocking period, plates were heated at 60 °C for 10 min in a dry oven to melt gelatin and then, they were rinsed three times to remove any unbound blocking reagent. Two sets of ELISAs were performed by using monoclonal anti-\textit{Y. pestis} LcrV protein and hyperimmunized serum, which was obtained from repeated injections of F1-V fusion protein to adult female C57BL/6 mice. Plates were incubated with primary antibodies in PBS-T with 1% (v/v) goat serum (1:10000) overnight at 4 °C. PBS-T was used to wash plates three times followed by the addition of 100 μL of PBS-T (1% (v/v) goat serum) containing AP-conjugated goat anti-mouse IgG(H&L) antiserum at a 1:1000 dilution. After 2 h of incubation at room temperature, the plates were washed four times with PBS-T and then, 100 μL of sodium carbonate (50 mM) and magnesium chloride (2mM) buffer (pH 9.3) containing 1 mg/mL phosphatase substrate was added. Plates reacted from 20 to 40 min at room temperature. Optical density (OD) of each well was measured at 405 nm using a Spectramax 190 Plate Reader (Molecular Devices, Sunnyvale, CA). The results were normalized with the data for the protein (only) solution in PBS and reported as relative epitope availability.

4.3.7 Protein Incubation in Saturated Solutions of Monomers

Saturated solutions of the SA, CPH, and CPTEG diacids were obtained by adding an excess of each diacid in deionized water and phosphate buffer. All the data presented in the Results and Discussion section is based on the use of PBS, except for the
secondary structure data from circular dichroism, which is based on deionized water due to the high background absorbance of the PBS. The solutions were filtered with 0.22 µm syringe filter after incubation for 48 h (37°C and 100 rpm). The pH of the SA, CPH, and CPTEG monomer saturated solutions was 4.2, 5.5, and 6.5, respectively. Stock solutions of F1-V fusion protein and recombinant F1B2T1-V1 were made in phosphate buffer (0.1 M, pH 7.4) and deionized water at a concentration of 1 mg/mL. The final solutions were obtained by adding the protein solution to the diacid solution at a volume ratio of 15:85 to achieve a final protein concentration of 150 µg/mL. These samples were incubated for 0 and 7 days at 37°C and shaken at 100 rpm for stability analysis. All experiments were performed in triplicate.

4.3.8 Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE):

Primary Structure

The primary structure of each protein was evaluated using SDS-PAGE. Separate samples that had been incubated for 0 or 7 days were centrifuged (10,000 rpm, 10 min) and 25 µL of each sample was mixed with equal volumes of non-reducing (SDS (10% w/v), Tris-HCl (1M, pH 6.8), glycerol (3 mM), bromophenol blue (0.01% w/v)) and reducing sample buffers (SDS (10% w/v), Tris-HCl (1M, pH 6.8), glycerol (3 mM), bromophenol blue (0.01% w/v), and β-mercaptoethanol (0.05% v/v)). Samples were mixed and heated at 95 °C for 5 min. The next steps of the SDS-PAGE protocol experiments were previously described in the Protein Release section.
4.3.9 Circular Dichroism (CD): Secondary Structure

The secondary structure of the antigens was monitored after 0 and 7 days of incubation by far UV CD at a wavelength range of 190-260 nm. Samples in water were used to avoid high absorbance. The spectra were collected using Jasco J-170 software (Easton, MD). The spectra of the monomer solutions were subtracted from the protein spectra. The spectra shown are the average of three experiments.

4.3.10 Hydrogen-Deuterium Exchange Monitored by Fourier-Transform Infrared (FTIR) Spectroscopy: Tertiary Structure

The F1B2T1-V10 protein is a small molecular mass protein composed of 390 amino acids of which only one is a tryptophan and only thirteen are tyrosine. The low content of these two amino acids makes it difficult to study the tertiary structure of this protein using spectroscopic techniques (i.e., fluorescence spectroscopy). ¹H/²H exchange kinetics allows the characterization of protein stability in different experimental conditions. FTIR spectra of proteins in the presence of deuterium are used for estimation of slowly and rapidly exchanging amide protons and information about hydrogen bond stability of amide protons can be obtained from these exchange rates. Specifically, changes in tertiary structure of proteins can be followed by monitoring changes in the amide II band intensity (1600-1500 cm⁻¹) over time when proteins are exposed to deuterium. This approach was used to determine changes in the tertiary structure of F1-V fusion protein and F1B2T1-V10. After 0 and 7 days of incubation with monomers, 500 µL of each of protein samples were placed in glass vials and an equal volume of deuterium (Sigma Aldrich) was added. Immediately after addition of...
deuterium, the headspace of the glass vials was flushed with argon and all vials were stored in an argon-flushed desiccator. At 0, 1, 2, 3, and 4 h, 80 µL of each sample were removed from vials with a syringe and immediately placed on silica nitride substrates; the samples were dried overnight at room temperature in order to obtain protein films to collect FTIR spectra. Films made from protein only and deuterium solutions were used as controls for each time interval; deuterium spectra were subtracted from sample spectra. Spectra were obtained using an FTIR spectrometer using a Nicolet Nexus 470 (Madison, WI), equipped with a liquid nitrogen cooled MCT/A detector. For each sample, 256 scans were recorded at a resolution of 4 cm\(^{-1}\). Omnic 5.2 software was used to collect the data and to obtain the areas under the amide I (1700-1600 cm\(^{-1}\)) and amide II regions. The amide II region was normalized to the amide I region to eliminate possible fluctuations in intensity \(^{41}\). Amide II/amide I ratios were expressed as fractions of non-exchanged protons and used to follow changes in tertiary structure.

4.3.11 Statistical Analysis

All data was statistically analyzed by using a student’s t-test with the statistical software JMP\textsuperscript{®} 7 (Cary, NC). Comparisons between treatments were made and p-values of less than or equal to 0.05 were considered significant.

4.4 Results and Discussion

Preservation of protein structure, and thus, immunogenicity, is important to be able to induce effective immune responses when proteins are used as vaccine immunogens\(^9\text{-}^{11}\). Previous studies have shown that mechanisms of protein alteration are protein-
specific. In order to design adequate protein carriers for vaccination, it becomes necessary to select polymer formulations that can stabilize the hierarchical structures of specific proteins of interest and maintain their antigenicity. The experiments described in this work were performed in order to determine the effect of polyanhydride chemistry on the stabilization of proteins (i.e., F1-V fusion protein and recombinant F1_{B2T1}-V10) that have the potential to be used in designing vaccines against *Y. pestis*.

**4.4.1 F1_{B2T1}-V10 Protein Construction and Purification**

The F1_{B2T1}-V10 antigen is encoded by a 1,170-bp DNA construct as described above. Of the three immune-stimulating domains from the F1 capsular protein, B1, B2 and T1, B1 and B2 represent B cell stimulating epitopes and T1 is a T cell stimulating epitope. It also includes a coding region of the LcrV antigen, with the immunomodulatory domain encoded by amino acids 133-175 deleted. Following purification of the F1_{B2T1}-V10 protein, it was analyzed by western blot analysis using anti-V antisera (**Figure 4.2**). This Figure also compares the purified F1_{B2T1}-V10 protein with the full length F1-V fusion protein obtained from the Biodefense and Emerging Infections (BEI) Research Resources Repository (catalog number NR-4525, lot number FIN-0229). This Figure depicts the molecular size difference between the two proteins and demonstrates that the selected anti-sera reacted with both proteins.
Figure 4.2 Electrophoretic analysis of F1$_{B2T1}$-V10 and F1-V. Lanes 1 and 4: induced whole cell lysate expressing F1$_{B2T1}$-V10; Lanes 2 and 5: uninduced whole cell lysate control; Lanes 3 and 6: purified F1-V (BEI). Left panel: Coomassie blue stained gel; right panel: immunoblot. The blot was developed with anti-V antiserum and alkaline phosphatase-labeled donkey anti-goat conjugate antiserum.

4.4.2 Microparticle Fabrication and Protein Release

F1-V fusion protein and F1$_{B2T1}$-V10 were encapsulated in 20:80 CPH:SA and 50:50 CPTEG:CPH microparticles in order to study the release kinetics of these two proteins. The specific polymer chemistries were chosen based on previous work suggesting that these chemistries may stabilize globular proteins $^{24,25}$. Microparticles were fabricated using the cryogenic atomization method and visualized by SEM. Figure 4.3 shows electron photomicrographs of F1-V-loaded 20:80 CPH:SA and F1$_{B2T1}$-V10-loaded 50:50 CPTEG:CPH microparticles with morphologies (size and surface topography) that are consistent with previous work on polyanhydride microparticles encapsulating other proteins $^{7,25,38}$. The size of the majority of the microparticles was between 2 and 15 µm or 6 and 10 µm for the 20:80 CPH:SA and 50:50 CPTEG:CPH, respectively.
After encapsulation, the F1-V and F1_{B2T1}-V10 antigens were released from both 20:80 CPH:SA and 50:50 CPTEG:CPH microparticles. The release profiles for both proteins are similar and depend on the microparticle chemistry (Figure 4.4). The profile of proteins released from 20:80 CPH:SA microparticles (Figure 4.4(a)) showed an initial burst release of ~20% with an eventual release of ~75% of the total encapsulated protein after 32 days. These release profiles are consistent with previous work.
evaluating other combinations of proteins and polyanhydride chemistries with similar hydrophobicities. It can be observed from Figure 4.4(b) that the 50:50 CPTEG:CPH microparticles display a faster release of the proteins with more than 90% of the total encapsulated protein released during the first 32 days, which is consistent with previous work on ovalbumin release from 50:50 CPTEG:CPH microparticles.

Figure 4.4 Protein release kinetics from polyanhydride microparticles: (●) Cumulative fraction of F1-V fusion protein, and (■) rF1\textsubscript{B2T1}-V10 released from 20:80 CPH:SA (a) and 50:50 CPTEG:CPH (b) microparticles. Error bars represent standard error of two different experiments run with triplicate samples.
4.4.3 Protein Antigenicity following Release

Initial studies investigated the antigenic stability of the commercially available F1-V fusion protein and the recombinant F1\textsubscript{B2T1}-V10 that had been released during the initial first 72 h of incubation from 20:80 CPH:SA and 50:50 CPTEG:CPH microparticles. Figure 4.5 shows the effect of polymer chemistry on protein antigenicity, as measured by a protein-specific ELISA using monoclonal anti-\textit{Y. pestis} LcrV protein. It can be observed that antigenicity of the recombinant F1\textsubscript{B2T1}-V10 decreased significantly when released from 20:80 CPH:SA microparticles with a greater than 50% reduction in the optical density (OD) value compared with the unencapsulated protein. The antigenicity of the F1-V fusion protein after release from the 20:80 CPH:SA microparticles was statistically indistinguishable from that of the unencapsulated protein. In contrast, both proteins retained their antigenicities when released from 50:50 CPTEG:CPH microparticles. Similar results were obtained from protein-specific ELISA using hyperimmunized serum indicating maintenance of multiple immunological epitopes (data not shown). The deleterious effect of the CPH:SA chemistry on F1\textsubscript{B2T1}-V10 antigenicity may be attributed to several reasons. First, it is known that there is a decrease in the pH when poly(SA) degrades \textsuperscript{14}, exposing proteins to an acidic microenvironment that may be detrimental to protein structure and function \textsuperscript{7,14,38}. Second, of the three polyanhydrides studied, CPH is the most hydrophobic polymer following by SA, which makes 20:80 CPH:SA more hydrophobic than 50:50 CPTEG:CPH \textsuperscript{38}. Hydrophobicity has been shown to induce non-covalent protein aggregation \textsuperscript{7,14}, so this phenomenon may also contribute to the reduction of protein antigenicity. On the other hand, the presence of ethylene glycol in the CPTEG-containing polymers results in an amphiphilic and less acidic microenvironment that has been shown to preserve protein activity \textsuperscript{25,38}. 
When comparing the two antigens, a reduction in antigenicity was observed for $F_{1B2T1-V10}$, suggesting that the omission of specific peptides in this novel protein construct may have resulted in a more fragile protein.

Figure 4.5 Antigenic analysis after encapsulation and release from polyanhydride microparticles. Error bars represent standard deviation of three different experiments with duplicate samples in each. Groups identified with the same letter are not statistically different ($p$-value = 0.3).

4.4.4 Incubation of Antigen with Monomer Solutions

An indirect way to mimic the microenvironment inside polymeric devices during fabrication and release is to study the stability of proteins in the presence of saturated...
monomer solutions. To better understand the contribution from each of the polymer degradation products to the detrimental effect observed on the released antigens, the F1-V fusion protein and the F1$_{B2T1}$-V10 were exposed to the degradation products of SA, CPH, and CPTEG polymers for zero and seven days. The antigenicities of F1-V and F1$_{B2T1}$-V10 were also assessed by ELISA and using a monoclonal anti-Y. pestis LcrV and the results are shown in Figure 4.6. Similar ELISA results were obtained using hyperimmunized serum to evaluate protein antigenicity (data not shown). For the F1-V fusion protein, a statistically significant decrease in its antigenicity was observed in the presence of the SA monomer whereas for the F1$_{B2T1}$-V10 protein there was a loss in antigenicity (characterized by a 60-80% reduction in the OD values) when incubated in the presence of either SA or CPH monomer solutions. These results are consistent with the argument that acidity has a more significant impact on the antigenicity of both Y. pestis antigens, and in addition, there is a further detrimental effect on the antigenicity of F1$_{B2T1}$-V10 due to hydrophobic interactions. Collectively, the data indicates that the presence of the CPTEG monomer preserves antigenicity of fragile antigens. A detailed analysis of protein structure alterations was next performed to provide a more detailed evaluation of protein structure as it related to loss of antigenicity and to identify the structural levels at which the changes are occurring.
Figure 4.6 Antigenic analysis after 0 and 7 days of incubation at 37°C of: (a) F1-V fusion protein and (b) F1<sub>B2T1</sub>-V10 protein with polyanhydride degradation products. Error bars represent standard deviation of three different experiments with duplicate samples in each. Groups identified with the same letter are not statistically different (p-value > 0.05).

The primary structure of F1-V (MW ~53 kDa) and F1<sub>B2T1</sub>-V10 (MW ~37 kDa) incubated with the polyanhydride degradation products was analyzed by SDS-PAGE under reducing conditions (Figure 4.7). In the top panel, a dominant band at 53 kDa is
observed and this characteristic band was consistently observed after seven days of incubation in saturated monomer solutions indicating that no degradative changes in the primary structure of the F1-V fusion protein occurred nor is there any evidence of increased protein aggregation. The bottom panel of Figure 4.7 shows the SDS-PAGE results for F1_{B2T1}-V10. At day 0, similar banding patterns are observed for protein samples taken from PBS and all the anhydride monomer solutions as evidenced by the presence of the intact protein at ~37 kDa and the similar banding pattern for the less intense bands. After seven days of incubation in the CPH monomer solution, however, much of the parent protein was altered or degraded based on a less intense band at 37 kDa, while a more intense band around 150 kDa is detectable. The ability to detect a higher molecular weight F1_{B2T1}-V10 polypeptide band is likely a consequence of protein aggregation by non-covalent interactions, which may be attributed to the hydrophobicity of the CPH monomer \(^{14, 25}\). When incubated with SA, a less intense dominant band (~37 kDa) was observed, but other bands with similar intensities were also present at lower molecular weights (e.g., around 37, 25, and 15 kDa). Keeping in mind that the same amount of protein was loaded in each lane of the gel, the presence of lower molecular mass bands may be indicative of protein hydrolysis \(^{14}\). This may be a consequence of the acidic microenvironment produced by the SA monomer. Consistent with the antigenicity studies, no changes occurred in the protein primary structure when incubated with CPTEG (Fig. 7, lanes 3 and 4). These changes in primary structure in the F1_{B2T1}-V10 incubated in SA monomer solutions indicate that the loss in antigenicity may be a direct consequence of primary structure changes associated with protein degradation.
Figure 4.7 SDS-PAGE under reducing conditions results for F1-V (top) and F1_{B2T1-V10} (bottom) incubated at 37 °C in saturated polyanhydride monomer solutions in PBS. Approximately, 3 µg of protein was loaded in all the lanes. Lane 1: molecular mass ladder (from top to bottom: 250, 150, 100, 75, 50, 37, 25, 20, 15, and 10 kDa); lane 2: protein in PBS; lane 3: CPTEG day 0; lane 4: CPTEG day 7; lane 5: CPH day 0; lane 6: CPH day 7; lane 7: SA day 0 and lane 8: SA day 7.

The CD spectra for F1-V and F1_{B2T1-V10} incubated in polyanhydride monomer solutions at day zero showed two minima occurring at 209-211 nm and 217-222 nm, where the first minima is dominant, indicating substantial coil and α-helix content.
together with comparably less β-sheet structure (Figure 4.8). In the case of the F1-V fusion protein, the minima are better defined and they occur at a lower molar circular dichroism absorbance (Δε) showing a more defined and complex secondary structure. These results are in agreement with studies by Powell et al. for the full length F1-V fusion protein 27. From Figure 4.8(b) and Figure 4.8(d) it can be observed that after seven days of incubation, the two characteristic minima cannot be distinguished and a clear reduction is observed in the Δε absorbance for F1-V incubated in SA monomer and F1B2T1-V10 incubated in SA and CPH monomers. It is also important to note that the stronger positive band between 190 and 195 nm is also characteristic of β-sheet content 42. This maximum is clearly reduced after incubation with SA monomer for both antigens and also after incubation with CPH monomer for the F1B2T1-V10 protein. These two observations suggest that a severe unfolding of the protein may be occurring in the presence of these monomer solutions. In the case of the protein incubated with SA monomer, the changes described are observable at day zero itself, suggesting a strong acidity-dependent unfolding of both proteins. This change in the secondary structure of the protein can be attributed to the acidity of the SA monomer, which is consistent with previous work showing that the F1 portion of the protein is not stable in acidic environments 27, 31, 43. In particular, Goodin et al. 31 showed that the F1-V protein exhibits a strong tendency to multimerize with reduction of pH 31. Based on previous observations and the results from this study, it becomes extremely important to identify suitable carriers that are able to maintain adequate pH conditions to stabilize Y. pestis antigens.
Figure 4.8 CD spectra of (a) and (b) F1-V fusion protein and (c) and (d) F1$_{B2T1}$-V10 protein in the presence of CPTEG, CPH, and SA monomers at (a) and (c) day 0 and (b) and (d) day 7. All the water spectra correspond to the specific protein in solution used as a control to identify structural changes.

Exchange rates of amide hydrogen for deuterium were used to follow changes in tertiary structure of the F1-V fusion protein and the recombinant F1$_{B2T1}$-V10 protein. These rates depend on the stability of protein secondary structure and the accessibility of N-H groups to solvent $^{41}$. The FTIR spectra F1$_{B2T1}$-V10 incubated in the solution of SA monomer showed a clear decrease in the absorbance in the amide II region with increased exposure to deuterium (spectra not shown). Similar results were obtained for
the F1-V fusion protein incubated in the presence of SA monomer (Figure 4.9(a)) but with a less pronounced reduction in the non-exchanged fraction. Reduction in the absorbance of the amide II region suggests a conformational change in the presence of SA monomer. The fraction of non-exchanged ratio was obtained by normalizing the intensity of the amide II band to the amide I band and is expected to be around one for tightly folded proteins. Data obtained at time zero indicate that the exchange is greater in the solutions of SA monomer as shown by the decrease in the fraction of non-exchanged hydrogen ions (Figures 4.9(a) and 4.9(c)). This data confirms a conformational change in both antigens in the presence of SA and this change in tertiary structure may be attributed to the change in the secondary structure previously observed and to the acidity of this monomer. On the other hand, the structures of F1-V and F1_{B2T1-V10} are largely unperturbed after the initial incubation with CPH and CPTEG monomers. After seven days of incubation, a clear decrease in the non-exchange ratio is observed in the presence of SA monomer, which is consistent with the structural changes that were observed during the initial incubation. A decrease in the fractional non-exchange is observed when F1_{B2T1-V10} is incubated in CPH monomer for seven days (consistent with the change in the secondary structure) while the protein incubated with the CPTEG monomer maintains its tertiary structure (Figure 4.9(d)). No statistical decrease in the fractional non-exchange was observed after seven days of incubation of F1-V with CPH and CPTEG monomers (Figure 4.9(b)). The changes in secondary and tertiary structure of the F1-V fusion protein and the F1_{B2T1-V10} recombinant protein in the presence of SA monomer may be sufficient to cause the observed alterations in protein antigenicity.
Figure 4.9 Tertiary structure characterization of F1-V fusion protein and F1p-V10 recombinant protein. Fraction non-exchanged hydrogen ions during a 4 h incubation of F1-V in anhydride monomers (a) day 0 and (b) day 7 with deuterium; fraction non-exchanged hydrogen ions during a 4 h incubation rF1_{B2T1}-V10 in anhydride monomers (c) day 0 and (d) day 7 with deuterium. Specific protein in PBS (○), SA (■), CPH (▲), and (☆) CPTEG (☆). All the PBS curves correspond to the specific protein incubated with PBS used as control to determined structural changes. Error bars represent standard deviation from triplicates.

As discussed before, other protein carriers have been studied (i.e., poly(glycolic acid), poly(lactic acid) and their copolymers), but these are not highly suitable for protein
stabilization because their bulk erosion mechanism generates a low pH microenvironment that has a detrimental effect on protein antigenicity. However, with the combined surface and bulk erosion mechanisms exhibited by polyanhydrides, water ingress into the bulk is controllable and in most cases is not significant; therefore, these low pH microenvironments would mostly be located on the surface of polymeric particles during release helping to maintain the structural levels and antigenicity of proteins. Further, the solubility in water of the hydrophobic anhydride monomers studied in this work is lower than that of the corresponding ester monomers (like lactic acid and glycolic acid). This lower solubility is one of the factors leading to a higher pH microenvironment with the anhydride degradation products as compared to the ester degradation products, thus favoring greater protein stability.

In order to avoid the side effects generated by the use of live and killed whole cell vaccines, an increasing number of polypeptide subunit and recombinant protein vaccines have been designed. However, these next generation vaccines generally are poorly immunogenic and often fail to provide protection against pathogens. Therefore, it is essential to design carriers that maintain structural integrity and immunogenicity of these subunit proteins in order to avoid the induction of inadequate immune responses. Our results show that primary, secondary, and tertiary structure as well as the antigenicity of two Y. pestis vaccine antigens is affected by the acidic pH microenvironment within saturated solutions of SA. The F1B2T1-V10 structural conformation was shown to be sensitive to acidic conditions and indeed, some precipitation of the F1B2T1-V10 protein was observed after incubation in a solution of the SA monomer. This can be attributed to the fact that the pH of the monomer solution
approaches the isoelectric point of the protein (pI ~ 5) of F1\textsubscript{B2T1}-V10 recombinant protein, resulting in aggregation\textsuperscript{25,45}. In addition, changes in the structure and antigenicity of F1\textsubscript{B2T1}-V10 were also observed in the presence of the CPH monomer solution; these changes were attributed to the hydrophobicity of the monomer as opposed to the pH. In contrast to globular proteins that exhibit a stable structure\textsuperscript{7,14}, F1\textsubscript{B2T1}-V10 appears to be affected by multiple mechanisms of instability when incubated with CPH or SA monomers. The amphiphilic CPTEG-containing polymers provided the most stabilizing environments for both the F1-V fusion protein and the F1\textsubscript{B2T1}-V10 recombinant protein because of the less acidic pH microenvironment and their amphiphilic nature. These results indicate that amphiphilic chemistries are promising carriers and adjuvants for \textit{Y. pestis} antigens.

### 4.5 Conclusions

This work identified polyanhydride monomers with chemical properties that maintained the structural integrity of two protein antigen candidates for \textit{Y. pestis}. The results provide promising insights for the rational design of new and effective vaccine delivery vehicles based on biodegradable and biocompatible polyanhydrides. The importance of selecting the appropriate polymer chemistry that will maintain structural integrity resulting in a biologically stable protein (e.g., antigen) was demonstrated. The antigenicity of proteins released from the amphiphilic CPTEG:CPH microparticles was preserved, suggesting that these polymers are promising carriers for \textit{Y. pestis} antigens. The increased protein stabilization afforded by the chemistry and microenvironment of polyanhydrides makes this polymer system likely to provide a means to deliver nascent
immunogens that have maintained multiple immunological epitopes to facilitate vaccine efficacy in a genetically diverse population.

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4.7 References


CHAPTER 5

Sustained Delivery of Functional Antibody from Amphiphilic Polyanhydride Nanoparticles

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5.1 Abstract

It has been suggested that antibodies (e.g., anti-serum or monoclonal antibodies) could be used in early, passive immune treatments for HIV as well as to help fight off an infection. As with the development of protein pharmaceutics, one of the most challenging tasks in passive immunotherapies is to deal with the physical and the chemical instabilities of the protein (i.e., antibodies), which invariably leads to loss of biological activity. In order to avoid these problems, it is necessary to design delivery vehicles that will minimize the degradation, maximize the in vivo activity, and provide controlled release of the encapsulated protein.

In this study, the use of polyanhydride nanoparticles is suggested for the delivery of two antibodies in order to show their ability to release stable therapeutic payloads. Tetanus antitoxin and anti-TNF-α monoclonal antibody were encapsulated and released from polyanhydride nanoparticles. These nanoparticles provide the ability to control antibody release kinetics and additionally, preserve antibody functionality and bioactivity upon fabrication and release. The amphiphilic 50:50 CPTEG:CPH nanoparticles demonstrated the best combination of characteristics compatible for either anti-serum or mAb preservation and release, making it an ideal candidate for a future used as a delivery system for therapeutic proteins.
5.2 Introduction

Protein-based drugs have emerged as a promising class of therapeutics\(^1\). There are currently \(~200\) protein-based medicines (e.g., therapeutic proteins and peptides, monoclonal antibodies, etc) on the market, and \(~400\) in development\(^2\). Specifically, antibodies have become a major class of protein-based therapeutic agents\(^3\). About a fifth of new drugs in clinical testing today are antibodies\(^4\).

Passive antibody therapy has been widely used to provide immunity against disease, or help fight off infections\(^5,6\). Antibodies against microbial, cellular and soluble targets produce and exploit various effector functions for therapeutic purposes: opsonization, activation of complement, antibody-dependent cell cytotoxicity, virus and toxin neutralization, direct antimicrobial activity, etc\(^3,6\). Several diseases are targets for therapeutic antibodies that are clinically approved or currently being tested in clinical trials. These include transplant rejection, rheumatoid arthritis, Crohn’s disease, osteoporosis, asthma, arthritis, Herpes simplex virus, HIV and different types of cancer\(^3,6-9\). Delivery of antibodies is typically performed intravenously, with a need for multiple and large doses in order to achieve effective systemic levels\(^5,6\). The route of administration and the large doses required make antibody therapies expensive. Therefore, there is a clear need to reduce the required number of doses without compromising the bioavailability and effectiveness of the treatment.

In this regard, polymer matrices have been exploited as controlled release vehicles to deliver therapeutic levels of antibodies at the intended site of action\(^10-13\). Direct local delivery at diseased sites, spatial control over antibody distribution, reduced toxicity, co-administration with other therapeutic agents (i.e., antibiotics, small molecule drugs), and
control over release kinetics are some of the advantages of using polymeric matrices to for the controlled delivery of antibodies. However, there are still several disadvantages with the use of polymeric devices for the delivery of antibodies, including poor loading capabilities that result in low antibody bioavailability, reduced systemic antibody exposure, and poor antibody stability within the delivery device. In order to achieve good therapeutic performance, it is necessary that the polymer chemistry and the device fabrication methods do not inactivate the antibody; it is ideal that antibody release correlates with preservation of its bioactivity.

Biodegradable polyanhydride particles have emerged as promising carriers for vaccine delivery because of their ability to release antigen in a controlled manner as they degrade, which can eliminate the need for multiple doses. Polyanhydrides undergo surface erosion, allowing no moisture penetration into the bulk, thus making payload release kinetics more predictable. Another advantage of polyanhydrides is that, due to the limited water solubility of their degradation products, a much less acidic microenvironment is produced compared to that of degrading polyesters; this is beneficial to the stability of the (protein) payload. Biodegradable polyanhydride micro and nanoparticles have been shown to stabilize and provide sustained release of several proteins used in vaccine formulations against different diseases (i.e., tetanus, pneumonic plague, anthrax). These particles also have several other attractive properties, such as the ability to act as an adjuvant and enhancing the immune response, while providing a platform which can be programmed to degrade over different times, depending on the chemistry.
In this study, the use of polyanhydride nanoparticles is investigated for the delivery of two therapeutic antibodies in order to show their ability to release stable payloads. Polyanhydrides based on 1,6-bis(p-carboxyphenoxy)hexane (CPH), sebacic acid (SA), and 1,8-bis(p-carboxyphenoxy)-3,6-dioxacotane (CPTEG) monomers were used in this work. The antibodies studies include tetanus anti-toxin, a full anti-sera that is used to for the treatment of tetanus\textsuperscript{26,27}, and anti-TNF-α, a monoclonal antibody applied to treat Crohn’s disease and rheumatoid arthritis by binding to soluble and membrane TNF-α in order to neutralize its activity\textsuperscript{4,6,28}. The antibodies were encapsulated and released from polyanhydride nanoparticles and a systematic analysis of protein stability upon encapsulation and release was performed. The studies described herein demonstrate that the amphiphilic environment provided by CPTEG-containing polyanhydrides preserved antibody functionality suggesting that this platform can be effectively used as a delivery system for therapeutic proteins.

5.3 Materials and Methods

5.3.1 Materials

Materials needed for monomer synthesis and polymerization, as well as for nanoparticle fabrication, including sebacic acid (99%), p-carboxy benzoic acid (99+%), and 1-methyl-2-pyrrolidinone, anhydrous (99+%), were purchased from Aldrich (Milwaukee, WI); 4-p-hydroxybenzoic acid, 1,6-dibromohexane, 1-methyl-2 pyrrolidinone, and tri-ethylene glycol were purchased from Sigma Aldrich (St. Louis, MO); 4-p-fluorobenzonitrile was obtained from Apollo Scientific (Cheshire, UK); potassium carbonate, dimethyl formamide, toluene, sulfuric acid, acetic acid,
acetonitrile, acetic anhydride, methylene chloride, pentane, and petroleum ether were purchased from Fisher Scientific (Fairlawn, NJ). Phosphatase substrate was purchased from Aldrich (St. Louis, MO). Polyacrylamide 4-20% Tris-HCl pre-cast gradient gels, unstained protein standards, pre-stained broad range molecular weight standards, and Flamingo Gel Stain were purchased from BioRad Laboratories (Richmond, CA). Bicinchoninic acid (BCA) and micro BCA protein assay kits and Slide-A-Lyzer dialysis cassettes (10,000 MW cut-off membranes) were obtained from Pierce Biotechnology Inc. (Rockford, IL).

5.3.2 Monomer and Polymer Synthesis

Diacids of CPH and CPTEG were synthesized as described previously\textsuperscript{29,30}. SA and CPH prepolymer were synthesized by the methods described by Shen et al\textsuperscript{31} and Conix et al\textsuperscript{30}, respectively. Subsequently, 20:80 CPH:SA and 50:50 CPTEG:CPH copolymers were synthesized by melt polycondensation as respectively described by Kipper et al\textsuperscript{15} and Torres et al\textsuperscript{29}. The chemical structure was characterized with \textsuperscript{1}H NMR and the molecular mass was determined using gel permeation chromatography (GPC)\textsuperscript{15,29}.

5.3.3 Nanoparticle Fabrication

Two percent (w/w) tetanus anti-toxin or anti-TNF-\(\alpha\) (clone XT3.11, BioXCell Fermentation/Purification Services, West Lebanon, NH) encapsulated nanoparticles were fabricated by the anti-solvent nanoencapsulation method recently published by Ulery et al\textsuperscript{23}. Nanoparticle morphology and size distribution were characterized by
scanning electron microscopy (SEM, JEOL 840A, JEOL Ltd., Tokyo, Japan) and quasi-elastic light scattering (QELS, Zetasizer Nano, Malvern Instruments Ltd., Worcester, UK), respectively.

5.3.4 In vitro Protein Release

Tetanus anti-toxin and anti-TNF-α loaded nanoparticles were placed in microcentrifuge tubes with 0.1 mM phosphate buffer saline (PBS, pH 7.6). Samples were sonicated to uniformly distribute the nanoparticles and placed an incubator at 37 °C with constant agitation. Supernatants were sampled over time to determine the amount of released antibody with the micro-BCA assay. Removed volumes were replaced with fresh PBS to maintain perfect sink conditions. Data is presented as cumulative fraction of released antibody, which was determined by dividing the amount released at each time point by the total amount of encapsulated antibody into the nanoparticles\textsuperscript{17,20,21}. A second method employing dialysis tubes was utilized to avoid accumulation of polymer degradation products within the medium surrounding the particles; however, no differences were observed in the release kinetics profiles and resulted antibody stability (data not shown).

For antigenicity and functionality analysis, antibodies were released for 72 h. Following this time, release sample supernatant was removed and Vivaspin 500 centrifugal concentrators (Sartorius Stedim Biotech, Aubagne, France) were used to concentrate the released samples and antibody concentrations were measured using the micro BCA assay. Sample concentrations were adjusted uniformly for the antigenicity and functionality assays, as well as for the structural analysis.
5.3.5 Tetanus Anti-toxin Functionality after Release

5.3.5.1 Tetanus Toxoid Specific Enzyme Linked Immunosorbent Assay (ELISA)

High protein binding 96-well Costar microtiter plates (Corning Life Sciences, Lowell, MA) were coated overnight with 100 µL of phosphate buffer saline (PBS) at a pH of 7.4 containing 10 µg/mL of tetanus toxoid. PBS containing 0.05% Tween 20 (PBS-T) and 2% (w/v) gelatin as a non-specific blocking agent (BD Biosciences, San Jose, CA) was used to block plates for at least 2 h at room temperature. After the blocking period, plates were heated at 60 °C for 10 min in a dry oven to melt gelatin and rinsed three times to remove any unbound blocking reagent. 100 µL of tetanus anti-toxin released from polyanhydride nanoparticles at a concentration of 20 µg/mL (diluted in PBS-T with 1% (v/v) fetal calf serum (FCS)) were added to each of the tetanus toxoid pre-coated wells. Unencapsulated tetanus anti-toxin (protein only in PBS-T 1% FCS) at the same concentration was used as positive control to compare the functionality of the released anti-toxin. Plates were incubated with anti-toxin samples overnight at 4 °C. After incubation, plates were washed three times with PBS-T and 100 µL of biotin-conjugated anti-horse IgG (1:2000 in 1% (v/v) FCS PBS-T) was added to each well and incubated for 4 h at room temperature (RT). Plates were washed three times with PBS-T and 100 µL of alkaline phosphatase conjugated streptavidin (diluted 1:1000 in PBS-T) was added to each well. After 2 h of incubation at RT, the plates were washed four times with PBS-T and then, 100 µL of sodium carbonate (50 mM) and magnesium chloride (2mM) buffer (pH 9.3) containing 1 mg/mL phosphatase substrate was added. Plates reacted for 1 h at RT. The optical density (OD) of each well was measured at 405 nm using a Varian Cary 50 Microplate Reader (Varian, Inc., Sunnyvale, CA). The results were normalized
with the data for the unencapsulated antitoxin solution and reported as % residual functionality, as shown in Equation 5.1:

\[
% \text{ residual functionality} = \left( \frac{OD \text{ sample}}{OD \text{ unencapsulated}} \right) \times 100 \tag{Eq. 5.1}
\]

5.3.5.2 Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE):

Primary Structure

The primary structure of tetanus anti-toxin was evaluated using SDS-PAGE. Samples released from polyanhydride nanoparticles as well as unencapsulated protein were incubated with equal volumes of reducing sample buffer (SDS (10% w/v), Tris-HCl (1M, pH 6.8), glycerol (3 mM), bromophenol blue (0.01% w/v), and β-mercaptoethanol (0.05% v/v)). Samples were mixed and heated at 95 °C for 5 min after which they were loaded onto 4-20% Tris-HCl pre-cast gels and run at 120 V and 4°C for 90 min. Five µL of unstained protein standard were used as reference to determine the molecular weight of the samples. The gels were incubated in fixative solution (40% ethanol, 10% acetic acid) at 4°C for 3 h. Next, they were stained with fluorescent flamingo gel stain overnight, and washed with a 0.01% Tween 20 solution to reduce non-specific fluorescence. A Typhoon 8600 (GE Healthcare, Piscataway, NJ) fluorescence scanner was used to obtain the images of the gels. Experiments were performed in triplicate.

5.3.5.3 Circular Dichroism (CD): Secondary Structure

The secondary structure of the tetanus anti-toxin was monitored after release from polyanhydride nanoparticles by far UV CD (Jasco J-710 Spectropolarimeter, Easton,
MD)) at a wavelength range of 190-260 nm\textsuperscript{18-21}. The spectra were collected using Jasco J-170 software (Easton, MD). The PBS spectra were subtracted from the protein spectra. The spectra shown are the average of three experiments.

5.3.5.4 \textit{Fluorescence Spectroscopy: Tertiary Structure}

Fluorescence spectra were collected using a Varian Cary Eclipse Fluorescence Spectrometer (Varian, Inc.). The spectra (300-500 nm) were collected at an excitation wavelength of 280 nm that corresponds to tyrosine and tryptophan residues\textsuperscript{19,20}. The PBS spectral emission values were subtracted from each sample. The emission spectrum was analyzed for shifts in peak intensity and wavelength, which are indicative of alterations in protein tertiary structure.

5.3.6 \textit{Anti-TNF-\alpha Functionality and Bioactivity after Release}

5.3.6.1 \textit{TNF-\alpha Specific Enzyme Linked Immunosorbent Assay (ELISA)}

The ability of the released anti-TNF-\alpha to recognize and bind to TNF-\alpha was determined by ELISA. The ELISA protocol was similar to the previously described ELISA method for tetanus toxoid. Plates were initially coated with recombinant murine TNF-\alpha (PeproTech, Rocky Hill, NJ) at a concentration of 5 \( \mu \)g/mL. Released antibody samples as well as unencapsulated anti-TNF-\alpha were added at a concentration of 10 \( \mu \)g/mL. The results were normalized with the data for the unencapsulated anti-TNF-\alpha antibody solution and reported as \% residual functionality, as shown in \textbf{Equation 5.1}. 
5.3.6.2 Cell Culture

L929 (murine fibroblasts) cells obtained from ATCC (Manassas, VA) were maintained in culture at 37°C in a 5% CO₂ humidity using high glucose DMEM medium (Invitrogen, Carlsbad, CA) supplemented with 10% heat-inactivated FBS (Valley Biomedical, Winchester, VA), 100 IU/mL penicillin and 10 µg/mL streptomycin (Mediatech, Herndon, VA). After 5-7 days of culturing, confluent cells were washed and resuspended in culturing.

5.3.6.3 TNF-α Cytotoxicity Assay

L929 cells were incubated in 96-well flat-bottomed Costar microtiter plates at a concentration of 3.5 x 10⁴ cells per well overnight at 37°C. To determine the optimal TNF-α cytotoxic dose, cells were incubated with serial dilutions of TNF-α (from 1000 to 0.9 pg/mL) for 24 h at 37°C in the presence of actinomycin D (2 µg/mL)³²,³³. The MTT assay was utilized to determine cell viability. Optical density (OD) was measured at 570 and 690 nm. Data is presented as % cytotoxicity (Supplemental Figure 5.1A), as determined by Equation 5.2:

\[
\% \text{ cytotoxicity} = 100 \times \left[ \frac{OD \text{ control} - OD \text{ sample}}{OD \text{ control}} \right]
\]

An optimal TNF-α killing dose of 500 pg/mL was determined based on 90% cytotoxicity in the L929 cells.
5.3.6.4 Titration of anti-TNF-α Antibody Neutralization Activity against TNF-α

The optimal dose of recombinant TNF-α selected from cytotoxicity assays (500 pg/mL) was pre-incubated for 4 h with serial dilutions of anti-TNF-α antibody (clone XT3.1) (from 10 µg/mL to 39 ng/mL), and added to L929 cells in 96-well microtiter plates. Pre-incubation of cytokine with the specific neutralizing antibody prior to addition to responding cells prevents cytokine binding to its receptor. After 24 h of culture, cell viabilities were measured as described above. Data is presented as % protection (Supplemental Figure 5.1B), as determined by Equation 5.3, in which the positive control are cells cultured in the absence of TNF-α.

\[
\% \text{ protection} = 100 \times \left[ \frac{OD \text{ sample}}{OD \text{ positive control}} \right]
\]

An antibody dose of 5 µg/mL was found to achieve ~100% protection from TNF-α killing.

5.3.6.5 Released anti-TNF-α Antibody Neutralization Bioactivity

In these experiments, treatments including anti-TNF-α released from polyanhydride nanoparticles, anti-TNF-α-loaded polyanhydride nanoparticles, and unencapsulated antibody were pre-incubated for 4 h with 500 pg/mL of recombinant TNF-α. Concentrations of released antibody as well as mass of anti-TNF-α-loaded nanoparticles were adjusted to achieve the optimal dose of anti-TNF-α antibody required to neutralize cytokine cytotoxicity, as determined in the experiment described above. The amount of anti-TNF-α-loaded nanoparticles necessary was determined by using the release
profiles during the first 4 h of release as a reference. After 24 h of culture, cell viabilities were measured as described above. Data is presented as % protection (Equation 5.3).

5.3.7 Statistical analysis

Statistical analysis was carried out using a one-way model ANOVA with JMP® 7 (Cary, NC) to account for comparison-wise error. Comparisons between treatments were made with Tukey’s HSD to determine statistical significance.

5.4 Results

5.4.1 Polymer and nanoparticle characterization

\(^1\)H NMR spectroscopy was used to characterize the polymer structure and the spectra were consistent with previously published data\(^{15,29}\). The synthesized 20:80 CPH:SA copolymer had an average M\(_w\) of 15,000 g/mol with a polydispersity index (PDI) of 2.1 while the 50:50 CPTEG:CPH copolymer had a M\(_w\) of 8,500 g/mol with a PDI of 1.7. These values were obtained from \(^1\)H NMR and corroborated with GPC and are consistent with previous work\(^{15,17,21,23,24,29}\).

Tetanus antitoxin and anti-TNF-α monoclonal antibodies were encapsulated in 20:80 CPH:SA and 50:50 CPTEG:CPH nanoparticles using an anti-solvent nanoencapsulation method. These two chemistries were chosen based on previous work that demonstrated that their ability to stabilize globular proteins\(^{20,34}\) as well as recombinant antigens such as F1-V\(^21\), and because they exhibit chemistry-specific adjuvant properties in vitro\(^{22-24}\) and in vivo\(^{14,16}\). The morphology of the antibody-loaded nanoparticles was visualized by SEM and were consistent with previous work\(^{14,23,24}\). QELS was used to determine
nanoparticle size distribution; average diameters for the 20:80 CPH:SA chemistry were 240 ± 44 nm and those for the 50:50 CPTEG:CPH particles were 176 ± 29 nm.

5.4.2 *In vitro* antibody release kinetics is dictated by polyanhydride chemistry

*Figure 5.1* show the release profiles of tetanus antitoxin and anti-TNF-α. The release of both antibodies is controlled by the polymer chemistry-dependent degradation kinetics of the nanoparticles. A slightly faster antibody release kinetics was observed from the 50:50 CPTEG:CPH particles with 80 to 90% of the protein released after 25 days compared to 70 to 75% of protein released from the 20:80 CPH:SA nanoparticles during the same period of time. The difference in the release profile is related to a higher initial burst from the 50:50 CPTEG:CPH nanoparticles, which is consistent with previous work with other protein antigens. This behavior is a consequence of the amphiphilic nature of CPTEG:CPH copolymers, which exhibit a combination of bulk and surface erosion, while the hydrophobic 20:80 CPH:SA exhibits surface erosion. However, the kinetics of the initial burst was different for each antibody; in the case of tetanus antitoxin the initial burst for both chemistries (~20% for 20:80 CPH:SA and ~35% for 50:50 CPTEG:CPH) occurs during the first three hours of release (*Figure 5.1A*), while for anti-TNF-α antibody a higher initial burst was observed from the 50:50 CPTEG:CPH particles (~ 35%) after 24 h of *in vitro* release (*Figure 5.1B*). This indicates that the nature of the protein and its interactions with the polyanhydride chemistries influenced the obtained release profile.
Figure 5.1 Cumulative release fraction of (A) tetanus antitoxin and (B) anti-TNFα antibody released from 20:80 CPH:SA (△) and 50:50 CPTEG:CPH (●) nanoparticles. Error bars are representative of two independent experiments performed in duplicate.

5.4.3 Amphiphilic 50:50 CPTEG:CPH particles released functional tetanus antitoxin

The stability of the released tetanus antitoxin was evaluated and is shown in Figure 5.2. No detrimental effect on anti-serum functionality was observed when the protein was exposed to the nanoparticle fabrication conditions, suggesting that the use of organic solvents and other processing steps in the nanoparticle fabrication does not impair protein stability. Functionality of the released anti-serum was evaluated by ELISA and the analysis demonstrates that tetanus antitoxin released from 50:50 CPTEG:CPH nanoparticles retained ~100% of its functionality, while antitoxin released from 20:80 CPH:SA nanoparticles preserved only 60% of its functionality.
Figure 5.2 Residual functionality of tetanus antitoxin released from polyanhydride nanoparticles. A tetanus antitoxin-specific ELISA was used to determine changes in functionality of the released protein compared to unencapsulated antibody and antibody exposed to nanoparticle fabrication conditions. Data are normalized to the unencapsulated antibody control. Error bars represent standard deviation of three independent experiments performed in triplicate. * indicates groups that are statistically significant (p ≤ 0.05) from the unencapsulated antibody control.

A detailed analysis of protein structure alterations was performed to understand the loss of functionality upon release from the 20:80 CPH:SA nanoparticles and to identify the structural levels at which the changes are occurring. A summary of this analysis is presented in Figure 5.3. SDS-PAGE was utilized to evaluate changes in primary structure. Tetanus antitoxin is mainly composed of immunoglobulin G (IgG) molecules and therefore its two main characteristics bands appear at ~50 kDa, which is characteristic of the heavy chains, and at ~25 kDa, representing the light chains. Figure
5.3A shows that the two main characteristic bands were observed for the unencapsulated tetanus antitoxin control together with some secondary bands at higher molecular weights, which correspond to other proteins that compose the anti-serum. While the two main bands were observed for antitoxin released from both 50:50 CPTEG:CPH and 20:80 CPH:SA nanoparticles, the bands were significantly weaker in intensity when compared with the unencapsulated antitoxin. Besides, the 50 kDa band was observed at a slightly higher molecular weight and other bands at higher molecular weights were observed, which may be indicative of protein aggregation. These observations are more remarkable in the antitoxin released from the 20:80 CPH:SA nanoparticles.

The CD spectra for tetanus antitoxin showed a minimum between 217 and 219 nm (Figure 5.3B) indicating a predominance of β-sheet conformation, as previously described by Cathou et al. While nanoparticle fabrication conditions preserved the secondary structure, antitoxin released from 50:50 CPTEG:CPH nanoparticles showed a slight shift of the minimum to the left (~ 215 nm). A more pronounced shift to the left together with a significant increase in molar ellipticity (Δε) was observed when tetanus antitoxin was released from 20:80 CPH:SA nanoparticles, signifying a loss in secondary structure.

Fluorescence spectroscopy was used to analyze changes in the tertiary structure of the protein. While the position of the peak is preserved for tetanus antitoxin released from both polyanhydride chemistries, antitoxin peak intensity was lower for the protein release from the 20:80 CPH:SA nanoparticles, indicating protein unfolding (Figure
5.3C). The nanoparticle fabrication conditions and the 50:50 CPTEG:CPH nanoparticles did not have any detrimental effects on the tertiary structure of the antitoxin.

Figure 5.3 Structural integrity of tetanus antitoxin was best preserved by the amphiphilic 50:50 CPTEG:CPH nanoparticles while protein released from the hydrophobic 20:80 CPH:SA nanoparticles showed changes in primary, secondary and tertiary structure. Representative data from three independent experiments is presented. A) Primary structure analysis by SDS-PAGE, B) Secondary structure by CD spectra, and C) Tertiary structure by fluorescence spectroscopy.
5.4.4 Functionality of anti-TNF-α was partially lost upon release for 72 h from both polyanhydride chemistries

Anti-TNF-α monoclonal antibody was released and its functionality upon release was evaluated using ELISA. The anti-TNF-α (clone XT3.1) used in this study was selected because it has been used for in vitro and in vivo neutralization of TNF-α. Figure 5.4 shows that ~40% loss of functionality was observed when the anti-TNF-α monoclonal antibody was released from 20:80 CPH:SA and 50:50 CPTEG:CPH particles. Antibody functionality was maintained when exposed to nanoparticle fabrication conditions.

![Bar graph showing residual functionality of anti-TNF-α](image)

**Figure 5.4** Functionality of anti-TNF-α partially lost upon release for 72 h from both polyanhydride chemistries. Residual functionality of anti-TNF-α released from polyanhydride nanoparticles was measured using an ELISA and compared to unencapsulated antibody and antibody exposed to nanoparticle fabrication conditions. Data are normalized to the unencapsulated antibody control. Error bars represent standard deviation of three independent experiments performed in triplicate. * indicates groups that are statistically significant (p ≤ 0.05) from the unencapsulated antibody control.
5.4.5 Anti-TNF-α-loaded polyanhydride nanoparticles are capable of releasing bioactive antibody avoiding the detrimental effect of accumulation of degradation products

An in vitro TNF-α neutralization assay was employed to assess the ability of anti-TNF-α-loaded nanoparticles to preserve the biological function of the monoclonal antibody directly after its release. In addition, a comparison between the effect of any structural changes that may have occurred to antibodies during the 72 h release experiment, and bioactivity directly after release was performed. Because there was no statistical difference in the amount of anti-TNF-α released between the two nanoparticle chemistries utilized in these studies in the first 4 h (as shown in Figure 5.1B), direct comparisons between the various groups can be made. Antibody-loaded nanoparticle mass was adjusted in order to obtain an antibody concentration of 5 µg/mL in the assay well during the 4 h of incubation. The accumulated antibody released during 72 h as well as the unencapsulated antibody and antibody exposed to nanoparticle fabrication conditions controls were also added at a concentration of 5 µg/mL to allow for direct comparisons between the groups.

As observed in Figure 5, the functionality trends showed in Figure 5.4 correlate with the bioactivity of this monoclonal antibody, protecting L929 cells from the cytotoxic effect of recombinant TNF-α. Only 60% protection was achieved during 72 h of release from both chemistries. However, better protection was obtained when anti-TNF-α-loaded nanoparticles were directly used in the assay indicating that accumulation of polyanhydride degradation products in the media surrounding the particles has a detrimental effect on antibody bioactivity. This observation was chemistry-independent (Figure 5.5). Protection levels obtained with the direct incubation of antibody-loaded
nanoparticles were similar to or higher than the 90% that was also observed for the unencapsulated antibody and antibody exposed to nanoparticle fabrication conditions controls.

**Figure 5.5** Anti-TNF-α-loaded polyanhydride nanoparticles are capable of releasing bioactive antibody, while accumulation of polyanhydride degradation products affects antibody bioactivity. Percentage protection from TNF-α cytolytic activity achieved by anti-TNF-α antibody. L929 cells were cultured for 24 h in the presence of TNF-α and unencapsulated antibody, antibody exposed to nanoparticle fabrication conditions (fabrication conditions), or antibodies released from polyanhydride nanoparticles. The viability of the cells was quantified by the MTT assay and represented as percentages against control cells cultured in the absence of TNF-α. Error bars indicate standard error of three independent experiments performed in triplicate. Treatments with different letters are significantly different from one another at \( p \leq 0.05 \), while * indicates statistical significance between the two nanoparticle groups \( p \leq 0.003 \).
5.5 Discussion

Sustained delivery of antibodies using biodegradable polyanhydride nanoparticles can reduce the high costs associated with antibody-related therapies. However, antibody instability leading to loss of biological activity upon encapsulation and release from these delivery vehicles is a formidable challenge in the design of effective antibody-based therapeutic formulations. The data presented herein demonstrate that amphiphilic polyanhydride nanoparticle-based delivery platforms can be used to provide sustained delivery of functional antibodies (i.e., polyclonal or monoclonal).

Bioavailability and therapeutic indices for many therapeutic antibodies are strongly dependent on the circulating half-life and this can be altered by using controlled-release methods, based on the geometry, size and chemistry of the delivery devices, the appropriate antibody loading, and delivery route. The nanoparticle-based delivery system studied herein demonstrated polymer chemistry-dependent sustained release of antibodies for almost one month, as shown in Figure 5.1. In the present study, two specific polyanhydride chemistries (i.e., 20:80 CPH:SA and 50:50 CPTEG:CPH) were studied; however, we have previously demonstrated for several protein antigens that varying the co-monomer ratio, results in release profiles ranging from weeks to months.

While sustained release of antibodies over time results in longer circulating half-life, it is essential that the released protein is functional and bioactive. Formulations of antibodies in many polymer vehicles often require exposure to conditions that may be deleterious to protein structure and function. In this work, the nanoparticle fabrication conditions utilized to encapsulate antibodies into polyanhydride nanoparticles (e.g., exposure to organic solvent, sonication, and vacuum drying) had no effect on the
structure, functionality, and bioactivity of both tetanus antitoxin and anti-TNF-α monoclonal antibodies (Figures 5.2, 5.3, 5.4 and 5.5). This is in agreement with similar results with other proteins exposed to these conditions\textsuperscript{17,18,20,21,25,34}.

Intravenous immunoglobulin (IVIg) is a FDA-approved therapeutic comprising of polyclonal antibodies pooled from serum donors and administered to patients\textsuperscript{3,5} for treatment of AIDS and toxin-mediated diseases\textsuperscript{5,6}. However, since new developments allow for the production of unlimited types of monoclonal antibodies (mAbs), the use of therapeutic formulations based on mAbs have increased due to their high specificity\textsuperscript{3,5}. Since therapies based on both polyclonal antibodies (i.e., tetanus antitoxin) and mAbs (i.e., anti-TNF-α) are currently in use, the present work included a detailed analysis of release and stability of both types of antibodies.

As shown in Figures 5.2 and 5.3, the amphiphilic 50:50 CPTEG:CHP copolymer nanoparticles released stable tetanus antitoxin and preserved the functionality of the antitoxin. In contrast, tetanus antitoxin released from the hydrophobic 20:80 CPH:SA nanoparticles showed ~40% loss in antibody functionality (Figure 5.2). This detrimental effect of the hydrophobic 20:80 CPH:SA carrier on antibody functionality was shown to correlate with changes in the primary, secondary, and tertiary structures of the protein (Figure 5.3). Protein aggregation was observed when protein was released from both nanoparticle chemistries (Figure 5.3A), presumably due to the presence of the hydrophobic CPH\textsuperscript{17} in both copolymer systems; this behavior has been observed for other protein antigens\textsuperscript{19,20,34}. In addition, a decrease in the β-sheet content was observed (i.e., left-shift of spectral minima as well as decrease of peak intensity at 200 nm which is characteristic of β-sheet content\textsuperscript{35}) for the protein released from 20:80
CPH:SA particles, as shown in Figure 5.3B. These changes in the secondary structure may lead to protein unfolding, as suggested by the fluorescence spectroscopy data (Figure 5.3C).

Released monoclonal anti-TNF-α antibody from both polyanhydride nanoparticles chemistries showed a ~40% loss of functionality (Figure 5.4) that was also reflected in its biological activity (Figure 5.5). The results presented in Figure 5.5 also showed that exposure of antibody to polymer degradation products (e.g., cumulative release over 72 h) had a detrimental effect on the biological activity of anti-TNF-α mAb. However, delivery of anti-TNF-α mAb by directly adding antibody-loaded nanoparticles to the assay wells improves the bioactivity of this mAb as reflected in the higher protection percentages (Figure 5.5) (i.e., higher neutralization) from the cytotoxic effect of TNF-α in L929 cells.

Collectively, these results indicate that the amphiphilic 50:50 CPTEG:CPH nanoparticles provide sustained release of functional tetanus antitoxin and anti-TNF-α antibody. Similar results have been observed previously with this amphiphilic system when used as a delivery vehicle for other proteins including globular proteins\textsuperscript{17,20}, acid-stabilized proteins\textsuperscript{20}, and \textit{Yersinia pestis} antigens\textsuperscript{21}.

5.6 Conclusions

One of the most difficult challenges to overcome in antibody-based therapies is to provide sustained delivery of therapeutic doses of bioactive antibodies. The studies reported herein demonstrate the potential use of amphiphilic polyanhydride nanoparticles as an effective antibody delivery platform. These nanoparticles provide
the ability to sustain antibody release kinetics for about four weeks, thus increasing antibody circulation half-life. Additionally, these particles are capable of preserving antibody functionality and bioactivity upon fabrication and release. Current studies are focused on investigating formulations based on these particles for in vivo neutralization of the inflammatory effect due to TNF-α after endotoxin challenge.

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5.8 References


5.9 Supplemental data

Supplemental Figure 5.1 Optimization of TNF-α cytotoxic dose and anti-TNF-α neutralization dose. A 500 pg/mL dose of TNF-α was sufficient to achieve around 95% of cytotoxicity in L929 cells, while 5 µg/mL of either anti-TNF-α antibody was necessary to protect close to 100% of cells. A) Titration of cytolytic activity of the recombinant TNF-α, B) neutralization of TNF-α by different doses of antibody. The viability of the cells was quantified by MTT assay and represented as percentages against control cells cultured in the absence of TNF-α. Error bars represents standard deviation of three independent experiments performed in triplicate.
CHAPTER 6

Protein Adsorption on Biodegradable Polyanhydride Microparticles

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6.1 Abstract

The *in vitro* adsorption of plasma proteins on polyanhydride microparticles based on sebacic acid (SA), 1,6-bis(p-carboxyphenoxy)hexane (CPH), and 1,8-bis(p-carboxyphenoxy)-3,6-dioxaoctane (CPTEG) was studied. Three model proteins from bovine serum (albumin (BSA), immunoglobulin G (IgG) and fibrinogen (Fg)) were used. The adsorption was studied using X-Ray Photoelectron Spectroscopy and gel electrophoresis. 2-D electrophoresis was used to study the adsorption of plasma proteins from bovine serum. Differences in the amount of protein adsorbed were detected as a function of: (i) copolymer composition and (ii) specific protein studied. A direct correlation between polymer hydrophobicity and protein adsorbed was observed and higher quantities of Fg and IgG were absorbed. *In vitro* release studies were performed with ovalbumin-encapsulated microparticles that were incubated with Fg; these studies showed a reduction in the amount of ovalbumin released from the microparticles when Fg is adsorbed on the surface. An understanding of protein adsorption patterns on parenteral delivery devices is valuable in optimizing their *in vivo* performance.
6.2 Introduction

The use of biodegradable polymeric devices as drug carriers and adjuvants has promise because these materials can sustain the release of protein antigens over time in addition to exhibiting immunomodulatory properties that can be controlled by selecting the correct polymer chemistry.\textsuperscript{1,2} Polyester microparticles, specifically those composed of lactic acid and glycolic acid (e.g., poly (lactide-co-glycolide), PLGA), have been extensively studied and used as delivery devices for biomedical applications.\textsuperscript{3,4} Different proteins have been encapsulated into PLGA microparticles because they provide a sustained release for these proteins and their degradation products are easily absorbed by neighboring cells.\textsuperscript{5-7} However, these degradation products can create an acidic microenvironment that has been shown to perturb the structure of encapsulated proteins.\textsuperscript{5,7,8} In addition, PLGA is a bulk-eroding polymer that, in aqueous environments, can expose the encapsulated protein to a high moisture environment that may lead to aggregation.\textsuperscript{9,10}

In order to reduce moisture-induced aggregation, researchers have studied surface eroding polymers such as polyanhydrides.\textsuperscript{11,12} Many polyanhydride degradation products do not result in a decrease in the local pH that is as severe as that due to PLGA degradation (due to the lower solubility of polyanhydride degradation products in water). Thus, polyanhydrides provide a favorable environment for protein stabilization and release. In this regard, polyanhydrides have been shown to maintain the primary, secondary and tertiary structure of various proteins (i.e. ovalbumin, lysozyme, tetanus toxoid) as well as preserve their biological activity.\textsuperscript{13-15} Additionally, \textit{in vitro} release
studies have shown that it is possible to control the release kinetics of proteins, ranging from days to months, by choosing the appropriate polyanhydride chemistry.\textsuperscript{11-15}

When these drug carriers are injected, their surface will be in contact with blood. It is well known that plasma proteins in the blood adsorb to these carriers within a few minutes after contact.\textsuperscript{16-19} The biodistribution and biocompatibility of these carriers, and therefore their effectiveness as vaccine adjuvants, can be influenced by the adsorption of these plasma proteins. For example, proteins adsorbed on polymer microparticles can determine the immunological recognition of these carriers by the mononuclear phagocytic system (MPS).\textsuperscript{17-19} Some plasma proteins can act as opsonins (e.g., immunoglobulin or complement factors) that can accelerate the recognition and elimination of drug carries by macrophages of the MPS.\textsuperscript{17,19} In case these opsonins are missing or other proteins such as albumins are adsorbed, a reduction in the uptake of these carriers by the MPS is observed, thus increasing their blood half-life.\textsuperscript{19,20} Other type of cells and immune pathways can be regulated by the presence of specific proteins; for example, adsorption of fibrinogen has shown to play an important role in the adhesion of platelets and neutrophils, and also this protein participate in the coagulation cascade.\textsuperscript{21-23} It is known that the particle surface properties (e.g., hydrophobicity and charge) and protein properties (e.g., hydrophobicity, charge, molecular mass, and conformational stability) play an important role in determining the amount of protein adsorbed on different types of material surfaces.\textsuperscript{16,20,24-26} Typically, the first protein layer formed on the specific surface will determine the subsequent interaction between proteins and the material surface. To describe the formation of these protein layers it is important to study both the protein-surface affinities and the
competitive or cooperative interactions between the different plasma proteins. In particular, it is known that abundant and rapidly adsorbed proteins can be displaced by trace proteins that arrive at the surface slowly (Vroman effect); this can affect the development of time-dependent competitive protein adsorption.\textsuperscript{27,28}

Plasma protein adsorption on different material surfaces (e.g., modified silica) has been extensively studied for tissue engineering using films as model devices and focusing on non-degradable materials;\textsuperscript{22,29-32} but there are few systematic studies of protein adsorption phenomena on degradable materials. In this study, microparticles of polyanhydride copolymers based on sebacic acid (SA) and 1,6-bis(\(\rho\)-carboxyphenoxy)hexane (CPH) have been used.\textsuperscript{1,13,33} The hydrolytic degradation rates of polyanhydrides can be altered by simple changes in the polymer backbone by choosing appropriate monomers.\textsuperscript{34} For example, in order to enhance the hydrophilicity of the monomer to obtain a faster degradation rate, triethylene glycol (TEG) can be incorporated into the CPH backbone resulting in poly(1,8-bis(\(\rho\)-carboxyphenoxy)-3,6-dioxaoctane) (CPTEG), which when copolymerized with CPH results in a copolymer with amphiphilic properties.\textsuperscript{35}

The main purpose of this study was to determine how different degradable polymer chemistries affect protein adsorption. In this work, three model proteins from bovine serum were chosen for in vitro experiments: bovine serum albumin (BSA), which is the most abundant protein (60\%) in plasma;\textsuperscript{22,30} immunoglobulin G (IgG), which is an opsonin and is the most abundant antibody in plasma;\textsuperscript{18,22,30} and fibrinogen (Fg), which in addition to its opsonization properties, is a “sticky” protein that adsorbs strongly on different surfaces.\textsuperscript{17,21,23} In order to study adsorption, X-Ray Photoelectron Spectroscopy
(XPS) and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using a fluorescent gel dye were used. To determine the effect of competitive adsorption, polyanhydride microparticles were incubated in bovine serum and 2-D electrophoresis was used to qualitatively determine the specific protein(s) adsorbed. Finally, in order to determine the effect of surface adsorption on protein release profiles, *in vitro* release studies were performed with ovalbumin (Ova)-encapsulated microparticles that were incubated with Fg, and the release kinetics was characterized with SDS-PAGE.

### 6.3 Materials and methods

#### 6.3.1 Materials

Chemicals needed for monomer synthesis and polymerization, sebacic acid (99%), *p*-carboxy benzoic acid (99+%), and 1-methyl-2-pyrrolidinone anhydrous (99+%), were purchased from Aldrich (Milwaukee, WI); 4-*p*-hydroxybenzoic acid, 1,6-dibromohexane, 1-methyl-2 pyrrolidinone, and tri-ethylene glycol were purchased from Sigma Aldrich (St. Louis, MO); 4-*p*-fluorobenzonitrile was obtained from Apollo Scientific (Cheshire, UK); potassium carbonate, dimethyl formamide, toluene, sulfuric acid, acetic acid, acetonitrile, acetic anhydride, methylene chloride, and petroleum ether were purchased from Fisher Scientific (Fairlawn, NJ). BSA and ovalbumin were purchased from Sigma Aldrich (St. Louis, MO); bovine IgG was purchased from Bethyl Corporation (Montgomery, TX); and bovine Fg and bovine serum were obtained from Aniara Corporation (Mason, OH). Materials for SDS-PAGE and 2-D electrophoresis, which included 12% Tris-Glycine pre-cast gels, unstained protein standards, Flamingo gel
stain, 11 cm Immobilized pH gradient (IPG) strips (pH 3-10, non-linear), and 4-15% polyacrylamide gels were purchased from BioRad Laboratories (Richmond, CA).

### 6.3.2 Polymer synthesis and characterization

CPH and CPTEG diacids were synthesized as previously described.\(^{36,37}\) SA and CPH prepolymer were synthesized by the methods described by Shen et al.\(^{38}\) and by Conix.\(^{36}\) Subsequently, CPH:SA and CPTEG:CPH copolymers were synthesized by melt polycondensation as respectively described by Kipper et al.\(^{39}\) and Torres et al.\(^{37}\) The chemical structure of the polymers was characterized with \(^1\)H NMR and the molecular weight was determined using gel permeation chromatography (GPC). These methods were described in our previous work and the results are consistent with previous work.\(^{37,39}\)

### 6.3.3 Microparticle fabrication and characterization

Microparticles of different polymer compositions were made using cryogenic atomization.\(^{40}\) Briefly, 100 mg of the polymer was weighed and dissolved in methylene chloride. The polymer solution was then pumped through an 8700–1200 MS ultrasonic atomizing nozzle (SonoTek Corporation, Milton, NY) into 200 mL of frozen ethanol (with an excess of liquid nitrogen). 50:50 CPH:SA and CPTEG-based copolymer compositions were fabricated at 4°C (inside a walk-in cooler) while the other polymers were fabricated at room temperature. All the compositions were stored at -80 °C for three days. For CPTEG-based compositions, after the first 24 h, ~200 mL of cold ethanol was added to reduce aggregation; the solutions were stirred at 300 rpm for 15
min and placed back in the freezer at –80 °C. After three days, vacuum filtration was used to collect the microparticles and they were dried overnight under vacuum.\textsuperscript{15} Microparticle samples were coated with an ultrathin layer of gold by low vacuum sputter coating and Scanning Electron Microscopy (SEM) (JEOL 840 A, JEOL Peabody, MA) was used to observe the morphology of the microparticles.

\textbf{6.3.4 Protein adsorption}

20\% w/v suspensions of polymer microparticles were prepared in phosphate buffer saline (PBS) (0.1 M, pH 7.4). BSA, IgG, and Fg suspensions in PBS were prepared with a final concentration of 1 mg/mL. Then, 250 μL of the protein solutions were added to 750 μL of the microparticle suspensions to obtain a final protein concentration of 250 μg/mL. The solution was mildly vortexed for 1 min and incubated for 5 min at 37 °C. After incubation, the solution was centrifuged at 12000 x g for 10 min. Three washes with PBS were performed and the microparticles were dried under vacuum overnight.

\textbf{6.3.5 X-Ray Photoelectron Spectroscopy (XPS)}

XPS analysis was used to determine the amount of protein adsorbed on the surface of the microparticles. XPS data was acquired using a PHI 5500 Multi-technique system (Physical Electronics, Inc., Chanhassen, MN). Samples were mounted using 3M double-sided Scotch tape. Standard aluminum source was used with carbon as reference for charge correction. Blank microparticles were used as controls. High-resolution C1s peaks were collected and fitted used CasaXPS software (RBD Instruments, Bend, OR). Binding energies were referenced to the aliphatic hydrocarbon peak at 285.0 eV.\textsuperscript{31,41}
The total protein adsorbed is presented as µg of protein per area (cm²), which was obtained using the mean diameter of the microparticles. The area of the C1s peaks that contributes to the nitrogen content was divided by the total area of the carbon peak in order to determine the atomic concentration fraction that is related to the fraction of protein that is adsorbed on the microparticles. This was then used to estimate the total mass (µg) of protein adsorbed.

6.3.6 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

To determine the amount of protein adsorbed and to corroborate the results obtained with XPS, SDS-PAGE was performed. In order to extract the protein adsorbed on the polymer microparticles, 600 µL of a non-reducing sample buffer (SDS (10% w/v), Tris-HCl (1 M, pH 6.8), glycerol (3 mM), bromophenol blue (0.01% w/v)) were added to 20 mg of microparticles. The samples were vortexed and heated for 5 min at 70 °C. After cooling at room temperature and centrifuging for 10 min at 12,000 × g, the supernatant was collected and each solution was filtered with 0.20 µm syringe filters to eliminate any remaining microparticles. The samples were loaded into 12% Tris-Glycine pre-cast gels and run for 90 minutes at 140V. 5 µL of unstained protein standard were used as reference to determine the molecular mass of the samples. The gels were incubated in fixative solution (40% ethanol, 10% acetic acid) at 4°C for 3 h. Next, they were stained with fluorescent flamingo gel overnight, after which the gels were washed with 0.01% Tween 20 solution to eliminate excess in the background.

A Typhoon 8600 (GE Healthcare, Piscataway, NJ) fluorescence scanner with a green laser (532 nm) and a 555 nm longpass emission filter was used to obtain the
images of the gel and ImageQuantTL (GE Healthcare, Piscataway, NJ) software was used to quantify the intensity of the bands in the gels. In order to quantify the unknown samples, a standard curve with concentrations ranging from 2500 ng/mL to 10 ng/mL was obtained for the different proteins using the same SDS-PAGE protocol used for the unknown samples. The main characteristic bands for each of the proteins were used to obtain the standard curves, and the same bands were used for protein quantification. This fluorescence technique with the overnight staining protocol used in these experiments exhibits a limit of detection of 1 ng. Linear quantitation was obtained with the described protocol with correlation coefficients in the range of 0.97 to 0.99. The quantified adsorbed protein was normalized to the initial amount of protein used in the initial incubation. The total protein adsorbed is presented as µg protein per area (cm²).

6.3.7 Qualitative identification of serum proteins by 2-D electrophoresis

Microparticle suspensions (20% w/v) were prepared in PBS and 2.5 mL of bovine serum supplemented with bovine fibrinogen (10 µg/mL) was added. The samples were incubated at 37°C for 5 min with constant mixing (100 rpm). Three washing steps with PBS were performed as described in the Protein adsorption section. For protein desorption, a solution containing 10% (w/w) SDS and 2.3% (w/w) dithioerythritol was applied and samples were heated at 95 °C for 10 min. Particles were centrifuged for 10 min at 12000 x g and the supernatant was applied for the 2-D electrophoresis analysis.

The first dimension of the electrophoresis was done using 11 cm IPG strips (pH 3-10). The first dimension separation was performed in the IPGPhor system (GE
Healthcare, Piscataway, NJ) following a slow voltage ramping protocol: 50 V for 10 h, 500 V for 1 h, 1000 V for 1 h, and 8000 V for 4 h. For the second dimension of the separation analysis, strips were loaded in 4-15% polyacrylamide gels and run for 2 h at 140 °C. Gels were fixed, stained and scanned as described in the SDS-PAGE section. A 2-D separation was performed using bovine serum samples that were used as a control. A qualitative analysis was performed by comparing the gels of the desorbed proteins with those obtained from bovine serum and ImageQuantTL (GE Healthcare, Piscataway, NJ) software was used to identify the main bands on the gels.

6.3.8 In vitro protein release

The effect of protein adsorption on the release kinetics of protein-loaded polymer microparticles was studied. Ovalbumin (ca. 4 wt%) was encapsulated into polymer microparticles fabricated by cryogenic atomization as described previously. Twenty mg of protein-loaded microparticles were incubated with fibrinogen at a final concentration of 10 µg/mL by the procedure described before. Ova-encapsulated microparticles not subjected to protein adsorption underwent the same wash protocol in order to be used as control and to obtain differences in the release profiles that are only caused by the presence of adsorbed protein on the surface.

15 mg of Ova-encapsulated microparticles were suspended in 1 mL of phosphate buffer (0.1 M, pH 7.4) and incubated at 37°C and 100 rpm. Samples of 750 µL were taken at different intervals of time and replaced with fresh PBS. The release samples were stored at 4°C for measurements of protein concentration by SDS-PAGE as described before. The experiments were performed in triplicate. The cumulative release...
was normalized by the total protein encapsulated, which was determined as previously described.\textsuperscript{15}

6.4 Results and discussion

6.4.1 Microparticle fabrication and characterization

Figure 6.1 shows SEM images of 10:90, 20:80 and 50:50 CPTEG:CPH and poly(SA) microparticles fabricated by cryogenic atomization. It was observed that the surface morphology of the microparticles varied with the polymer composition; Determan et al.\textsuperscript{13} suggested that this change in surface morphology is caused by hydrophobicity (i.e., the higher the CPH content, the smoother the surface of the microparticles). In Figure 6.1, we observe this trend with the different CPTEG:CPH compositions; the surface of the microparticles becomes smoother with increasing CPH content. The mean diameter for all the microparticles was 10-16 µm, which is consistent with previous work\textsuperscript{15,40}.

Table 6.1 XPS analysis of atomic concentrations and ratios of elements present on control blank polymer microspheres.

<table>
<thead>
<tr>
<th>Microsphere compositions</th>
<th>C%</th>
<th>N%</th>
<th>O%</th>
<th>O/C%</th>
<th>N/C%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Poly SA</td>
<td>76.23</td>
<td>0.00</td>
<td>23.77</td>
<td>0.31</td>
<td>0.00</td>
</tr>
<tr>
<td>20:80 CPH:SA</td>
<td>77.54</td>
<td>0.00</td>
<td>22.42</td>
<td>0.29</td>
<td>0.00</td>
</tr>
<tr>
<td>50:50 CPH:SA</td>
<td>76.37</td>
<td>0.00</td>
<td>23.18</td>
<td>0.30</td>
<td>0.00</td>
</tr>
<tr>
<td>10:90 CPTEG:CPH</td>
<td>72.75</td>
<td>0.24</td>
<td>27.01</td>
<td>0.37</td>
<td>0.0033</td>
</tr>
<tr>
<td>20:80 CPTEG:CPH</td>
<td>76.28</td>
<td>0.26</td>
<td>23.46</td>
<td>0.31</td>
<td>0.0034</td>
</tr>
<tr>
<td>50:50 CPTEG:CPH</td>
<td>75.30</td>
<td>0.29</td>
<td>24.34</td>
<td>0.32</td>
<td>0.0039</td>
</tr>
</tbody>
</table>
**Figure 6.1** SEM images of microparticles fabricated by cryogenic atomization: (a) 10:90 CPTEG:CPH, (b) 50:50 CPTEG:CPH, (c) Poly(SA), and (d) 20:80 CPTEG:CPH. Scale bar: (a), (c), and (d) 50 µm, and (b) 10 µm.

### 6.4.2 XPS analysis

The detection of proteins at interfaces using XPS generally involves the detection of nitrogen in the proteins; when the substrate (i.e., polymer surface) contains the same atoms as the adsorbed proteins (but at different atomic concentrations), changes in the N/C ratio can be used to evaluate the adsorption of proteins.\(^{32}\) Blank polymer microparticles were used as controls in order to determine the presence of any nitrogen
Table 6.1 shows the elemental atomic concentration and elemental ratios on the surface of the blank microparticles. It was observed that microparticles containing CPTEG show the presence of nitrogen atoms at ca. 0.2% (atomic concentration), which was attributed to the chemicals used in the polymer synthesis.

Incubation of microparticles with the three different model proteins was performed at 37 °C for 5 min. This short incubation period is representative of in vivo conditions because it has been shown that the removal of up to 90% of intravenously applied devices from circulation in the blood stream occurs within a few minutes. Therefore, the adsorption of plasma proteins should occur during this time. Incubation was also performed for 30 min (data not shown) and no difference in the amount of adsorbed protein was identified (by XPS analysis); these results are in agreement with literature models for protein adsorption.

Figure 6.2 shows XPS results (presented as µg of protein adsorbed per cm²) of (a) CPH:SA and (b) CPTEG:CPH microparticles for BSA, IgG and Fg. An increase in the nitrogen content is observed for all the polymer chemistries after incubation with protein providing evidence for the adsorption of protein on the microparticle surface. A direct correlation between the amount of protein adsorbed and the CPH content is present in both polymer systems. This trend correlates with the hydrophobicity of the polymer surface. Previous contact angle experiments performed by Lopac et al. have shown that the hydrophobicity of these systems increases when the CPH content increases. On the other hand, an increase in amphiphilicity (i.e., increasing CPTEG content from 10-50%) results in a decrease in the amount of protein adsorbed by more than 50% in the case of IgG and Fg; these results are consistent with other studies that showed that
incorporation of PEG on different surfaces avoids plasma protein adsorption reducing macrophage uptake and increasing carrier blood half-life.\textsuperscript{16,18,20,47} From Figure 6.2, it is also clear that higher amounts of Fg (1.2 µg/cm\textsuperscript{2}) and IgG (0.9 µg/cm\textsuperscript{2}) are adsorbed on the different microparticle compositions; other plasma protein adsorption studies have shown similar trends after \textit{in vitro} incubation with serum.\textsuperscript{18,31} This is important because in addition to the total amount of adsorbed protein, the type of protein adsorbed could affect the \textit{in vivo} behavior of these drug carriers.\textsuperscript{18,22,30,47-49} Because of their opsonization properties, the adsorption of IgG and Fg can enhance phagocytosis by the MPS while the adsorption of BSA would reduce phagocytosis, thus increasing the blood circulation time of the microparticles.\textsuperscript{18,41,47-49} Long circulation time periods are desirable for protein delivery vehicles in order to allow uptake and presentation of the encapsulated protein by antigen presenting cells (APCs).

\textbf{Table 6.2} Quantification of components fitted to high-resolution C1s spectra.

<table>
<thead>
<tr>
<th>Protein-adsorbed microspheres</th>
<th>Percentage area of C1s components</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C1</td>
</tr>
<tr>
<td>10:90 CPTEG:CPH</td>
<td></td>
</tr>
<tr>
<td>Blank</td>
<td>68.9</td>
</tr>
<tr>
<td>BSA</td>
<td>64.7</td>
</tr>
<tr>
<td>50:50 CPH:SA</td>
<td></td>
</tr>
<tr>
<td>Blank</td>
<td>74.9</td>
</tr>
<tr>
<td>IgG</td>
<td>64.3</td>
</tr>
<tr>
<td>50:50 CPTEG:CPH</td>
<td></td>
</tr>
<tr>
<td>Blank</td>
<td>64.8</td>
</tr>
<tr>
<td>Fg</td>
<td>57.9</td>
</tr>
</tbody>
</table>
Figure 6.2 Total protein adsorbed (µg/cm²) on polyanhydride microparticles obtained from XPS analysis: (a) CPH:SA and (b) CPTEG:CPH. Error bars represent standard error of three different experiments with duplicate samples in each. * represent p-value < 0.05 obtained from statistical student-t test.

High-resolution XPS analysis was used to probe the presence of proteins on the surface of the polymer microparticles. High-resolution C1s spectra were fitted with four main components according with their characteristic binding energy representing
aliphatic hydrocarbon at 285.0 eV (C1), ether and amine groups at 286.5 eV (C2), 
carbonyl and amide groups at 288.2 eV (C3), and ester and carboxylic acid groups at 
289.1 eV (C4). Analysis of C1s spectra allows for more direct comparison of 
protein adsorption based on increases in the amide and carboxylic acid groups (C3 and 
C4 components respectively). Both the C3 and the C4 components contribute to the 
identification of the adsorbed protein; however, the C3 component has been shown to 
be more significant. This is consistent with the XPS analysis of lyophilized proteins 
used as controls. Table 6.2 shows percentage areas obtained from high-resolution C1s 
fitting spectra for three different microparticle compositions as an example for each of 
the proteins studied. From inspection, there is a substantial increase in the C3 
component (from 3.4% to 10.0%) for 50:50 CPH:SA microparticles after adsorption of 
IgG. For 10:90 CPTEG:CPH microparticles, an increase in the C3 and C4 components 
is observed after incubation with BSA protein, from 4.8% and 5.0% to 6.9% and 6.3% 
respectively. It is instructive to note that for 10:90 CPTEG:CPH microparticles (which 
have the highest CPH content), the amount of BSA adsorbed is lower than that of IgG in 
50:50 CPH:SA, indicating that the adsorption of plasma proteins depends on the specific 
protein. For 50:50 CPTEG:CPH microparticles, a small increase of 0.8% is observed 
after incubation with Fg, which is the “stickiest” protein studied, confirming that the 
reduction of the CPH content (or the increase of CPTEG content) lowers the adsorption 
of plasma proteins.
6.4.3 SDS-PAGE

SDS-PAGE using a fluorescent gel stain dye was performed in order to confirm the results obtained by XPS analysis. Non-reducing conditions were used; bands at 66, 150 and 330 kDa were expected for BSA, IgG and Fg respectively. The bands for each of the proteins appeared in the correct molecular weight (images not shown); lower molecular weight bands appeared for the CPH:SA system (especially for BSA) indicating that the proteins underwent hydrolysis. This phenomenon is attributed to the decrease in pH caused by the presence of SA in combination with the exposure to high temperatures during the incubation period and the sample preparation process; this observation is consistent with previous results.\textsuperscript{13,14}

**Figure 6.3** shows the amounts of adsorbed protein per total surface area for (a) CPH:SA and (b) CPTEG:CPH microparticles. Once again, an adsorption pattern based on microparticle hydrophobicity is observed; the amount of protein adsorbed increases with CPH content and decreases with CPTEG or SA content. These results were confirmed statistically with a student-t test that showed significant differences ($p < 0.05$), especially between the microparticles containing a high fraction of CPH (i.e. 50:50 CPH:SA and 10:90 CPTEG:CPH) and those containing more SA or CPTEG (i.e., poly(SA) or 50:50 CPTEG:CPH). These results are consistent with the XPS analysis (**Figure 6.2**). The SDS-PAGE results also confirm differences in the amount of protein adsorbed according to the type of protein studied. Consistent with the results shown in **Figure 6.2**, the data shown in **Figure 6.3** indicated that BSA is the least adsorbed protein with an average mass of protein adsorbed of 0.3 $\mu$g/cm$^2$ followed by IgG (1.1 $\mu$g/cm$^2$) and Fg (1.8 $\mu$g/cm$^2$).
Figure 6.3 Total protein adsorbed (µg/cm²) on polyanhydride microparticles obtained by SDS-PAGE of (a) CPH:SA, and (b) CPTEG:CPH. Error bars represent standard error of three different experiments with duplicate samples in each. * represent p-value < 0.05 obtained from statistical student-t test.
6.4.4 Competitive protein adsorption

The three proteins used in this study are present in plasma at different concentrations and, as mentioned before, the type of protein adsorbed will have an effect on the biodistribution of these carriers. This makes it important to determine how these proteins adsorb in “competition”. In order to evaluate this “competitive effect”, 2-D electrophoresis was used to determine how proteins from bovine serum (Fg supplemented) competitively adsorbed on polymer microparticles. Figure 6.4 shows the 2-D electrophoresis gels of proteins from bovine serum adsorbed on: (a) 50:50 CPH:SA and (b) 50:50 CPTEG:CPH microparticles. The strongly detectable proteins on the 2-D gels were albumin, immunoglobulin (light and heavy chains), IgM, antithrombin III, and fibrinogen. Some proteins (e.g., Apo E) were observed only for the most hydrophobic surface. From Figure 6.4, it is clear that for most of the proteins (e.g., IgG (light and heavy), albumin, IgM, fibrinogen) the bands present on gel (a) are more intense than the corresponding bands on gel (b); this is consistent with the results from the XPS and SDS-PAGE studies, indicating high protein adsorption on hydrophobic surfaces (i.e., 50:50 CPH:SA). This study also corroborated the protein-rejection properties of PEG (i.e., 50:50 CPTEG:CPH) shown previously in this study and others.16,20

Considering that albumin represents more than 60% of the total plasma proteins, one may expect this protein to be the most adsorbed; however, this is not the case for the two chemistries studied. In addition to protein-surface compatibility, this observation may be attributed to structural changes in albumin after adsorption on microparticle surfaces, as reported previously.25,53 This phenomena may also be a consequence of the Vroman effect in which displacement of abundant proteins by less abundant ones
occurs as well as the displacement of “sticky” proteins occurs by other trace proteins, explaining why fibrinogen was not found on the surface in high amounts even after it was supplemented back in the serum.\textsuperscript{27,28} Displacement often occurs within seconds after incubation\textsuperscript{47} and this is why we may be able to detect only the irreversibly adsorbed protein layer after 5 min of incubation.

**Figure 6.4** 2-D electrophoresis pattern of protein adsorbed on (a) 50:50 CPH:SA and (b) 50:50 CPTEG:CPH microparticles after incubation with bovine plasma. 1: IgM; 2: albumin; 3: antithrombin III; 4: fibrinogen \( \gamma \); 5: fibrinogen \( \beta \); 6: IgG \( \gamma \); 7: Apo E; 8: IgG light chains; 9: Apo A1.
6.4.5 In vitro protein release

The adsorption of plasma proteins on the surface of polyanhydride microparticles may affect the release kinetics of protein-loaded microparticles. In order to study this effect, 50:50 CPTEG:CPH microparticles loaded with 4% ovalbumin (48 kDa) were fabricated and incubated with fibrinogen (330 kDa) as described before. An in vitro release study was performed for 28 days. SDS-PAGE was chosen to quantify the release kinetics because as fibrinogen desorbed over time, it was possible to differentiate between the two proteins. Figure 6.5 shows two representative gels from this study; samples taken after 2 h of release (lane 1) and from one to seven days (lane 3 to lane 9) are shown for (a) Ova-encapsulated microparticles, and (b) Ova-encapsulated microparticles that were incubated with Fg. It is important to observe that only a band at ca. 48 kDa (characteristic of Ova) is present in both gels indicating that desorption of Fg is not occurring, confirming that a short time of incubation is enough to obtain an irreversible layer of adsorbed protein and that the wash steps performed in the incubation process eliminated any reversible layers of adsorbed protein.

Figure 6.5 also indicates a clear difference in the intensity of the characteristic band between gels (a) and (b), suggesting a reduced amount of released protein, caused by the presence of fibrinogen on the surface of the microparticles. Confirming these results, Figure 6.6 shows the cumulative Ova release profile from 50:50 CPTEG:CPH microparticles; the fraction of Ova released was obtained by normalizing the mass of released protein by the total amount of protein encapsulated. A decrease in the rate of Ova released is observed when Fg is adsorbed on the surface of the microparticles. The initial burst, which is 50% when no Fg is adsorbed to the surface, decreases to 26%
when Fg is adsorbed on the microparticle surface. Similar results were obtained from an

*in vitro* release study for 50:50 CPH:SA microparticles (data not shown).

**Figure 6.5** SDS-PAGE of 50:50 CPTEG:CPH microparticles: (a) Ova-encapsulated, and

(b) Ova-encapsulated/Fg-adsorbed. Lane 1: sample after ~2 h of release; lane 2: MW

standard ladder; lane 3 to lane 9: release samples at day 1 through day 7 respectively.
The release kinetics of proteins from polyanhydride microparticles is controlled by the degradation of the polymer, \textsuperscript{13,15} which in turn is dependent upon the hydrophobicity of the polymer. As we have shown in this work, the polymer hydrophobicity affects the adsorption/desorption kinetics of plasma proteins. In the case of bulk erodible polymers, as the erosion of the polymer progresses, more internal porous structures will be exposed, thus enlarging the available polymer surface area and providing additional sites (i.e., new porous walls) for protein adsorption;\textsuperscript{54} therefore the potential desorption of the adsorbed protein after water penetration is countered by the re-absorption of the protein on these new sites. Surface erodible materials are generally hydrophobic and prevent water penetration into the bulk. The interaction of plasma proteins is stronger with hydrophobic surfaces, leading to a higher adsorption/desorption ratio (i.e., higher net adsorption rate) as surface erosion progresses; this net adsorption of proteins on the “degraded” particle may lead to a slower release of encapsulated protein. Our experiments support this line of thought and the presence of plasma proteins on the surface of polymer microparticles does reduce the amount of protein released from biodegradable materials that are surface or bulk erodible. This may have positive implications if the desired rates of release of the protein are slower than what is observed without any surface adsorption of proteins, such as for vaccine delivery applications. However, caution must be exercised in that depending on the specific protein that is adsorbed, the microparticles could become less prone to phagocytosis. Our studies provide a systematic and rational framework to study these complex phenomena and will have value in the molecular design of adjuvants for vaccine delivery and of constructs and scaffolds for tissue engineering.
Figure 6.6 Ovalbumin protein released from 50:50 CPTEG:CPH microparticles. Error bars represent standard deviation of two different experiments with duplicate samples each.

6.5 Conclusions

The in vitro adsorption of three model plasma proteins on polyanhydride microparticles was studied. The adsorption of plasma proteins can affect the in vivo behavior of these polymeric devices in different ways. These studies demonstrated a direct correlation between polymer hydrophobicity and amount of protein adsorbed. The type of protein also plays an important role in the adsorption process. The release kinetics of protein from loaded microparticles is slowed down by the presence of adsorbed protein on the surface. The determination and knowledge of these patterns is important to understand the in vivo behavior of drug delivery devices. Therefore, these
studies provide a rational approach for the correct selection of polymer chemistry in order to design suitable carriers for therapeutic proteins and vaccine antigens.

6.6 Acknowledgments

The authors acknowledge financial support from NSF (EEC 0552584) and the Grow Iowa Values Fund. The authors thank Dr. Robert Doyle for his assistance with the use of the confocal microscope and Dr. Michael Wannemuehler and Dr. Maria Torres-Gonzalez for discussions on the design and development of these experiments. We thank Elise Schiltz, an undergraduate at Iowa State University, who participated in this project.

6.7 References


CHAPTER 7

Chemistry-Dependent Adsorption of Serum Proteins on Polyanhydride Microparticles Affects Uptake and Activation of Dendritic Cells: Identification of Complement Receptor 3-Mediated Particle Uptake

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7.1 Abstract

The delivery of antigen-loaded microparticles to DCs may benefit by optimizing the surface of the microparticles themselves exploiting material properties and introducing signals that mimic pathogen properties. However, after being in contact with serum, proteins will quickly adsorb onto the surface of microparticles and considerably modify their surface characteristics. In this work, we described the chemistry-dependent serum protein adsorption patterns on polyanhydride particles and their implications in the molecular interactions with dendritic cells (DCs). The chemistry-dependent activation of DCs by non-serum coated particles was corroborated with the enhanced expression of MHC II and CD40 on DCs after incubation with 50:50 CPTEG:CPH particles, and the increased secretion of IL-6, TNF-α, and IL-12p40 by 50:50 CPH:SA particles. Presence of opsonins (i.e., complement component 3 (C3) and IgG) was able to trigger the adjuvant properties of these vaccine carriers by enhancing the properties that induce maturation of DCs (i.e., internalization, cell surface molecules expression and cytokine secretion) in a chemistry-dependent manner. Utilizing DCs derived from complement receptor 3 mice (CR3−/− mice), CR3 mediated-internalization of both non-serum- and serum-coated particles was identified. These studies provide valuable insights into the rational design of targeted vaccine platforms to induce robust immune responses and improve vaccine efficacy.
7.2 Introduction

The design of vaccine adjuvants capable of activating innate immunity is important for the induction of protective immune responses that will result in the production of efficacious vaccines. A key step in the activation of the innate immune system is the recognition of pathogen-associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs) on the surface of antigen presenting cells (APCs), including dendritic cells (DCs). DCs can internalize and process soluble antigens, resulting in maturation and directing further interactions with other immune cells, including naïve T cells. The use of polymer particles to deliver antigen, either encapsulated or bound to the surface, has been shown to enhance antigen presentation compared to the administration of soluble antigen alone.

The interaction of antigen-loaded microparticles with DCs may benefit from engineering the microparticle surface by exploiting material properties and introducing motifs that mimic the surface of pathogens. For example, it has been demonstrated that cationic surfaces greatly enhance uptake. On the other hand, the presence of ligands, which bind to specific cellular receptors, increases internalization. After contact with serum, microparticles may undergo significant changes in their surface properties due to the rapidly adsorption of serum proteins.

Polyanhydride microparticles have been shown to possess immunomodulatory properties, which, when combined with their ability to stabilize and provide sustained release of protein antigens, make them excellent candidates as adjuvants for the design of single dose vaccine. Our previous work has shown that serum protein adsorption patterns on polyanhydrides microparticles are correlated to their surface
properties (i.e., hydrophobicity) suggesting that the adsorption of serum proteins can be tailored by controlling the particle surface chemistry \(^{13}\). Proteins like IgG and complement factors (i.e., opsonins) have been identified at the surface of microparticles, which may influence recognition and uptake by APCs \(^{9,13,21}\). Naturally, pathogens like *Mycobacterium tuberculosis*, *Legionella pneumophila*, and *M. leprae* coat themselves with serum proteins, which facilitate phagocytosis by host cells by interaction with specific cell surface receptors such as complement, Fc\(\gamma\), and mannose receptors \(^{22-27}\). Therefore, understanding the biological consequences of serum protein adsorption to microparticles on the interaction of particles with APCs and APC activation may provide vital insights for the rational design of improved biomaterial-based adjuvants.

This study was designed to evaluate the adsorption of mouse serum proteins on to the surface of polyanhydride microparticles and to understand their effect on microparticle uptake by DCs and DC activation. Polyanhydrides based on sebacic acid (SA), 1,6-bis(p-carboxyphenoxy)hexane (CPH), and 1,8-bis(p-carboxyphenoxy)-3,6-dioxaoctane (CPTEG) were evaluated in this study. The studies described herein identified the role of complement component C3 adsorbed on the surface of polyanhydride microparticles in receptor-mediated uptake and activation of DCs.

### 7.3 Materials and Methods

#### 7.3.1 Materials

Chemicals needed for monomer synthesis and polymerization, sebacic acid (99%), \(p\)-carboxy benzoic acid (99\%\)), and 1-methyl-2-pyrrolidinone anhydrous (99\%), were purchased from Aldrich (Milwaukee, WI); 4-\(p\)-hydroxybenzoic acid, 1,6-dibromohexane,
1-methyl-2 pyrrolidinone, and triethylene glycol were purchased from Sigma Aldrich (St. Louis, MO); 4-p-fluorobenzonitrile was obtained from Apollo Scientific (Cheshire, UK); potassium carbonate, dimethyl formamide, toluene, sulfuric acid, acetic acid, acetonitrile, acetic anhydride, methylene chloride, and petroleum ether were purchased from Fisher Scientific (Fairlawn, NJ). Materials for SDS-PAGE and 2D electrophoresis, which included 12% tris-glycine precast gels, unstained protein standards, Flamingo gel stain, 11-cm immobilized pH gradient (IPG) strips (pH 3–10, nonlinear), and 4–15% polyacrylamide gels were purchased from BioRad Laboratories (Richmond, CA). Phosphatase substrate was purchased from Aldrich (St Louis, MO). β-mercaptoethanol, *E. coli* lipopolysaccharide (LPS) O111:B4, and rat immunoglobulin (rat IgG) were purchased from Sigma Aldrich. The materials required for the DC culture medium include: granulocyte macrophage colony stimulating factor (GM-CSF), purchased from PeproTech (Rocky Hill, NJ); HEPES buffer, RPMI 1640, penicillin-streptomycin, and L-glutamine, purchased from Mediatech (Herndon, VA); and heat inactivated fetal calf serum, purchased from Atlanta Biologicals (Atlanta, GA). Materials used for flow cytometry included: intracellular (IC) fixation buffer, purchased from eBioscience (San Diego, CA); unlabeled anti-CD16/32 FcγR, purchased from Southern Biotech (Birmingham, AL); unlabeled hamster IgG, fluorescein isothiocyanate (FITC) conjugated anti-mouse MHC II (I-A/I-E) (clone M5/114.15.2), PE conjugated anti-mouse MHC Class I (H-2Kd/H-2Dd)(clone 34-1-2S), allophycocyanin (APC) anti-mouse CD40 (clone 1C10), phycoerythrin-Cy7 (PE/Cy7) anti-mouse CD86 (clone GL-1), Alexa Fluor® 700 anti-mouse CD11c (clone N418); and corresponding isotypes: FITC-conjugated rat IgG2bκ; PE-conjugated rat IgG2a (clone eBR2a), APC rat IgG2ak (clone eBR2a), PE/Cy7
conjugated rat IgG2b (clone KLH/G2b-1-2), Alexa Fluor® 700 conjugated Armenian hamster IgG (clone eBio299Arm). All of these reagents were purchased from eBioscience. Cadmium selenide quantum dots (QDs) (emission at 630nm) were a gift from Dr. Aaron Clapp at Iowa State University.

### 7.3.2 Monomer and Polymer Synthesis

Diacids of CPH and CPTEG were synthesized as described previously. SA and CPH prepolymer were synthesized by the methods described by Shen et al. and Conix et al., respectively. Subsequently, 50:50 CPH:SA and 50:50 CPTEG:CPH copolymers were synthesized by melt polycondensation as respectively described by Kipper et al. and Torres et al. \(^{29}\). \(^1\)H NMR spectroscopy was used to characterize the polymer structure and the resultant spectra were consistent with previously published data. The synthesized 50:50 CPH:SA copolymer had an average \(M_w\) of 12,000 g/mol with a polydispersity index (PDI) of 2.0 while the 50:50 CPTEG:CPH copolymer had a \(M_w\) of 8,000 g/mol with a PDI of 1.8. These values were obtained from \(^1\)H NMR and corroborated with GPC and are consistent with previous work.

### 7.3.3 Microparticle Fabrication and Characterization

Cryogenic atomization was used to fabricate 50:50 CPH:SA and 50:50 CPTEG:CPH microparticles, as described elsewhere. Briefly, 100 mg of the polymer was weighed and dissolved in methylene chloride. For QD-loaded microparticles, QDs were added to the dissolved polymer and dispersed by sonication at 40 Hz for 30 s. The polymer solution was then pumped through an 8700–1200 MS ultrasonic atomizing
nozzle (SonoTek Corporation, Milton, NY) into 200 mL of frozen ethanol (with an excess of liquid nitrogen). Microparticles were fabricated at 4 °C. Compositions were stored at -80 °C for 3 days. For 50:50 CPTEG:CPH, after the first 24 h, 200 mL of cold ethanol was added to reduce aggregation; the solutions were stirred at 300 rpm for 15 min and placed back in the freezer at -80 °C. After 3 days, vacuum filtration was used to collect the microparticles and they were dried overnight under vacuum. Scanning electron microscopy (SEM) (JEOL 840 A, JEOL Peabody, MA) was used to observe the morphology of the microparticles. The particle size distribution was obtained from SEM images using ImageJ image analysis software (National Institutes of Health, Bethesda, MD)\textsuperscript{13, 16, 19, 20}. An average of 200 particles per image was analyzed. Quasi-elastic light scattering (QELS) was used to determine the ζ-potential of the particles, as described previously\textsuperscript{3}. Particle morphology and size were consistent with previous work\textsuperscript{14, 16-20}. An average size of 6 ± 4 µm and 5 ± 3 µm was calculated for 50:50 CPH:SA and 50:50 CPTEG:CPH particles, respectively.

7.3.4 Mice

C57BL/6 mice were purchased from Harlan Sprague Dawley (Indianapolis, IN) and CR3\textsuperscript{−/−} mice (C57BL/6 background) were obtained as a generous gift from Dr. Mary Ann McDowell (University of Notre Dame). All mice were housed under specific pathogen-free conditions where all bedding, caging, and feed were sterilized prior to use. All animal procedures were conducted with the approval of the Iowa State University Institutional Animal Care and Use Committee.
7.3.5 Adsorption of Mouse Serum onto Microparticles

13.3% w/v suspensions of polymer microparticles were prepared in phosphate buffered saline (PBS) (0.1 M, pH 7.4). Mouse serum was obtained from cardiac puncture of euthanized C57BL/6 mice and storage in aliquots at -20 °C until use. Particle suspensions were incubated with fresh mouse serum to a 1:4 final volume ratio of particle suspension to serum. The suspension was mildly vortexed for 1 min and incubated for 30 min at 37 °C. After incubation, the particle suspension was centrifuged at 12,000 x g for 10 min to pellet the particle-protein complexes. The pellet was re-suspended in PBS, transferred to a new vial, and centrifuged again (at the same conditions) to pellet the particle-protein complexes; this procedure was repeated three times. After the third washing step the supernatant did not contain any detectable amount of proteins based on BCA protein detection assay and SDS-PAGE analysis. Microparticles were dried under vacuum overnight for at least 2 h.

7.3.6 Determination of Protein Adsorption Patterns

Proteins were eluted from the particles by applying a solution containing 10% (w/w) SDS and 2.3% (w/w) dithioerythritol for 2-D electrophoresis analysis and reducing sample buffer (SDS (10% w/v), Tris-HCl (1M, pH 6.8), glycerol (3 mM), bromophenol blue (0.01% w/v)) for SDS-PAGE analysis. Samples were heated at 95 °C for 10 min and particles were centrifuged for 10 min at 12,000 x g and the supernatant was used to perform SDS-PAGE and 2D electrophoretic analysis. 2D electrophoresis was used to qualitatively and semi-quantitatively identify the type of proteins adsorbed to polyanhydride particles. The first dimensional separation was
performed in the IPGPhor system (GE Healthcare, Piscataway, NJ) using 11-cm IPG strips (pH 3–10) following a slow voltage ramping protocol: 50 V for 10 h, 500 V for 1 h, 1000 V for 1 h, and 8000 V for 4 h $^{13,32}$. For the second dimension of the separation analysis, IPG strips were loaded in 4–15% polyacrylamide gels and run for 2 h at 140 V. The gels were incubated in fixative solution (40% ethanol, 10% acetic acid) at 4°C for 3 h. Next, they were stained with fluorescent flamingo gel stain (BioRad Laboratories, Richmond, CA) overnight, and washed with a 0.01% Tween 20 solution to reduce nonspecific fluorescence $^{13,16}$. A Typhoon 8600 (GE Healthcare, Piscataway, NJ) fluorescence scanner was used to obtain the images of the gels. Experiments were performed in triplicates. A qualitative analysis was performed by comparing the gels of the desorbed proteins with those obtained from mouse sera and Progenesis SameSpots (Nonlinear Dynamics Inc., Durham, NC) software was used to identify the main bands on the gels by comparison on their molecular weight and isoelectric point position on the gel. The fluorescence volume of each protein spot was quantified using ImageQuantTL (GE Healthcare, Piscataway, NJ) and normalized with the total fluorescence volume of the gel. Data for each protein spot is presented as percentage of fluorescence volume.

**7.3.7 Identification of Complement Component C3 and C3a**

Sandwich ELISA was used for identification of specific components in the protein mix recovered from microparticle surfaces. A mouse complement component C3 ELISA kit from Kamiya Biomedical Company (Seattle, WA) was utilized for quantitative determination of C3 in samples recovered from particle surfaces. Protocol was followed exactly as described by the company.
Complement activation was assessed by measuring the concentration of C3a in the serum supernatants after incubation with the polyanhydride particles. A mouse complement component 3a (C3a) ELISA kit from Kamiya Biomedical Company was utilized for this purpose.

7.3.8 DC Culture and Stimulation

DCs were grown as described previously and stimulated with either 200 ng/mL lipopolysaccharide (LPS; a positive control), 125 µg/mL of QD-loaded or blank 50:50 CPH:SA and 50:50 CPTEG:CPH microparticles or left untreated (NS; non-simulated, negative control). Treatments were applied to the DCs incubated in DC culture medium (RPMI containing 1% L-glutamine, 1% penicillin–streptomycin solution, 2% HEPES, 0.5% gentamicin, 0.1% β-mercaptoethanol, and 10% heat inactivated fetal bovine serum (FBS) supplemented with GMCSF (10 ng/mL)) on day nine post-harvest and incubated for 48 h. DCs were >90% positive for DC marker CD11c. For internalization studies, a released QD control was subtracted from each treatment group to account for QD release due to particle degradation. For this control, after 48 h of particle incubation in DC culture medium, particles were centrifuged and the supernatants containing the released QDs were added to DCs for 48 h to account for any fluorescence caused by the uptake of released QDs as opposed to internalization of QD-loaded particles. FluoSpheres® carboxylate-modified polystyrene microspheres (PS, 2µm, 580/605 nm, Invitrogen, Carlsbad, CA) were used as controls for internalization experiments.
7.3.9 Flow Cytometry Analysis

Flow cytometric analysis of surface molecule expression was performed to assess for the expression of MHC I, MHC II, CD40, and CD86 as previously described \(^3, 14, 33, 34\).

7.3.10 Cytokine Assays

After stimulation for 48 h with nanoparticles, cell-free supernatants were assayed for IL-1\(\beta\), IL-10, TNF-\(\alpha\), IL-6, and IL-12p40 using a multiplex cytokine assay in conjunction with a Bio-Plex System (BioRad, Hercules, CA).

7.3.11 Statistical Analysis

The statistical software JMP\textsuperscript{®}7 was used to analyze the cell surface marker, cytokine, and internalization data. One-way ANOVA and Tukey’s HSD were used to determine statistical significance among treatments and p-values < 0.05 were considered significant.

7.4 Results

7.4.1 Chemistry-dependent protein adsorption patterns: immunoglobulin G (IgG) and complement component C3 adsorption on polyanhydride particles surface

It is well known that cationic particles show greater adhesion with cell membranes and therefore it is important to determine the surface charge of polymeric particles since this property could influence their uptake. Measurements of \(\zeta\)-potential using QELS resulted in similar values (-22 ± 5.5 mV) for both particle chemistries and is consistent with previous work \(^3\). The presence of deprotonated carboxylic groups may account for
the negative surface charge of the polyanhydride particles. After incubation with mouse serum, the average ζ-potential was -5 ± 6 mV, which is an indication of the presence of serum proteins on the surface. This value for the ζ-potential is consistent with other work in which BSA adsorbed on the surface of negatively charged polystyrene particles reduced their highly negative ζ-potential ⁹.

SDS-PAGE and BCA protein quantification assay were used to determine quantitatively the adsorption of serum proteins onto polyanhydride microparticles. From SDS-PAGE results (data not shown), more intense bands are observed for 50:50 CPH:SA gels, which is indicative of a higher amount of proteins adsorbed on the surface of these microparticles. This pattern, corroborated with BCA assay, is consistent with previous work that showed that the hydrophobic nature of the CPH:SA system results in increased adsorption of specific proteins ¹³. In contrast, the presence of ethylene glycol motifs in the CPTEG:CPH system lead to a reduced protein adsorption from mouse serum ¹³, ³⁵. This repulsive effect of formulations containing poly(ethylene glycol) has been demonstrated previously and showed the reduction of opsonin (i.e., IgG and C3) adsorption ³⁵. Figures 7.1A and 7.1B show representative images of 2D gels for proteins eluted from 50:50 CPH:SA and 50:50 CPTEG:CPH particles, respectively. In order to identify the main proteins adsorbed on microparticles, gels obtained from eluted proteins from particles were compared with reference map and databases of mouse serum ³⁶. From Figures 7.1A and 7.1B, the main proteins identified on the surface of both 50:50 CPH:SA and 50:50 CPTEG:CPH particles were albumin and IgG; however, complement component C3 is mainly present on the surface of 50:50 CPH:SA particles. Table 7.1 shows a detailed analysis of the main proteins identified on polyanhydride
microparticles and it is presented as percentage of overall protein amount adsorbed to the particles (this analysis was made based on fluorescence volume). While opsonins were the predominant type of protein bound to 50:50 CPH:SA particles, other proteins including apolipoproteins were present on 50:50 CPTEG:CPH particles.

![Diagram A](image1)
![Diagram B](image2)
![Diagram C](image3)
![Diagram D](image4)

**Figure 7.1.** Chemistry-dependent protein adsorption patterns: immunoglobulin G (IgG) and complement component C3 adsorption on polyanhydride particles surface.
Representative 2-D gels of proteins adsorbed on 50:50 CPH:SA (A) and 50:50 CPTEG:CPH (B) particles. (C) Complement component C3 was adsorbed on the surface of 50:50 CPH:SA microparticles, as measured through an anti-mouse complement C3 ELISA. Data is presented as µg of C3/cm$^2$. Values are the mean of three independent experiments; error bars correspond to standard error of means. * represents p-value ≤ 0.05, as obtained from a student t-test. (D) 50:50 CPH:SA microparticles induced C3 cleavage (i.e., complement activation) as measured by the appearance of C3a in mouse serum supernatant after incubation with microparticles. Data is normalized with control serum that was similarly incubated in the absence of any particles (negative control). Values are means of three independent experiments; error bars correspond to standard error of mean. * represents p-values ≤ 0.05.

It has been shown that encapsulation of different payloads can change surface properties (i.e., ζ-potential) of polyanhydride particles depending on the nature of the payload and how it distributes inside the particle $^{17}$. The effect of antigen encapsulation on the patterns of serum proteins adsorbed on polyanhydride particles was evaluated by using ovalbumin (Ova) as a model antigen. Encapsulation of Ova did not change the chemistry-dependent pattern of proteins adsorbed to the microparticles (data not shown).

The amount of C3 adsorbed to the microparticles was quantified in the samples of eluted proteins (Figure 7.1C). An average of 0.12 µg/cm$^2$ and 0.38 µg/cm$^2$ were calculated for C3 adsorption on 50:50 CPH:SA and 50:50 CPTEG:CPH particles, respectively. Cleavage of C3 was evaluated by assessing the presence of C3a in serum supernatant after incubation with polyanhydride particles. The data presented in Figure 7.1D shows that higher amounts of C3a were generated after incubation of 50:50 CPH:SA particles in mouse serum.
Table 7.1. Representative percentages of the most abundant protein species adsorbed on 50:50 CPH:SA and 50:50 CPTEG:CPH particles$^{a,b}$.

<table>
<thead>
<tr>
<th>Adsorbed protein</th>
<th>50:50 CPH:SA</th>
<th>50:50 CPTEG:CPH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Albumin</td>
<td>23.2</td>
<td>28.7</td>
</tr>
<tr>
<td>IgG γ chain</td>
<td>32.5</td>
<td>25.4</td>
</tr>
<tr>
<td>IgG light chain</td>
<td>4.1</td>
<td>5.3</td>
</tr>
<tr>
<td>IgM</td>
<td>7.2</td>
<td>10.4</td>
</tr>
<tr>
<td>Complement component C3</td>
<td>25</td>
<td>7.9</td>
</tr>
<tr>
<td>Apolipoprotein A-I</td>
<td>1.4</td>
<td>2.5</td>
</tr>
<tr>
<td>Apolipoprotein E</td>
<td>0.3</td>
<td>3.8</td>
</tr>
<tr>
<td>Apolipoprotein H</td>
<td>0.0</td>
<td>8.5</td>
</tr>
<tr>
<td>Transferrin</td>
<td>0.0</td>
<td>2.3</td>
</tr>
</tbody>
</table>

$^a$ Data for each protein spot is presented as percentage of fluorescence volume. The fluorescence volume of each protein spot was quantified using ImageQuantTL and normalized with the total fluorescence volume of the gel, which include all protein spots.

$^b$ Data is representative of three independent experiments.

7.4.2 Internalization of non-serum and serum$^+$ polyanhydride particles is mediated by complement receptor 3

The ability of the polyanhydride particles to be internalized by DCs was tested in order to determine the effect of adsorbed proteins to enhance particle internalization.

When 50:50 CPH:SA particles were incubated with DCs, ~20% of cells were observed to contain particles, while incubation of 50:50 CPTEG:CPH particles with DCs resulted in less than 10% particle-positive cells (Figure 7.2). The low internalization observed for the amphiphilic 50:50 CPTEG:CPH microparticles was consistent with previous work from our laboratories $^{34, 37}$. The effect of serum protein adsorption on particle internalization was assessed. When serum-coated microparticles were added to the DC cultures, it was observed that 20 % more DCs had internalized 50:50 CPH:SA particles
which may be a consequence of the interaction of opsonins (i.e., IgG and C3) with cellular receptor like FcR and C3 receptors. To evaluate this hypothesis, internalization of sham-coated and serum-coated particles by DCs deficient in CR3 was tested. Strikingly, internalization of both sham-coated and serum-coated polyanhydride particles was significantly reduced independent of particle chemistry. Similar levels of internalization of polystyrene particles by WT and CR3−/− DCs were observed (Figure 7.2).

**Figure 7.2.** Internalization of polyanhydride particles is mediated by complement receptor 3. Percentages of DCs with internalized microparticles after incubation with sham-coated and and serum-coated 50:50 CPH:SA and 50:50 CPTEG:CPH particles was measured by flow cytometry. Data is represented as mean ± standard error of three
independent experiments performed in triplicate. * indicates significant differences between WT and CR3\(^+\) within a group, while # indicates significant differences between sham-coated and serum-coated particles within a chemistry.

7.4.3 Serum proteins adsorbed on particle surfaces influence the expression of MHC II and co-stimulatory molecules on DCs in a chemistry dependent manner

We have previously demonstrated the chemistry-dependent activation of DCs by polyanhydride particles \(^{14,34}\). The results shown in Figure 7.3A corroborate these findings with enhanced expression of MHC II when DCs are incubated with 50:50 CPTEG:CPH particles compared to that obtained with 50:50 CPH:SA particles. Both polymer chemistries equally enhanced the surface expression of the co-stimulatory molecules CD86 and CD40 (Figures 7.3B and 7.3C). Interestingly, the increase in internalization observed for serum-coated 50:50 CPH:SA particles results in the up-regulation of both MHC II and CD40 (Figures 7.3A and 7.3C).

Analysis of particle\(^+\) and particle\(^-\) populations of DCs from WT mice showed a direct correlation between particle internalization and the surface expression of CD40 was observed. In contrast, enhancement of MHC II and CD86 surface expression on DCs was not dependent on nanoparticle internalization, as evidenced by no significant difference in expression levels between particle\(^-\) and particle\(^+\) populations (Supplemental Figure 7.1). These observations are consistent with previous work showing the relationship between polyanhydride nanoparticle internalization and activation of murine DCs \(^{34}\).
Figure 7.3. Serum proteins adsorbed on particles influence the expression of MHC II and co-stimulatory molecules on DCs in a polymer chemistry-dependent manner. After stimulation with either non-serum or serum+ microparticles for 48 h, DCs were harvested from WT mice and analyzed by flow cytometry for surface expression of (A) MHC II, (B) CD86, (C) CD40. LPS and non-stimulated cells (NS) were used as positive and negative controls, respectively. Data are expressed as the mean ± standard error of three independent experiments performed in triplicate. * represents significant difference compared to the NS group at p-value ≤ 0.05, while # represents significant difference between sham-coated and serum-coated particles within a group at p-value ≤ 0.05.
7.4.4 Reduced internalization of non-serum and serum adsorbed particles by CR3⁺ DCs results in reduction in the expression of MHC II and CD40 molecules

Results showed in Figure 7.4 demonstrates that the reduction in particle internalization by CR3⁺ DCs results in changes in DC activation profiles. A significant decrease in the expression of MHC II and CD40 was also observed (Figures 7.4A and 7.4C). Expression of CD86 on CR3⁺ DCs was not statistically significant from that observed in WT DCs for all the particle groups, indicating that its expression is independent of CR3-mediated internalization (Figure 7.4B).

7.4.5 Serum proteins adsorbed on 50:50 CPTEG:CPH microparticles enhance secretion of pro-inflammatory cytokines (i.e., IL-6 and TNF-α) and IL-12p40

In addition to antigen presentation and T cell activation, DCs also modulate immune responses via the cytokines they secrete. After 48 h of incubation with particles of either chemistry, enhanced secretion of IL-1β was observed (Figure 7.5A). However, only 50:50 CPH:SA particles increased the secretion of IL-6, IL-12p40, and TNF-α in comparison with the non-stimulated cells (Figures 7.5B, 7.5C, and 7.5D). This is in agreement with previous work that showed that the hydrophobic 50:50 CPH:SA particles significantly influenced DC cytokine production14,33. Moreover, the secretion of IL-6, IL-12p40 and TNF-α by DCs incubated with serum-coated 50:50 CPTEG:CPH particles was significantly elevated (Figures 7.5B, 7.5C, and 7.5D).
Figure 7.4. Reduced internalization of non-serum and serum adsorbed particles by CR3$^{-/-}$ DCs results in reduction in the expression of MHC II and CD40 molecules. After stimulation with either non-serum or serum$^+$ microparticles for 48 h, DCs were harvested from WT and CR3$^{-/-}$ mice and analyzed by flow cytometry for surface expression of (A) MHC II, (B) CD86, (C) CD40. LPS and non-stimulated cells (NS) were used as positive and negative controls, respectively. Data are expressed as the mean ± standard error of three independent experiments performed in triplicate. * represents significant difference between non-serum and serum$^+$ within a group at p-value ≤ 0.05, while # represents significant difference between WT and CR3$^{-/-}$ within a group at p-value ≤ 0.05.
In general, no differences in the amounts of secreted cytokines by WT and CR3\(^{-/-}\) DCs were observed. The only exception was a decrease in the production of IL-12\(p40\) by CR3\(^{-/-}\) DCs when cultured with serum-coated 50:50 CPH:SA particles (Supplemental Figure 7.2). It has been previously shown that secretion of IL-12\(p40\) is enhanced by particle internalization\(^{34}\); as the internalization of 50:50 CPH:SA particles is considerably reduced by CR3\(^{-/-}\) DCs, the reduction in secretion of IL-12\(p40\) after incubation with this particles may be related with this decrease in internalization.

7.5 Discussion

Biodegradable polymeric particles have been extensively studied as carriers for the delivery of antigens and drugs\(^{1,14,15,39,40}\). APCs are cells that would encounter and engage with polymeric nanoparticles in a manner involving the interaction of polymeric structures on the surface of the particle with membrane receptors on the APC. This interaction will initiate particle uptake and influence the magnitude and polarity of the immune resultant response\(^{1,2}\). Adsorption of specific serum proteins to the surface of polymeric particles alters their recognition and uptake by APCs\(^{12,13,41}\). The data presented herein demonstrate that the chemistry-dependent adsorption of serum proteins on polyanhydride particles (Figure 7.1) influences their internalization and the activation of murine DCs (Figures 7.2, 7.3, 7.4 and 7.5 and Supplemental Figures 7.1 and 7.2).
Figure 7.5. Serum proteins adsorbed on 50:50 CPTEG:CPH microparticles enhance secretion of IL-6, TNF-α and IL-12p40. After stimulation with either non-serum or serum* microparticles for 48 h, supernatants were harvested and assayed for (A) IL-1β, (B) IL-6, (C) IL-12p40, and (D) TNF-α. Data is represented as mean concentration of cytokines ± standard error of three independent experiments performed in triplicate. LPS was used as a positive control stimulant with mean values of 1,041 pg/mL for IL-1β, 44,329 pg/mL for IL-6, > 50,000 pg/mL for IL-12p40 and TNF-α. * represents significant difference compared to the NS group at p-value ≤ 0.05, while # represents significant difference between non-serum and serum* within a group at p-value ≤ 0.05.
Particle chemistry and hydrophobicity play an integral role in particle internalization by DCs, as well as DC activation profiles (i.e., cell surface marker expression and cytokine production) \(^3, 8, 14, 33, 34\). These properties also determine patterns of serum protein adsorption on polyanhydride particles. While the hydrophobic 50:50 CPH:SA particles adsorb higher amounts of serum proteins, including IgG and complement component C3, the presence of ethylene glycol in the amphiphilic 50:50 CPTEG:CPH reduced serum protein adsorption (Figure 7.1). Identification of C3 coating on polyanhydride particles is important since it is known to mediate cellular uptake of particles via complement receptor-mediated mechanisms \(^35, 42\). The inhibitory effect of EG-containing particles on protein adsorption has been previously observed and is related to longer particle circulation times \(^9, 35, 42, 43\).

Identification of C3 is of special interest because of the potential of using the complement cascade as a danger signal of innate immunity, which is a desirable goal in the design of adjuvants \(^44\). Complement can also promote antigen-specific immune responses enhancing both humoral \(^45\) and T cell immunity \(^46\). Just like on pathogen surfaces, instantaneous complement activation by the alternative pathway can be achieved by deposition of C3 on the surface of polymer particles that can promote cleavage to form C3a and C3b fragments, which have biological functions \(^44, 47\). In this study, we demonstrate that polyanhydride chemistries (i.e., 50:50 CPH:SA and 50:50 CPTEG:CPH) activate complement, as shown in Figure 7.1D in which the presence of the C3a fragment was identified in serum after incubation with polyanhydride particles.

The molecular mechanisms and interactions of these particulate adjuvants with dendritic cells have been an area of study in our laboratories \(^3, 14, 33, 34, 37\) and we have
identified polymer-associated molecular patterns and descriptors responsible for the “pathogen-mimicking” activation of DCs$^{34,37}$; however, there is a value to study the effect of serum protein adsorption and its consequent outcomes (i.e., complement activation) on the molecular interactions with DCs. Non-serum coated 50:50 CPH:SA and 50:50 CPTEG:CPH particles were internalized by DCs but a higher percentage of cells with internalized particels (~22%) was identified when cells were co-cultured with 50:50 CPH:SA particles (Figure 7.2). These observations of chemistry-dependent internalization of polyanhydride particles are consistent with previous work$^{8,14,34}$. Serum-coated 50:50 CPH:SA particles were internalized at higher levels by DCs (~42% of particle$^+$ cells) and is hypothesized to be a consequence of greater adsorption of opsonins (i.e., IgG and C3) that could interact with Fcγ and complement receptors (i.e., CR3 and CR1), respectively, resulting in higher particle uptake$^4,38$. It is known that CR3 binds to C3bi, an inactive form of the C3b cleavage product of C3, fixed on particle surfaces, but CR3 may also cooperate with CR1, which binds to C3b or Fcγ receptors to facilitate antibody-dependent phagocytosis [48, 49]. Our data indicates that binding of C3 on the surface of 50:50 CPH:SA allows for cleavage of C3 into C3a and C3b fragments (Figure 7.1D), which suggest a role of CR3 receptor on the internalization of serum-coated particles. Using CR3$^{+/−}$ DCs a 90% reduction in particle$^+$ cells was observed, for this serum-coated particles. This data is consistent with previous work that demonstrated the role of CR3 receptor in the recognition of opsonized bacteria and zymosan particles$^{22,50,51}$. However, a similar inability to uptake non-serum coated particles for both polyanhydride chemistries was observed in CR3$^{+/−}$ DCs, demonstrating a CR3 receptor-
mediated uptake pathway for non-serum coated particles (Figure 7.2). This outcome demonstrates that C3 receptor plays a critical role in the internalization of polyanhydride particles. These observations are consistent with the pathogen-mimicking characteristics of polyanhydride particles since certain domains (i.e., lectin site) of the CR3 receptor have been related to the binding and phagocytosis of non-opsonic *Mycobacterium tuberculosis*, LPS, *Leishmania* lipophosphoglycan (LPG), and various particulate saccharides that include β-glucan and zymosan. Detailed studies in our laboratories have previously demonstrated the similar DC activation properties between polyanhydride particles and LPS identifying hydroxyl end group and oxygen backbone moieties as some of the important pathogen-mimicking structural descriptors. These structural descriptors may be also dictating the CR3-mediated internalization of polyanhydride particles described in this study, which appears to be specific for these chemistries as demonstrated by the high uptake levels of non-serum and serum-coated polystyrene particles by CR3⁺ DCs (Figure 7.2). The biodegradable nature of polyanhydride particles that allows the exposure of molecular patterns seems to be detrimental to dictate interactions with specific DC receptors (i.e., CR3) compare to those initiated by PS particles, which may be internalized by a different mechanism.

Besides particle internalization, the up-regulation of antigen presentation machinery and T cell co-stimulatory molecules as well as cytokine secretion by DCs are important and desirable characteristics of vaccine adjuvants in order to efficiently activate naïve T cells and promote the differentiation of B cells. A polymer chemistry-dependent enhancement in the expression of MHC II and co-stimulatory molecules (i.e., CD40 and CD86) and secretion of cytokines was observed in this study. (Figures 7.3, 7.4 and 7.5)
and is consistent with previous observations \textsuperscript{14, 33, 34, 37}. While 50:50 CPTEG:CPH particles enhanced the expression of MHC II, CD86 and CD40, 50:50 CPH:SA particles increased the secretion of IL-6, IL12-p40 and TNF-\(\alpha\). However, adsorption of serum proteins on the hydrophobic 50:50 CPH:SA particles resulted in higher expression of MHC II and CD40 (\textbf{Figures 7.3} and \textbf{7.4}). While the enhanced expression of CD40 was identified to be directly dependent on particle internalization, as shown in \textbf{Figure 7.4} and in previous work \textsuperscript{34}, the expression of MHC II was independent of particle internalization, but may be induced by CD40 triggering \textsuperscript{53}. On the other hand, serum protein adsorption on the amphiphilic 50:50 CPTEG:CPH particles increased the secretion of pro-inflammatory cytokines (i.e., IL-6 and TNF-\(\alpha\)) and IL-12p40 in comparison to non-serum-coated particles (\textbf{Figure 7.5}). The non-serum-coated particles did not induce the production of these cytokines, which is detrimental for the initiation of danger signals to trigger an adaptive immune response. Therefore, chemistry-dependent adsorption of serum proteins influences the expression of key surface markers and production of cytokines that are involved in DC maturation and antigen presentation.

Together, these results indicate that polyanhydride particles are capable of inducing DC activation and that polymer chemistry can be rationally designed to induce this mature DC phenotype; for example, the intrinsic characteristics of the amphiphilic 50:50 CPTEG:CPH (e.g., presentation of oxygen-rich molecular patterns to cells) particles can induce DC maturation (i.e., enhancement in MHC II, CD86, and CD40 expression) while the hydrophobicity of 50:50 CPH:SA particles can direct specific protein adsorption patterns that will also enhance its adjuvant properties. The specific interactions of particles with serum proteins and their consequences in the molecular interaction with
DCs documented in this study presents an intriguing opportunity for the rational design of vaccines possessing the capacity to induce diverse immune responses tailored for a target disease.

### 7.6 Conclusions

In this study, mouse serum protein adsorption patterns on the activation of DCs by polyanhydride particles were dictated by polymer chemistry. The polymer chemistry-dependent activation of DCs by non-serum coated particles was confirmed and the ability of adsorbed serum proteins to trigger the adjuvant properties of these particles was identified. The data indicates that complement receptor C3-mediated pathways are involved in the internalization of both non-serum- and serum-coated polyanhydride particles by DCs, highlighting the pathogen-mimicking characteristics of these particles. The receptor-mediated endocytosis induced by the direct interaction of adsorbed opsonins or intrinsic pathogen-mimicking patterns of polyanhydride particles with the complement receptor C3 may be exploited to design efficacious vaccines.

### 7.7 Acknowledgments

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7.8 References


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Supplemental Figure 7.1. Expression of CD40 is directly related to particle internalization by WT DCs but not the expression of MHC II or CD86. Percent of DC populations that internalized (particle\(^{+}\)) or did not internalize (particle\(^{-}\)) 50:50 CPH:SA and 50:50 CPTEG:CPH with or without serum proteins adsorbed on their surface that were also positive for (A) MHC II, (B) CD86, and (C) CD40. Data are represented as the mean ± standard error of three independent experiments each of which was performed in triplicate. * represents a statistically significant difference between nanoparticle\(^{+}\) and nanoparticle\(^{-}\) groups within a group at p-value < 0.05.
Supplemental Figure 7.2. Cytokine secretion profiles from CR3⁻⁻ DCs were consistent with those observed from WT DCs. After stimulation with either non-serum or serum⁺ microparticles for 48 h, supernatants were harvested and assayed for (A) IL-1β, (B) IL-6, (C) IL-12p40, and (D) TNF-α. Data is represented as mean concentration of cytokines ± standard error of three independent experiments performed in triplicate. LPS was used as a positive control stimulant with mean values of 1,041 pg/mL for IL-1β, 44,329 pg/mL for IL-6, > 50,000 pg/mL for IL-12p40 and TNF-α. * represents significant difference compared to the NS group at p-value ≤ 0.05, while # represents significant difference between non-serum and serum⁺ within a group at p-value ≤ 0.05.
CHAPTER 8

Mannose-Functionalized “Pathogen-Like” Polyanhydride Nanoparticles Target C-Type Lectin Receptors on Dendritic Cells

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8.1 Abstract

Targeting pathogen recognition receptors on dendritic cells (DCs) offers the advantage of triggering specific signalling pathways to induce a tailored and robust immune response. In this work, we describe a novel approach to targeted antigen delivery by decorating the surface of polyanhydride nanoparticles with specific carbohydrates to provide “pathogen-like” properties that ensure nanoparticle engage C-type lectin receptors on DCs. The surface of polyanhydride nanoparticles was functionalized by covalent linkage of di-mannose and lactose residues using an amine-carboxylic acid coupling reaction. Co-culture of functionalized nanoparticles with bone marrow-derived DCs significantly increased cell surface expression of MHC II, the T cell co-stimulatory molecules CD86 and CD40, the C-type lectin receptor CIRE and the mannose receptor CD206 over the non-functionalized nanoparticles. Both non-functionalized and functionalized nanoparticles were efficiently internalized by DCs, indicating that internalization of functionalized nanoparticles was necessary but not sufficient to activate DCs. Blocking the mannose and CIRE receptors prior to the addition of functionalized nanoparticles to the culture inhibited the increased surface expression of MHC II, CD40 and CD86. Together, these data indicate that engagement of CIRE and the mannose receptor is a key mechanism by which functionalized nanoparticles activate DCs. These studies provide valuable insights into the rational design of targeted nanovaccine platforms to induce robust immune responses and improve vaccine efficacy.
8.2 Introduction

The use of vaccine adjuvants to activate the innate immune system is crucial to vaccine effectiveness\(^1\). Adjuvants can be used to enhance the efficacy of single dose vaccines and reduce the required antigen dose. The use of biodegradable polymer nanoparticles as vaccine delivery vehicles allows for effective delivery of payloads by parental or mucosal administration by protecting the antigen from harsh physiological conditions and enabling transport across biological barriers (e.g., mucus membranes)\(^2\). Polyanhydride nanoparticles have shown excellent potential as vaccine carriers\(^3-6\). Encapsulation of protein antigens into polyanhydride particles stabilizes them and
provides sustained antigen release\textsuperscript{4,7}; these particles also enhance the immune response by acting as an adjuvant\textsuperscript{3}.

Dendritic cells (DCs) are antigen presenting cells (APCs) that play a major role in connecting the innate and adaptive immune systems, a key step to inducing protective immunity\textsuperscript{8}. DCs can sense and internalize antigen by a variety of mechanisms that trigger DC maturation and direct further interactions with other immune cells, including naïve T cells\textsuperscript{1,9,10}. Pattern recognition receptors (PRRs) on DCs detect the presence of a potential threat by interacting with pathogen-associated molecular patterns (PAMPs)\textsuperscript{10,11}. In particular, C-type lectin receptors (CLRs) are PRRs with highly conserved carbohydrate-recognition domains that bind sugar moieties (e.g., mannose, fucose, and glucan) on the surface of certain pathogens (e.g., \textit{Candida albicans}, \textit{Mycobacterium tuberculosis}, and \textit{Mycobacterium leprae}) in a calcium-dependent manner\textsuperscript{12,13}. Ligand recognition by Toll-like receptors, a type of PRR, promotes a cascade of intracellular signaling events\textsuperscript{14}. In addition, ligand recognition by CLRs leads to pathogen internalization via receptor mediated endocytosis and subsequent degradation and presentation of the pathogen as antigen to T cells in the context of MHC I or MHC II, or both\textsuperscript{13,15}. Engagement of CLR-associated signaling processes can induce diverse immune responses depending upon the type of carbohydrate ligand bound, the type of responding APC and the engagement of other PRRs on the APC\textsuperscript{12}. Thus, the targeting to and the activation of signaling pathways associated with CLRs (e.g., activation of NF-κB signaling pathway) can be a powerful strategy to tailor the immune response\textsuperscript{11,13,14}

Previous studies have demonstrated the potential of targeting CLRs to induce DC maturation and activation; these studies focused on the use mannoproteins from
pathogens\textsuperscript{16} or the conjugation of glycans directly to the antigen\textsuperscript{17}. Limited studies have explored the use of carbohydrate-functionalized vaccine carriers as targeting vehicles, with most studies using antibodies as ligands\textsuperscript{18}. This study outlines a novel approach to target the mannose receptor (CD206) and CIRE (CD209) on murine DCs by mimicking carbohydrate moieties found on the surface of pathogens\textsuperscript{19}. Polyanhydride nanoparticle surfaces were functionalized with di-mannose in order to study specific interactions with both CLRs known to recognize mannose residues\textsuperscript{10,13}. The polyanhydride system used in this study is an amphiphilic copolymer of 1,6-bis(p-carboxyphenoxy)hexane (CPH) and 1,8-bis(p-carboxyphenoxy)-3,6-dioxaoctane (CPTEG). The studies described herein demonstrate that mannose-functionalized polyanhydride nanoparticles induce receptor-mediated activation of DCs that may be exploited to enhance the adjuvanticity of this targeted delivery platform.

8.3 Materials and Methods

8.3.1 Materials

Chemicals needed for monomer synthesis and polymerization, as well as for nanoparticle fabrication, including sebacic acid (99\%), p-carboxy benzoic acid (99+\%), and 1-methyl-2-pyrrolidinone, anhydrous (99+\%), were purchased from Aldrich (Milwaukee, WI); 4-p-hydroxybenzoic acid, 1,6-dibromohexane, 1-methyl-2 pyrrolidinone, and tri-ethylene glycol were purchased from Sigma Aldrich (St. Louis, MO); 4-p-fluorobenzonitrile was obtained from Apollo Scientific (Cheshire, UK); potassium carbonate, dimethyl formamide, toluene, sulfuric acid, acetic acid,
acetonitrile, acetic anhydride, methylene chloride, pentane, and petroleum ether were purchased from Fisher Scientific (Fairlawn, NJ).

**8.3.2 Monomer and Polymer Synthesis**

CPTEG:CPH copolymers were synthesized by melt polycondensation as described previously\(^{20}\). The chemical structure was characterized with \(^1\)H NMR and the molecular mass was determined using gel permeation chromatography (GPC)\(^{20}\).

**8.3.3 Nanoparticle Fabrication**

An anti-solvent nanoencapsulation method recently published by Ulery et al.\(^ {21}\) was used to fabricate nanoparticles based on a 50:50 molar ratio of CPTEG:CPH copolymers (50:50 CPTEG:CPH). Briefly, polymer was dissolved in methylene chloride (15 mg/mL) held at 4\(^{\circ}\)C. The polymer solution was rapidly poured into a bath of pentane held at -40\(^{\circ}\)C at an anti-solvent to solvent ratio of 1:150. Particles were collected by filtration and dried under vacuum for 2 h. For fluorescence microscopy studies, FITC-dextran (1% (w/w)) was suspended and homogenized by sonication in the polymer solution prior to precipitation.

**8.3.4 Nanoparticle Surface Functionalization**

**8.3.4.1 Synthesis of Carboxylated-Lactose and Di-mannose**

\(\beta\)-1-O-allylated lactose\(^ {22}\) was prepared through mercury (II)-catalyzed allylation\(^ {23}\) of penta-O-acetyl-lactosylbromide. As expected, using excess amounts of NaIO\(_4\) (8 equivalents) under ruthenium-catalyzed Sharpless conditions\(^ {24,25}\) produced the desired
acid in 91% yield. Subsequent deacetylation under mild condition using K$_2$CO$_3$ provided the desired fully deprotected disaccharide (Figure 8.1A).

To obtain the dimannoside, the iterative synthesis of linear α-1,2-linked dimannose with a fluorous allyl group using fluorous-solid phase extraction (FSPE) served as the model\textsuperscript{26–28}. Carboxymethyl- di-mannose was obtained by ozonolysis of the alkene\textsuperscript{29}, followed by further oxidation with Jones reagent\textsuperscript{26}. Global deprotection under Birch reduction\textsuperscript{30} furnished the fully deprotected α-1,2-linked di-mannose (Figure 8.1B).

![Figure 8.1](image.png)

**Figure 8.1.** Schematic representation of the synthesis of (A) carboxy-functionalized lactose and (B) carboxy-functionalized di-mannose.
8.3.4.2 Surface Functionalization

Lactose and di-mannose were conjugated on the surface of polyanhydride nanoparticles by an amine-carboxylic acid coupling reaction\textsuperscript{28,30,31}. Lactose, a neutral sugar with the same molecular weight as di-mannose, was chosen as a control sugar because of its different stereochemical configuration compared to di-mannoside. Particles with glycolic acid groups on the surface (linker groups) and non-functionalized (NF) particles were used as controls. An average concentration of 9.6 x 10\textsuperscript{-4} mmol COOH/mg of NF nanoparticles was calculated by a theoretical approach using the end groups obtained from \textsuperscript{1}H NMR spectra and by deconvolution analysis of the C1s high-resolution spectra obtained from X-Ray Photoelectron Spectroscopy (XPS). The conjugation reaction was performed by incubating a nanoparticle suspension (100 mg/mL) with 10 equivalents of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC), 12 equivalents of N-hydroxysuccinimide (NHS), and 10 equivalents of ethylene diamine in distilled water. This first incubation was carried out at room temperature for 12 h at a constant agitation of 350 rpm. After the incubation period, particles were centrifuged at 10,000 rpm for 10 min and the supernatant was removed. A new incubation was performed with 12 eq. of EDC, 12 eq. of NHS and 10 eq. of the corresponding saccharide (i.e., lactose or di-mannose) in distilled water, using constant agitation at 350 rpm for 12 h at room temperature. Particles were sonicated before and after each incubation period to break aggregates. After the reaction was completed, nanoparticles were collected by centrifugation (10,000 rpm, 10 min) and dried under vacuum overnight.
8.3.4.3 Characterization of Functionalized Nanoparticles

The morphology and size of functionalized and non-functionalized nanoparticles were characterized by scanning electron microscopy (SEM, JEOL 840A, JEOL Ltd., Tokyo, Japan) and quasi-elastic light scattering (QELS, Zetasizer Nano, Malvern Instruments Ltd., Worchester, UK), respectively. For SEM, samples were vacuum dried and coated with gold before imaging with the electron microscope. The nanoparticles were suspended in cold water and sonicated for one minute before QELS readings were performed. QELS was also used to determine the ζ-potential of non-functionalized and functionalized nanoparticles in order to determine the presence of the disaccharides. XPS (PHI 5500 Multi-technique system, Physical Electronics, Inc., Chanhassen, MN) was used to determine the presence of lactose and di-mannose on the surface of polyanhydride nanoparticles. Binding energies were referenced to the aliphatic hydrocarbon peak at 285.0 eV$^{32}$. High-resolution C1s peaks were collected and fitted using CasaXPS software (RBS Instruments, Bend, OR). Finally, a modified and optimized high throughput version of a phenol-sulfuric acid assay$^{33}$ was used to quantify the amount of sugar attached to the nanoparticles. The modified assay was performed directly in 96-well plates and the incubation time was increased to 30 min to allow for equal reduction of the different sugars used to link to the nanoparticles. Non-functionalized nanoparticles were used as controls to account for polymer interference with the assay. A microplate reader was used to obtain the absorbance of standards and unknown samples using a wavelength of 490 nm. The total amount of sugar (μg/mg) was calculated by normalization with the nanoparticle mass.
8.3.5 *In vitro* DC Uptake and Activation

8.3.5.1 Mice

C57BL/6 (B6) mice were purchased from Harlan Sprague Dawley (Indianapolis, IN). All mice were housed under specific pathogen-free conditions where all bedding, caging, and feed were sterilized prior to use. All animal procedures were conducted with the approval of the Iowa State University Institutional Animal Care and Use Committee.

8.3.5.2 DC Culture and Stimulation

Dendritic cells were derived from the bone marrow precursor cells recovered from the femurs and tibias of B6 mice according to a previously published protocol\textsuperscript{34,35}. DCs were >90% positive for DC marker CD11c. For flow cytometry analysis, non-stimulated (NS) DCs and *Escherichia coli* lipopolysaccharide (LPS) (200 ng/mL) (Sigma Aldrich, St. Louis, MO) treated DCs were used as negative and positive controls, respectively. On day 9 of the culture, non-functionalized or functionalized nanoparticles were incubated with DCs for 48 h at a concentration of 0.125 mg/mL, a concentration chosen based on previously published studies\textsuperscript{35}. In order to investigate the role of mannose receptor engagement in DC activation by di-mannose-functionalized polyanhydride nanoparticles, DCs were incubated with anti-CD206 (Clone 310301, R&D Systems, Minneapolis, MN) and/or anti-CD209 (Clone LWC06, eBioscience, San Diego, CA) antibodies either individually or in combination at a final concentration of 10 µg/mL for 15 min at room temperature prior to addition of stimulants. Pre-incubation with anti-CD206 and/or anti-CD209 blocked the cross-linking of the mannose and/or CIRE receptors by sugar-functionalized nanoparticles. This treatment tested the hypothesis...
that functionalized nanoparticles enhance DC activation via increased mannose and/or CIRE receptor-mediated endocytosis.

8.3.5.3 Flow Cytometric Analysis

Flow cytometric analysis of surface molecule expression was performed as previously described\(^\text{34}\). Additional antibodies used in this study include FITC anti-mouse CD206 (MMR, clone MR5D3) and the isotype-specific control antibody FITC rat IgG2a κ (clone RTK2748) purchased from BioLegend (San Diego, CA), and biotin anti-mouse CD209 (CIRE, clone 5H10) purchased from eBioscience (San Diego, CA). As noted, the two antibodies used for FACS analysis are different from the antibodies used to block CLRs, thus preventing false negatives due to unavailable binding sites following treatment with the blocking antibody. Samples were acquired on a Becton-Dickinson FACSCanto\(^\text{TM}\) flow cytometer (San Jose, CA) and the data analyzed using FlowJo (TreeStar Inc., Ashland, OR). Percentage of cells expressing each cell surface marker were obtained by quantifying the peak shift from their respective isotype controls and the intensity of the expression was reported as mean fluorescence intensity (MFI).

8.3.5.4 Cytokine Assays

After stimulation for 48 h with non-functionalized or functionalized nanoparticles, cell-free supernatants were assayed for IL-1β, IL-10, TNF-α, IL-6, and IL-12p40 using a multiplex cytokine assay in conjunction with a Luminex 100 System (Flowmetrics, Austin, TX) as described elsewhere\(^\text{34,35}\).
8.3.5.5 Fluorescence Microscopy Analysis of Nanoparticle Uptake by DCs

To visualize nanoparticle interactions with DCs, a previously described experimental protocol for co-incubation, immunofluorescence preparation and microscopic observation was followed. In these experiments, an equivalent amount (200 µg/mL) of either non-functionalized or functionalized 1% FITC-dextran loaded nanoparticles were incubated with DCs, then washed repeatedly three times with pH 7.4 PBS to remove extracellular and non-adherent particles. DCs co-incubated with nanoparticles for 30 min were fixed and analyzed 2 h post uptake. At the indicated times, cultures were fixed for 20 min with 4% paraformaldehyde in pH 7.4 PBS, washed and incubated with 5 µg/mL tetramethylrhodamine-wheat germ agglutinin (WGA) for 10 min at 37°C. Stained and washed coverslips were mounted on glass slides using Pro-Long w/DAPI mountant (Invitrogen, Carlsbad, CA). Epifluorescence microscopy was performed using an Olympus IX-71 inverted microscope with blue, green, and red filter sets with a cooled CCD camera. Final image preparation along with morphometric analysis was performed as described previously by us employing thresholding and particle counting algorithms of ImageJ v1.36b (NIH, Bethesda, MD). Binary data was extracted from grayscale images collected from the FITC bandpass filter set using equivalent exposure settings from fixed samples of DCs various nanoparticle modifications. For each sample, images from 5 fields of view (FOV) were collected and compiled with all grayscale images, including the 5 FOVs from the various nanoparticle formulations. The compiled image set was then batch treated for background subtraction and thresholding using parameters set according to negative (no particle) control cultures that were performed
in parallel for each experiment and time point. Results presented are compiled from three separate biological replicates.

8.3.5.6 Statistical Analysis

The statistical software JMP® 7 was used to analyze the cell surface marker, cytokine, and internalization data. One-way ANOVA and Tukey’s HSD were used to determine statistical significance among treatments and p-values < 0.05 were considered significant.

8.4 Results

8.4.1 Polymer Synthesis

A 50:50 molar ratio copolymer of CPTEG and CPH was synthesized as previously described20. This formulation was chosen for these studies because previous studies have shown that it preserves protein structure and antigenicity in addition to exhibiting adjuvant properties4,5,7,34. 1H NMR spectroscopy was used to characterize the polymer structure and the spectra were consistent with previously published data20. The synthesized 50:50 CPTEG:CPH copolymer had a M_w of 8,500 g/mol with a polydispersity index (PDI) of 1.7. These values are consistent with previous work7,20.

8.4.2 Characterization of Functionalized Nanoparticles

Particle morphology and size of both non-functionalized and functionalized-nanoparticles were analyzed by SEM (data not shown). Some aggregation was observed after surface functionalization, which was attributed to the reaction conditions.
The aggregation was disrupted by sonicating the nanoparticles before use, as indicated by the size distribution profiles of the sonicated nanoparticles obtained by QELS (Table 8.1). No significant differences were observed between the size of the functionalized and the non-functionalized nanoparticles.

Table 8.1. Nanoparticle characterization. Non-functionalized and functionalized were characterized by QELS and zeta potential measurements. Particle size data represent the mean value ± standard deviation (SD) of dynamic light scattering data collected in three independent experiments. Zeta potential data represent the mean value ± SD of three independent readings. Change in zeta potential clearly indicates that sugar were efficiently conjugated to the 50:50 CPTEG:CPH nanoparticles surface. Sugar density on nanoparticles surface was determined by a colorimetric phenol-sulfuric acid assay. Amount of sugar was determined relative to standard curves; data is presented as mean ± SD of four independent experiments.

<table>
<thead>
<tr>
<th>Nanoparticle type</th>
<th>Average Particle Diameter (nm)</th>
<th>Average Particle ζ-Potential (mV)</th>
<th>Sugar Density (µg/mg of particle)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-functionalized</td>
<td>162 ± 43</td>
<td>-20 ± 0.6</td>
<td>----</td>
</tr>
<tr>
<td>Linker</td>
<td>231 ± 52</td>
<td>21 ± 1.5</td>
<td>----</td>
</tr>
<tr>
<td>Lactose</td>
<td>266 ± 44</td>
<td>26 ± 2.4</td>
<td>10.9 ± 6.8</td>
</tr>
<tr>
<td>Di-mannose</td>
<td>278 ± 32</td>
<td>28 ± 3.2</td>
<td>12.4 ± 5.5</td>
</tr>
</tbody>
</table>

Measurements of ζ-potential using QELS were employed to characterize the nanoparticle surface functionalization (Table 8.1). The non-functionalized nanoparticles exhibited a ζ-potential of -20 mV. In contrast, the various functionalized nanoparticles resulted in a positively charged ζ-potential (ranging from 20-26 mV). The positive charge observed after functionalization is attributed to the presence of the diamine linker on the surface of the particles; this was corroborated by analyzing the ζ-potential of
nanoparticles that were only reacted with the ethylene diamine linker but without glycolic acid or sugar. This control showed a slightly more positive ζ-potential in comparison to the linker and sugar modified particles. As expected, the reaction yield of sugar attachment to the linker is not 100%. Since the di-mannoside is a neutral sugar, the free amine groups present on the surface of the particles are responsible for the positive charge observed.

**Table 8.2.** An increase in the nitrogen content of functionalized nanoparticles correlates with the presence of the amine linker used in the amine-carboxylic acid reaction. X-ray photoelectron spectroscopy (XPS) analysis of atomic percentages and ratios of elements present on non-functionalized and functionalized 50:50 CPTEG:CPH nanoparticles.

<table>
<thead>
<tr>
<th>Nanoparticle type</th>
<th>% C</th>
<th>% N</th>
<th>% O</th>
<th>O/C</th>
<th>N/C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-functionalized</td>
<td>74.9 ± 1.5</td>
<td>1.2 ± 0.1</td>
<td>23.9 ± 2.3</td>
<td>0.320</td>
<td>0.016</td>
</tr>
<tr>
<td>Linker</td>
<td>65.5 ± 0.9</td>
<td>4.4 ± 0.5</td>
<td>18.1 ± 1.9</td>
<td>0.276</td>
<td>0.067</td>
</tr>
<tr>
<td>Lactose</td>
<td>76.3 ± 2.1</td>
<td>5.1 ± 0.4</td>
<td>20.9 ± 1.1</td>
<td>0.274</td>
<td>0.067</td>
</tr>
<tr>
<td>Di-mannose</td>
<td>73.9 ± 1.1</td>
<td>5.3 ± 0.7</td>
<td>23.3 ± 1.5</td>
<td>0.315</td>
<td>0.072</td>
</tr>
</tbody>
</table>

aData reported as mean ± standard deviation of four independent experiments performed in triplicate.

bRatio of the two elements as indicated.

The functionalized nanoparticles were characterized using XPS to quantitatively determine successful linker and sugar conjugation to the nanoparticle surface. **Table 8.2** shows the surface elemental atomic percentages and elemental ratios of non-functionalized and functionalized nanoparticles. Clear differences in the N/C ratio were
observed, showing a significant increase in nitrogen content (i.e., 0.01 in the non-functionalized particles to almost 0.07 in the functionalized particles). This increase in nitrogen content is directly related to the presence of the amine linker. The presence of nitrogen groups on the surface makes the surface composition of these particles similar to that of pathogens, which are known to have a high content of amine-containing compounds\textsuperscript{37}.

High-resolution C1s spectra were fitted with four main components according to their characteristic binding energies representing aliphatic hydrocarbon at 285.0 eV (C1), ether and amine groups at 286.5 eV (C2), carbonyl and amide groups at 288.2 eV (C3), and ester and carboxylic acid groups at 289.1 eV (C4)\textsuperscript{32}. Analysis of C1s spectra allows for more direct comparison of sugar attachment based on increases in the ether and amine groups (C2). Ether groups are characteristic of carbohydrate structures while the amine groups are indicative of the presence of the amine linker used for surface modification. Table 8.3 shows the average percentage areas obtained from high-resolution C1s fitted spectra. These data indicate a clear increase in the C2 component in the functionalized-nanoparticle groups, especially those functionalized with lactose and di-mannose. The C2 component percentage for the non-functionalized 50:50 CPTEG:CPH nanoparticles is \textasciitilde22\%, while that of the sugar-functionalized nanoparticles is \textasciitilde30\%. It is known that the outer surface of bacterial pathogens are comprised of various constituents bearing amine and hydroxyl groups (e.g., peptidoglycan, lipopolysaccharide, lipoproteins, and flagella)\textsuperscript{37}. Analysis of C1s (Table 8.3) and O1s (data not shown) spectra of functionalized nanoparticles showed a clear increase of amine, ether, and hydroxyl groups, resulting in a surface composition that is similar to...
that of pathogens\textsuperscript{37}. The total amount of sugar attached to the nanoparticle surface was quantified by the phenol-sulfuric acid assay. The results showed that similar amounts (12.6 ± 6.4 µg/mg of particle) of lactose and di-mannose were attached to the 50:50 CPTEG:CPH nanoparticles (Table 8.1).

**Table 8.3.** Increased surface presence of ether, amine and hydroxyl groups corroborated nanoparticle functionalization. Average percentage areas of aliphatic hydrocarbon (C1), ether and amine groups (C2), carbonyl and amide groups (C3), and ester and carboxylic acid groups (C4) were obtained from XPS high-resolution C1s fitted spectra.

<table>
<thead>
<tr>
<th>Nanoparticle type</th>
<th>C1</th>
<th>C2</th>
<th>C3</th>
<th>C4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-functionalized</td>
<td>59.7 ± 1.2</td>
<td>22.3 ± 0.7</td>
<td>8.7 ± 0.6</td>
<td>1.4 ± 0.1</td>
</tr>
<tr>
<td>Linker</td>
<td>58.3 ± 1.7</td>
<td>26.6 ± 0.7</td>
<td>7.1 ± 0.3</td>
<td>0.1 ± 0.2</td>
</tr>
<tr>
<td>Lactose</td>
<td>58.2 ± 0.8</td>
<td>30.1 ± 1.2</td>
<td>6.6 ± 0.6</td>
<td>0.6 ± 0.1</td>
</tr>
<tr>
<td>Di-mannose</td>
<td>58.1 ± 1.9</td>
<td>29.6 ± 1.0</td>
<td>6.9 ± 0.5</td>
<td>0.4 ± 0.1</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Data reported as mean ± standard deviation of four independent experiments performed in triplicate.

**8.4.3 Di-mannose-functionalized nanoparticles enhanced DC expression of MHC II, co-stimulatory molecules and CLRs**

Flow cytometry was used to determine if functionalized nanoparticles enhanced the activation status of DCs. In general, the results indicate that di-mannose functionalized nanoparticles significantly enhanced the expression of key surface markers that are associated with DC maturation and antigen presentation (i.e., MHC II, CD86, and CD40) and concomitantly increased the surface expression of CLRs (i.e., CD206 and CIRE) as
compared to non-functionalized nanoparticles (Figure 8.2). Another interesting observation from these studies is that the di-mannose functionalized nanoparticles induced higher expression of both CD206 and CIRE as compared to lactose-functionalized particles, confirming the specificity of both CIRE and CD206 for mannose residues as observed previously.  

**Figure 8.2.** Di-mannose-functionalized nanoparticles enhanced DC expression of MHC II, co-stimulatory molecules and C-type lectin receptors (CLRs). After stimulation with either non-functionalized (NF) or functionalized nanoparticles for 48 h, DCs were harvested and analyzed by flow cytometry for surface expression of (A) MHC II, (B) CD86, (C) CD40, (D) CIRE, or (E) CD206. LPS and non-stimulated cells (NS) were used as positive and negative controls, respectively. Data are expressed as the mean ± the SEM of four independent experiments performed in triplicate. * and # represent groups that are statistically significant (p ≤ 0.05) compared to the NS and NF groups, respectively. MFI = mean fluorescence intensity.
Soluble lactose and di-mannose and non-functionalized nanoparticles plus soluble sugar were used as controls to probe the benefit of the covalent linkage of the sugars to the nanoparticle surface. The addition of soluble sugars (i.e., lactose and di-mannose) to cultures of DCs with non-functionalized nanoparticles did not significantly change cell surface marker expression from levels induced by the non-functionalized nanoparticles alone (Supplemental Figure 8.1).

**8.4.4 Stimulation with functionalized nanoparticles enhanced pro-inflammatory cytokine secretion**

Although stimulation of DCs with either non-functionalized or functionalized nanoparticles modestly increased the secretion of IL-6 in comparison to the non-stimulated controls, the greatest amounts of IL-6 were produced by DCs stimulated with linker-modified nanoparticles (black bars in Figure 8.3). Elevated levels of TNF-α were secreted (gray bars in Figure 8.3) upon stimulation with the linker-functionalized particles. Neither IL-10 nor IL-1β was detected in the culture supernatants (data not shown). This observation is consistent with previously published work with other polyanhydride chemistries and different particle sizes. The level of IL-12p40 secreted into the culture medium by DCs stimulated with either non-functionalized or functionalized nanoparticles was not statistically different from the non-stimulated controls (data not shown).
Figure 8.3. Stimulation with functionalized nanoparticles enhanced secretion of pro-inflammatory cytokines from DCs. After stimulation with non-functionalized (NF) and functionalized nanoparticles for 48 h, supernatants were harvested and assayed for IL-6 (■) and TNF-α (■). Data is represented as mean concentration of cytokines ± the SEM of four independent experiments performed in triplicate. LPS was used as a positive control stimulant (> 50,000 pg/mL for IL-6 and 6,861 pg/mL for TNF-α) and non-stimulated cells (NS) were used as a negative control. * and # represent groups that are statistically significant (p ≤ 0.05) compared to the NS and NF groups, respectively.

8.4.5 Both non-functionalized and functionalized nanoparticles were efficiently internalized by DCs

Microscopic analyses were performed to determine if the enhanced activation of DCs by functionalized nanoparticles was associated with enhanced internalization.
Interestingly, all the functionalized nanoparticle groups were efficiently internalized following 30 min of co-incubation with DCs (Figure 8.4A). These qualitative findings were corroborated by morphometric analysis of the epifluorescent images of the FITC-loaded nanoparticles, which is presented as the total area ($\mu m^2$) of internalized nanoparticles. This metric allowed us to perform biologically relevant comparisons among all treatments. The morphometric analysis revealed that higher amounts of functionalized nanoparticles were internalized by DCs when compared to non-functionalized nanoparticles (Figure 8.4B).

**Figure 8.4.** Both non-functionalized and functionalized nanoparticles (green) were efficiently internalized by DCs after co-incubation for 30 min. Monolayers incubated for 2 h post-internalization with FITC-loaded nanoparticles were stained with WGA conjugated with tetramethylrhodamine to delineate the plasma membrane. (A)
Epifluorescent microscopy demonstrated that nanoparticle functionalization enhanced internalization when compared to non-functionalized (NF) nanoparticles. Representative epifluorescent images were captured and processed using identical exposure and ImageJ settings. (B) Quantitative morphometric analysis measuring nanoparticle uptake per field of view (FOV). Data is reported as the total area of fluorescence detected within each FOV (* represents groups that are statistically significant (p ≤ 0.05) compared to the NF group). Images shown are representative of four fields of view analyzed for each group and consistently observed over three independent experiments. Scale bar = 20 µm.

8.4.6 Mannose receptor-mediated activation of DCs by di-mannose functionalized nanoparticles

To test the specificity of the di-mannose functionalization for binding to CLRs, monoclonal antibodies were used to inhibit activation of the mannose receptor and CIRE on DCs stimulated with non-functionalized and di-mannose-functionalized nanoparticles (Figure 8.5). For all the surface markers evaluated (i.e., MHC II, CD40, CD86, CD209, and CD206), the mean fluorescence intensity (MFI) values for the non-stimulated DCs did not change over that of the controls when the monoclonal antibodies against the CLRs were added, indicating that no additional stimulation was provided by the antibodies used as blocking agents. After blocking with anti-CD209 or anti-CD206, a reduction in the expression of CIRE and CD206, respectively, was observed after stimulation with di-mannose-functionalized nanoparticles (as shown in Figures 8.5D and E). This result demonstrates that the use of specific monoclonal antibodies can block the up-regulation of the mannose and CIRE receptors induced by di-mannose-functionalized particles. Furthermore, when the anti-CD206 monoclonal antibody was used, the increased surface expression of MHC II, CD40, and CD86 was inhibited
(Figures 8.5A, B, and C). This outcome demonstrates that mannose receptor engagement by the functionalized nanoparticles plays a critical role in their adjuvant effect. In these experiments, the linker and lactose-functionalized particles were also added to separate DC cultures to control for the specificity of the blocking antibodies. After pre-treatment with the anti-CD206 monoclonal antibody, there was no significant inhibition in the expression of MHC II, CD40, and CD86 (Supplemental Figure 8.2), further corroborating the specificity of the mannose receptor mediating DC activation by the di-mannose-functionalized nanoparticles.

**Figure 8.5.** Mannose receptor-mediated activation of DCs by di-mannose functionalized nanoparticles is inhibited by receptor-specific monoclonal antibodies. DC cultures were incubated for 15 min with either α-CD206, α-CD209 or α-CD206 + α-CD209, or media (control, NS) and then were stimulated with either non-functionalized (NF) or di-mannose-functionalized nanoparticles for 48 h. DCs were harvested and analyzed via flow cytometry for changes in surface expression of (A) MHC II, (B) CD86, (C) CD40,
(D) CIRE, or (E) CD206. MFI (mean fluorescence intensity) ± the SEM of each of the markers of two independent experiments performed in triplicate is presented. * represents groups that are statistically significant (p ≤ 0.05) compared to the control treatment.

8.5 Discussion

Engineering nanoparticles that are targeted to specific receptors on APCs offers a novel approach for the rational design of effective vaccine adjuvants. The covalent linkage of specific carbohydrates to the surface of nanoparticles allows for the targeting of CLRs and activation of complex signaling pathways including crosstalk with other PRRs (e.g., TLRs and Fc receptors) that can direct the immune response. The data presented herein demonstrate the design and fabrication of novel di-mannose-functionalized polyanhydride nanoparticles that possess characteristically similar chemical compositions as that of pathogen surfaces. These “pathogen-like” nanoparticles were efficiently internalized by DCs (Figure 8.4) and concomitantly activated DCs (Figures 8.2 and 8.3) in vitro by specific interactions with the mannose receptor (Figure 8.5).

All the particles (i.e., non-functionalized and linker, lactose, and di-mannose-functionalized) enhanced the expression of MHC II, CD40, and CIRE (Figures 8.2A, 8.2C and 8.2D) when compared to non-stimulated DCs. While non-functionalized 50:50 CPTEG:CPH nanoparticles demonstrated moderate DC activation, di-mannose functionalization of the nanoparticles induced greater expression of MHC II, CD40, and CD86. MHC II, CD40, and CD86 play an important role in the induction of adaptive
immunity through activation of CD4$^+$ T cells, and it would be beneficial for an effective adjuvant to enhance the expression of MHC II and co-stimulatory molecules$^{1,9,34}$.

CIRE and the mannose receptor play an important role in antigen uptake, as well as in DC migration and initial interaction with T cells$^{9-11,13}$. As mentioned before, the activation of CLR-induced signalling processes can result in the induction of diverse immune responses$^{12}$. As shown in Figures 8.5D and 8.5E, by chemically conjugating di-mannose to the surface of polyanhydride nanoparticles, effective cross-linking of CLRs on DCs was achieved, resulting not only in a higher expression of both CIRE and CD206, but also in higher expression of other DC maturation markers (i.e., MHC II, CD40, and CD86) compared to the non-functionalized particles. High affinity binding of CLRs to their ligands is known to result in increased expression of the receptor on the cell surface$^{38}$. Additionally, the results presented in Supplemental Figure 8.1 demonstrate that the sugars must be covalently attached to the nanoparticles to enhance the activation of the DCs. Finally, the data presented in Supplemental Figure 8.2 further corroborates the binding of the di-mannose-functionalized nanoparticles to the mannose receptor in order to enhance the activation of DCs.

The expression of MHC II, CD86 and CD40 is essential for antigen presentation and activation of naïve T cells; however, it is not sufficient to induce an effective T cell response. In the context of a robust immune response, DCs need to secrete an appropriate profile of cytokines to enhance CD4$^+$ T cell activation. The data presented in Figure 8.3 demonstrate that DCs secreted IL-6 after stimulation with both non-functionalized and functionalized nanoparticles. IL-6 is important to both innate and adaptive immune response, as it contributes to systemic inflammation$^1$ and promotes
humoral immune responses\textsuperscript{39}. Linker-functionalized nanoparticles also induced the secretion of TNF-\(\alpha\). This cytokine is important in early DC maturation and further induction of T cell differentiation and, therefore, even small amounts can influence the bias and magnitude of an immune response. Production of these two cytokines by DCs has been previously associated with CLR-induced signaling events\textsuperscript{12,16}.

The observed enhancement in surface molecule expression (Figure 8.2) and IL-6 secretion (Figure 8.3) by DCs after stimulation with di-mannose functionalized nanoparticles was hypothesized to be related to an increase in particle internalization\textsuperscript{40}. However, as shown in Figure 8.4, all functionalized nanoparticles were efficiently internalized by DCs. In particular, both linker and di-mannose-functionalized nanoparticles were present in higher numbers inside the DCs than non-functionalized and lactose functionalized nanoparticles. These data indicate that particle internalization is necessary but not sufficient for enhanced DC activation. Previous work from our laboratories has demonstrated enhanced internalization of soluble antigen by monocytes when delivered with non-functionalized polyanhydride nanoparticles\textsuperscript{21}. Therefore, the results presented in Figure 8.4 suggest that the functionalized nanoparticles would also provide efficient antigen delivery.

The specific interaction of functionalized nanoparticles with CLR\(\text{s}\) documented in this study presents an intriguing opportunity for the rational design of nanovaccines possessing the capacity to induce diverse immune responses tailored for a target disease. While the increased activation of DCs with di-mannose functionalized particles was directly correlated with a receptor-ligand interaction, the enhanced internalization of linker-functionalized nanoparticles represents a non-specific interaction that may be
dictated by the positive charge of the functionalized particles. It is well known that cationic particles show greater adhesion with cell membranes\textsuperscript{41} and, therefore, may be an explanation for the enhanced cellular uptake of the linker-functionalized nanoparticles observed in this study. In addition, the non-specific interaction due to the linker-functionalized nanoparticles may closely mimic the interaction between DCs and endogenous danger signals. Indeed, cellular recognition of endogenous (e.g., hyaluronic acid, uric acid crystals) and exogenous (e.g., LPS, lipoteichoic acid) hydrophobic moieties is an important component of the danger hypothesis describing activation of innate immune responses\textsuperscript{42,43}. Non-functionalized polyanhydride nanoparticles present a hydrophobic surface to DCs in much the same manner as would apoptotic cells or endogenous stressors compared to the more robust responses induced by microbial PAMPs. Endogenous danger signals are known to stimulate a robust adaptive immune response in the absence of deleterious inflammatory responses\textsuperscript{42}, an attribute that would be valuable to replicate when developing safe and effective vaccines. Collectively, these results indicate that polyanhydride nanoparticles can be rationally functionalized to effectively enhance activation of APCs and lead to the design of efficacious targeted vaccine delivery platforms.

\textbf{8.6 Conclusions}

In this study, novel "pathogen-like" particles were fabricated by functionalizing the surface of polyanhydride nanoparticles with sugar residues. The ability of these novel particles to induce and enhance DC activation by specific interactions with the mannose receptor was demonstrated. Targeting CLR\textsuperscript{s} on DCs with ligands covalently linked to
polyanhydride nanoparticles has the potential to efficiently deliver antigens to DCs for effective processing and presentation to T cells. A specific ligand-receptor interaction (i.e., di-mannose-CD206) was identified by blocking the mannose receptor on DCs, which resulted in the inhibition of DC activation. This data indicates that mannose receptor signaling pathways are involved in the expression of surface molecules required for antigen presentation and T cell co-stimulation. The receptor-mediated endocytosis induced by the direct interaction of mannose residues with the mannose receptor may be exploited to design efficacious nanovaccines. The initial interactions of these novel di-mannose functionalized nanoparticles with CLR on DCs have been demonstrated in these studies; however, the biological consequences of these interactions still need to be evaluated and are a continued area of investigation in our laboratories.

8.7 Acknowledgments

The authors would like to acknowledge financial support from the ONR-MURI Award (NN00014-06-1-1176). This material is based upon work supported by the National Science Foundation under Grant No. EEC 0851519. The authors would also like to thank Shawn Rigby for his expertise in flow cytometry and James Anderegg of Ames Laboratory for his expertise in XPS.

8.8. References


19. Fernández, N.; Alonso, S.; Valera, I.; González Vigo, A.; Renedo, M.; Barbolla, L.; et al. Mannose-containing molecular patterns are strong inducers of


Covalent attachment of sugars to nanoparticles is necessary to enhance activation of DCs. After stimulation with either soluble lactose or di-mannose or soluble sugars plus non-functionalized (NF) nanoparticles for 48 h, DCs were harvested and analyzed by flow cytometry for surface expression of (A) MHC II, CD86, and CD40, and (B) CIRE and CD206. LPS and non-stimulated cells (NS) were used as positive and negative controls, respectively. Data are expressed as the mean ± the SEM of three independent experiments performed in duplicate. * represents groups that are statistically significant (p ≤ 0.05) compared to the NS group. MFI = mean fluorescence intensity.
Supplemental Figure 8.2. Expression of DC activation markers by linker and lactose-functionalized nanoparticles is not inhibited by receptor-specific monoclonal antibodies. DC cultures were incubated for 15 min with either α-CD206, α-CD209 or α-CD206 + α-CD209, or media (control, NS) and then were stimulated with either linker-functionalized (NF) or lactose-functionalized nanoparticles for 48 h. DCs were harvested and analyzed via flow cytometry for changes in surface expression of (A) MHC II, (B) CD86, (C) CD40, (D) CIRE, or (E) CD206. MFI (mean fluorescence intensity) ± the SEM of each of the markers of two independent experiments performed in triplicate is presented. * represents groups that are statistically significant (p ≤ 0.05) compared to the control treatment.
CHAPTER 9

Combining αGal Modification of Antigen and Polyanhydride Nanoparticle-Based Platforms for the Design of an Effective Plague Vaccine

A paper to be submitted for publication in PLoS ONE, 2011

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Keywords: αGal Modification, Polyanhydride Nanoparticles, Epitope Mapping, Nanovaccine, Yersinia pestis

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Abstract

Innovative vaccine platforms are needed in order to develop effective single-dose vaccines to control new and re-emerging diseases. These platforms should be able to direct antigen internalization and promote immunogenic responses by antigen presenting cells, as well as prevent rapid degradation of antigen, all these in order to augment, modulate and extend immune responses. In this work a transdisciplinary approach was utilized to rationally design efficacious vaccines against Yersinia pestis by combining two novel platforms, αGal-modification of the vaccine antigen (i.e., F1-V) and use of polyanhydride nanoparticles as vaccine delivery vehicles, which results in the induction of a robust immune response. High antibody titers with high avidity and with the ability to recognize epitopes that have been identified as protective were elicited by vaccine regimes containing soluble αGal-F1-V and encapsulated αGal-F1-V or F1-V. Using peptides arrays in combination with informatics tools we have introduced a novel strategy in understanding antigen-antibody interactions, generating information that allows for the rational selection of antigen (i.e., αGal-modification)- and adjuvant (i.e., polyanhydride nanoparticles)-based platforms for the design of efficacious vaccines.
9.2 Introduction

*Yersinia pestis*, the causative agent of pneumonic plague, is an antibiotic-resistant organism\textsuperscript{1,2}, easy to weaponize, and can significantly affect susceptible populations\textsuperscript{3}, making the development of an effective vaccine highly desirable. Previous studies have shown that immunization with the fusion protein, F1-V, provides protection in mice\textsuperscript{4} and cynomolgus macaques\textsuperscript{5}; however, this antigen failed to produce sufficient levels of protection in other non-human and human trials\textsuperscript{6,7}.

Despite the promising possibilities of the F1-V antigen, there remains a major hurdle in the treatment against plague and alternatives have been explored in order to improve vaccine efficacy and induce long-term, protective immunity. In this regard, targeting antigen presenting cells (APCs) offers an option capable of eliciting combined humoral and cellular responses, and has been applied to *Y. pestis*, showing protection in mice\textsuperscript{8}.

Targeting Fcγ receptors (FcγR) present on a variety of APCs (i.e., dendritic cells, Langerhans cells, and macrophages) is considered the most effective mechanism by which APCs identify and internalize antigens to induce an effective immune response\textsuperscript{9}. Antigen opsonization (i.e., formation of immune complexes) allows for the Fc portion of the antigen-bound antibodies to interact with FcγR\textsuperscript{9-12} and has been shown to increase immunogenicity of several antigens (e.g., tetanus toxoid, hepatitis B antigen, etc)\textsuperscript{10-12}.

Anti-αGal is the most abundant natural antibody in humans, constituting ~1% of serum IgG and interacts with the carbohydrate epitope Gal-α(1-3)-Gal-β(1,4)-GlcNAc-R (αGal) found on glycolipids and glycoproteins of most animals\textsuperscript{13,14}. This αGal epitope is synthesized in other animals by the glycosylation enzyme α1,3galactosyltransferase (α1,3GT); however, the α1,3GT gene was inactivated in humans and therefore human
cells are completely devoid of αGal epitopes, leading to the recognition of αGal antigens as foreign\textsuperscript{13,14}. As a result, any soluble antigen that has αGal epitopes will form immune complexes with anti-αGal and will be targeted for effective uptake by APCs\textsuperscript{15-18}. αGal modification has been shown to substantially increase the immunogenicity of proteins as diverse as bovine serum albumin (BSA)\textsuperscript{19}, viral hemagglutinin\textsuperscript{16}, and HIV gp120\textsuperscript{18}. Exploiting the natural occurring anti-αGal immune response to enhance immunity of the F1-V antigen is a strategy that may lead to reduced antigen dose and more efficacious immunization protocols resulting in cost-effective vaccines.

The use of vaccine adjuvants is another alternative to improve vaccine immunogenicity by providing pro-inflammatory signals and prolonging the persistence of vaccine antigens, which is of special importance to induce affinity maturation of B cells\textsuperscript{20}. Sustained exposure to antigen can be achieved by utilizing nanoparticle-based platforms; in fact, several biodegradable polymers have been studied as vaccine delivery vehicles\textsuperscript{20}. In particular, polyanhydride particles have demonstrated the ability to provide sustained release of stable protein antigens and to activate antigen presenting cells and modulate immune responses\textsuperscript{21-31}. Moreover, amphiphilic polyanhydride particles made of 1,6-bis(\textit{p}-carboxyphenoxy)hexane (CPH) and 1,8-bis(\textit{p}-carboxyphenoxy)-3,6-dioxaoctane (CPTEG) were able to stabilize the F1-V antigen\textsuperscript{21} and a vaccine formulation based on this copolymer provided long-term protection against \textit{Y. pestis} challenge in mice\textsuperscript{32}.

This study outlines a novel approach to design an effective single-dose vaccine against \textit{Y. pestis} by combining the polyanhydride nanoparticle-based platform with antigenically modified F1-V protein. It is hypothesized that the combination of these two
approaches will augment and accelerate antigen-specific immunity leading to the
development of a cost-effective plague vaccine that can reduce the need for multiple
injections and result in greater patient compliance. In this work, the F1-V antigen was
modified with αGal epitopes and to mimic the relevant human immune characteristics to
the αGal pathway, an α1,3GT gene knockout (KO) mouse model, which lacks αGal
epitopes and can produce anti-αGal similarly to humans, was used to evaluate the
efficacy of the proposed vaccine formulations. Peptide array and informatics analysis
tools were used to identify immunodominant epitopes recognized by the antibodies
elicited by the various vaccine regimens. The studies described herein demonstrate the
importance of integrating concepts from material science, protein chemistry,
immunology and computational analysis to rationally design the next generation of
adjuvants and vaccines for emerging and re-emerging diseases.

9.3 Materials and Methods

9.3.1 Materials

Chemicals needed for monomer synthesis and polymerization, as well as for
nanoparticle fabrication, including sebacic acid (99%), p-carboxy benzoic acid (99+%),
and 1-methyl-2-pyrrolidinone, anhydrous (99+%), were purchased from Aldrich
(Milwaukee, WI); 4-p-hydroxybenzoic acid, 1,6-dibromohexane, 1-methyl-2
pyrrolidinone, and tri-ethylene glycol were purchased from Sigma Aldrich (St. Louis,
MO); 4-p-fluorobenzonitrile was obtained from Apollo Scientific (Cheshire, UK);
potassium carbonate, dimethyl formamide, toluene, sulfuric acid, acetic acid,
acetonitrile, acetic anhydride, methylene chloride, pentane, and petroleum ether were
purchased from Fisher Scientific (Fairlawn, NJ). Goat anti-mouse IgG (H+L)-AP was purchased from Jackson ImmunoResearch (West Grove, PA). Phosphatase substrate was purchased from Aldrich (St Louis, MO). Polyacrylamide 4–20% Tris–HCl pre-cast gradient gels, unstained protein standards, pre-stained broad range molecular weight standards, and Flamingo Gel Stain were purchased from Bio-Rad Laboratories (Richmond CA). Bicinchoninic acid (BCA) and micro-BCA protein assay kits was obtained from Pierce Biotechnology Inc. (Rockford, IL).

9.3.2 Antigenic Modification of F1-V

Recombinant F1-V obtained from NIH Biodefense and Emerging Infections Research Resources Repository (BEI, Manassas, VA) was antigenically modified by chemical addition of αGal epitopes at lysine residues. Chemical addition of αGal epitopes to this vaccine antigen was performed at BioProtection Systems, Inc. (Ames, IA) using an efficient chemo-enzymatic synthesis of the αGal trisaccharide and conjugation\textsuperscript{33}. The modified F1-V was characterized by SDS-PAGE and western blot using either F1-V hyperimmunized mice sera, which were obtained from repeated injections of F1-V antigen to adult female C57BL/6 mice, or αGal (+) sera from α1,3GT KO mice, which were obtained after three intraperitoneal injections with rabbit red blood cells to induce production of anti-αGal antibodies.

9.3.3 Polymer Synthesis and Characterization

Synthesis of 1,6-bis(p-carboxyphenoxy)hexane (CPH) and 1,8-bis(p-carboxyphenoxy)-3,6-dioxaoctane (CPTEG) diacids was performed as described
previously. $^1$H NMR and gel permeation chromatography (GPC) were utilized to confirm polymer chemical structure and to measure molecular weight, respectively. The 50:50 CPTEG:CPH copolymer had a $M_n$ of 8,500 Da and a PDI of 1.70, consistent with previous work.\textsuperscript{21,22,29,34}

9.3.4 Nanoparticle Fabrication and Characterization

Both F1-V and αGal-modified F1-V (αGal-F1-V) encapsulated nanoparticles were fabricated by the anti-solvent nanoencapsulation method reported previously.\textsuperscript{31,32} Nanoparticle recovery was >70% and antigen encapsulation efficiency was >95%. Scanning electron microscopy (SEM, JEOL 840A, JEOL Ltd., Tokyo, Japan) and quasi-elastic light scattering (QELS, Zetasizer Nano, Malvern Instruments Ltd., Worcester, UK) were employed to investigate particle morphology and size, respectively.

9.3.5 In vitro Antigen Release Kinetics

F1-V and αGal-F1-V loaded nanoparticles were incubated with 0.1 mM phosphate buffer saline (PBS, pH 7.6) in microcentrifuge tubes. Samples were sonicated to uniformly distribute the nanoparticles and placed an incubator at 37 °C with constant agitation. Supernatants were sampled over time to determine the amount of released antigen using a microBCA protein assay. The removed volumes were replaced with fresh PBS following supernatant removal to maintain perfect sink conditions. Data is presented as cumulative fraction of released antigen, which was determined by dividing the amount released at each time point by the total amount of encapsulated protein into the nanoparticles.\textsuperscript{21,26,29}
9.3.6 Mice

The α1,3GT gene knockout (KO) mouse model was used to stimulate immunity to αGal and mimic human immunity to this epitope. Mice were obtained from BioProtection Systems, Inc. and housed under specific pathogen-free conditions where all bedding, caging, and feed were sterilized prior to use. All animal procedures were conducted with the approval of the Iowa State University Institutional Animal Care and Use Committee. An intraperitoneal injection regimen of rabbit red blood cells (RRBCs) was performed prior to immunization to induce production of anti-αGal antibodies. Animals received three injections of RRBCs (3 x 10^9 RRBCs/injection) at 14-day intervals. Seven days after the final RRBC injection, whole blood was collected from mice via the saphenous vein and serum was assessed for anti-αGal specific antibodies. Only mice whose sera showed optical density values (OD_{405nm}) higher than 5x background (PBS) were used in the study and animals were distributed randomly across the different immunization groups.

9.3.7 Vaccinations

Mice were vaccinated subcutaneously with the regimens described in Table 9.1 by suspension in pyrogen-free saline in a volume of 100 µL. Nanoparticles were sonicated briefly to disperse clumps prior to immunization using a 23 gauge needle. Control animals received 100 µL of saline alone. A booster dose of 5 µg of F1-V was subcutaneously administered to all mice 37 days after immunization. Blood samples were collected from the left saphenous vein prior to immunization, prior to boosting (pre-boost) at day 36, and five days after boosting (post-boost). Serum was collected by
centrifugation and stored at -20°C and assayed for anti-F1-V specific antibodies. These experiments were conducted in duplicate with a different batch of nanoparticles used for each experiment.

Table 9.1. Vaccination regimens.

<table>
<thead>
<tr>
<th>Experimental Group</th>
<th>Soluble F1-V (µg)</th>
<th>Soluble αGal-F1-V (µg)</th>
<th>Encapsulated F1-V (µg)</th>
<th>Encapsulated αGal-F1-V (µg)</th>
<th>50:50 CPTEG:CPH nanoparticles (µg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control-Saline</td>
<td>-----</td>
<td>-----</td>
<td>-----</td>
<td>-----</td>
<td>-----</td>
</tr>
<tr>
<td>S&lt;sub&gt;F1-V&lt;/sub&gt;</td>
<td>5</td>
<td>-----</td>
<td>-----</td>
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<td>-----</td>
</tr>
<tr>
<td>S&lt;sub&gt;αGal-F1-V&lt;/sub&gt;</td>
<td>-----</td>
<td>5</td>
<td>-----</td>
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<td>-----</td>
</tr>
<tr>
<td>E&lt;sub&gt;F1-V&lt;/sub&gt;</td>
<td>-----</td>
<td>-----</td>
<td>5</td>
<td>-----</td>
<td>500</td>
</tr>
<tr>
<td>E&lt;sub&gt;αGal-F1-V&lt;/sub&gt;</td>
<td>-----</td>
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<td>-----</td>
<td>5</td>
<td>500</td>
</tr>
<tr>
<td>S&lt;sub&gt;αGal-F1-V&lt;/sub&gt; + E&lt;sub&gt;αGal-F1-V&lt;/sub&gt;</td>
<td>-----</td>
<td>2.5</td>
<td>-----</td>
<td>2.5</td>
<td>500</td>
</tr>
<tr>
<td>S&lt;sub&gt;F1-V&lt;/sub&gt; + E&lt;sub&gt;F1-V&lt;/sub&gt;</td>
<td>2.5</td>
<td>-----</td>
<td>2.5</td>
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<td>-----</td>
<td>2.5</td>
<td>2.5</td>
<td>-----</td>
<td>500</td>
</tr>
</tbody>
</table>

*Quantities indicate the amounts of immunogen or adjuvant delivered to each mouse in the indicated group. S = soluble antigen; E = encapsulated antigen. Subscripts indicate amount of soluble or encapsulated antigen (in µg) administered per dose.
9.3.8 F1-V and LcrV Specific Enzyme-Linked Immunosorbent Assay (ELISA)

High protein binding 96-well Costar microtiter plates (Corning Life Sciences, Lowell, MA) were coated overnight with 100 µl of phosphate buffer saline (PBS) at a pH of 7.4 containing 0.5 µg/mL of F1-V or LcrV (BEI, Manassas, VA). PBS containing 0.05% Tween 20 (PBS-T) and 2% (w/v) gelatin as a non-specific blocking agent (BD Biosciences, San Jose, CA) was used to block plates for at least 2 h at room temperature. After the blocking period, plates were heated at 60 ºC for 10 min in a dry oven to melt gelatin, and rinsed three times to remove any unbound blocking reagent. Sera samples were diluted 1:100, then serially diluted two-fold in PBS-T with 1% (v/v) normal goat serum (NGS), and incubated overnight at 4ºC. PBS-T was used to wash the plates three times followed by the addition of 100 µL of PBS-T (1% (v/v) NGS) containing alkaline phosphatase (AP)-conjugated goat anti-mouse IgG(H&L) at a 1:1000 dilution. After 2 h of incubation at room temperature, the plates were washed four times with PBS-T and 100 µL of sodium carbonate (50 mM) and magnesium chloride (2 mM) buffer (pH 9.3) containing 1 mg/mL of phosphatase substrate was added. The plates were allowed to react for 20 min at room temperature. Optical density (OD) of each well was measured at 405 nm using a Spectramax 190 Plate Reader (Molecular Devices, Sunnyvale, CA). Endpoint titers were defined as the highest dilution with an OD value of at least 0.2, which was at least three times the background OD value. To determine the antibody isotype induced by the various immunization regimens, OD was determined by the same ELISA protocol as described before. Sera samples were diluted at 1:500 and alkaline phosphatase-conjugated goat anti-mouse IgG1, IgG2a, IgG2c, IgG3, and IgM (Jackson ImmunoResearch Laboratories, West Grove, PA) were used.
9.3.9 Antibody Avidity Assay

Antibody avidity analysis was performed as described previously\textsuperscript{25,32}. Briefly, ELISA plates were coated overnight with 0.5 \( \mu \)g/mL F1-V. Plates were washed with PBS-T and blocked for two hours with 2% gelatin in PBS-T. Serum samples were diluted 1:500 in 1\% NGS-PBS-T and incubated overnight at 4\(^\circ\)C. Plates were washed with PBS-T and 5M NaSCN diluted in 0.1M NaH\(_2\)PO\(_4\) buffer was added to the first column and two-fold serially diluted to 9.76 mM leaving two columns containing 0M NaSCN. The NaSCN was incubated for 15 min and the plates were washed five times with PBS-T. AP-conjugated goat anti-mouse IgG (H&L) diluted 1:1000 in 1\% normal goat serum in PBS-T was incubated for 2 h. Plates were washed and phosphatase substrate (1 mg/mL) in carbonate buffer (pH 9.3) was added. Changes in OD were measured at 405 nm using a spectrophotometer. Avidity index was defined as the concentration of NaSCN necessary to reduce the OD by 50\% compared to the wells treated with 0.1 M sodium phosphate.

9.3.10 Epitope Mapping by Peptide Arrays

For analysis of the binding specificity of the produced immune sera to F1 or V antigenic peptides, an ELISA assay was performed following a similar protocol to the one described previously. Two sets of overlapping peptides, one panel covering the full length of the F1 antigen (27 peptides) and the other covering the full length of the V antigen (53 peptides), were obtained from BEI. Immunlon 2HB 96-well plates were incubated with the peptides (5 \( \mu \)g/mL) and incubated overnight at 4\(^\circ\)C. Plates were blocked for 2 h at room temperature with 2.5\% skim milk in PBS-T. Sera samples were incubated at 1:200 dilution overnight at 4\(^\circ\)C and after three washing steps with PBS-T
AP-conjugated goat anti-mouse IgG(H&L) at a 1:1000 dilution was added. Plates were allowed to react for 2 h with the phosphatase substrate buffer described before and changes in OD were determined at 405 nm.

9.3.11 Statistical Analysis

JMP® software (SAS Institute, Cary, NC) was used to make comparisons between the various vaccine regimens using one-way ANOVA and Tukey’s HSD and p-values < 0.05 were considered significant.

9.3.12 Informatics Analysis

9.3.12.1 Clustering Analysis of Peptide Array Data

Cluster analysis was performed in order to identify antigenic peptides under the different treatment conditions which are significant for induction of robust immune response and to compare the immunization groups under study. In this hierarchical clustering method, the similarity between observations is assessed according to their relative proximity in the data space. Starting from the separate data points in the parameter space, $R^N$ (N=10 for the comparison of 80 peptides, and N=80 for 10 different immunization groups), the Euclidean distance, $d_E$, is calculated to classify clusters. This distance in the N-dimensional space is calculated according to the following equation.

$$d_E = \sqrt{\sum_{i=1}^{N} (x_i - y_i)^2}$$

The color coding of the peptide maps indicates the relative distance among the data, and the tree structures, which are referred to as dendrograms, show the hierarchical
grouping. Note that the order of the rows and columns were rearranged through the clustering.

9.4 Results

9.4.1 αGal modification of F1-V

Chemical addition of αGal epitopes to the F1-V antigen was performed using an efficient chemo-enzymatic synthesis of the αGal trisaccharide. SDS-PAGE was initially used to characterize the αGal-F1-V and compare it with the unmodified F1-V. Figure 9.1A shows a representative gel comparing the unmodified F1-V and the αGal-F1-V. While the unmodified F1-V lane shows a main band at ~53 kDa, which is consistent with previous work21, a higher molecular weight band was identified for the αGal-F1-V together with some less intense bands at 100 kDa. This slight increase in the antigen molecular weight is the indicative of successful attachment of αGal epitopes. Western blot analysis was performed to corroborate the modification of the F1-V antigen. When hyperimmunized F1-V sera were used to react with both proteins (Figure 9.1B), consistent bands similar to those observed in the SDS-PAGE gel were observed. These findings demonstrate that the αGal-modified F1-V conserved its primary structure after the modification process. On the other hand, when the blot was reacted against αGal(+) serum, the only bands observed were those that corresponded to the αGal-F1-V protein (Figure 9.1C), further confirming the successful attachment of αGal epitopes.
Figure 9.1 Electrophoretic and western blot analysis of the F1-V and αGal-F1-V antigens confirms attachment of αGal epitopes. A) Flamingo fluorescence stained gel; B) and C) immunoblots developed with anti-F1-V hyperimmunized serum and anti-αGal (+), respectively.

9.4.2 Nanoparticle Characterization and In vitro Antigen Release

Scanning electron photomicrographs of F1-V and αGal-F1-V loaded 50:50 CPTEG:CPH nanoparticles are shown in Figure 9.2. Particles showed similar spherical morphology and size (147 nm for F1-V loaded particles versus 169 nm for αGal-F1-V loaded particles), which was consistent with QELS analysis and with particle morphology and sizes observed in previous studies\textsuperscript{22,28,32}. The release kinetics of both antigens was monitored for more than 30 days and quantified using the micro-BCA assay. A higher initial burst was observed for the αGal-F1-V antigen (~2%) during the first two days resulting in ~75% of total protein released in 33 days compared to 68% of F1-V antigen in the same time period (Figure 9.2).
Figure 9.2 Slight faster release kinetics was observed for αGal-F1-V compared to the release of unmodified F1-V. (top) Scanning electron microphotographs of F1-V and αGal-F1-V-loaded 50:50 CPTEG:CPH nanoparticles. Scale bar: 5 µm. (bottom) Cumulative release fraction of (○) F1-V and (▲) αGal-F1-V antigens released from 50:50 CPTEG:CPH nanoparticles. Error bars are representative of two independent experiments performed in duplicate.

9.4.3 Nanovaccine formulations that include soluble and/or encapsulated αGal-F1-V induce enhanced antibody production with high avidity

Previous work has shown that αGal modification substantially increased the immunogenicity of diverse proteins like BSA\textsuperscript{19}, viral hemagglutinin\textsuperscript{16}, and HIV gp120\textsuperscript{18}. Therefore, it is hypothesized that αGal-modification of the F1-V antigen will generate a robust immune response. It has also been shown that high antibody titers correlate with protection against \textit{Y. pestis} challenge\textsuperscript{4,35}. Based on this, the quantity and quality of
antibodies produced after immunization with different vaccine formulations combining two platforms (i.e., αGal-modification of the antigen and amphiphilic polyanhydride nanoparticles as vaccine delivery vehicles) were assessed.

Prior to application of an antigen boosting dose, higher anti-F1-V antibody titers were obtained when 5 µg of αGal-F1-V was delivered solubly (SαGal-F1-V) compared to the same dose of unmodified F1-V (SF1-V) (**Figure 9.3A**). However, after boosting, no significant differences were observed in anti-F1-V titers between the two antigens. On the other hand, delivery of 100% of the antigen encapsulated in 50:50 CPTEG:CPH nanoparticles failed to induce antibody production since titers were below those of the soluble dose groups. When vaccine formulations comprised of 50% of the antigenic dose (2.5 µg) soluble together with 50% encapsulated into polyanhydride nanoparticles, high antibody titers were induced to the same level or higher than the 100% soluble groups. From this data, it is clear that the combination of encapsulated and soluble antigen is critical to the induction of high antibody titers, which is consistent with previous published data that used the same nanoparticle platform to deliver F1-V intranasally**32. Different antigen combinations were used to evaluate the effect of delivering either αGal-F1-V or unmodified F1-V soluble and/or encapsulated. **Figure 9.3A** shows that formulations containing soluble αGal-F1-V and unmodified F1-V or αGal-F1-V encapsulated (SαGal-F1-V + EF1-V and SαGal-F1-V + E αGal-F1-V) showed higher anti-F1-V antibody titers that were statistically significant when compared to the soluble F1-V formulation.

Since it has been previously shown that the LcrV protein (V antigen) is the causative agent of plague**4,8,35, anti-LcrV-specific ELISAs were carried out to determine if
antibodies produced after immunization with the various vaccine regimens recognize the V antigen. Results shown in Figure 9.3B demonstrated that all the vaccine formulations evaluated induced similar levels of anti-LcrV antibodies production with the exception of the 100% unmodified F1-V encapsulated group (E_{F1,V}). Serum analysis demonstrated that IgG1 was the dominant serum antibody isotype produced (Figure 9.3D).

**Figure 9.3** Nanovaccine formulations that include αGal-F1-V soluble and/or encapsulated stimulate enhanced antibody production with high avidity. (A) and (B) Anti-F1-V and anti-LcrV IgG antibody titers, respectively. Titers were determined by end-
point ELISAs 36 days post-vaccination (pre-boost) and post-boost (42 days after vaccination). (C) IgG antibody avidity pre and post-boosting dose. (D) Antibody isotype induced by various immunization regimens. Both avidity and optical density for isotypes were determined via ELISA at a 1:500 dilution. Data is presented as the mean ± SEM representative of two independent experiments. * = p ≤ 0.005 within the pre-boosting groups compared with the S_F1-V regimen, and # = p ≤ 0.05 within the post-boosting groups compared with the S_F1-V regimen.

In addition to antibody titer, the quality of the antigen-specific antibody, including avidity, also determines vaccine efficacy\textsuperscript{36}. In the present study, mice immunized with vaccine regimens containing αGal-F1-V antigen developed a high avidity F1-V-specific IgG response (Figure 9.3C). The formulation comprised of soluble and encapsulated αGal-modified F1-V (S_αGal-F1-V + E_αGal-F1-V) showed the highest avidity index 36 days post-vaccination after the boost. In contrast, mice vaccinated with S_F1-V and E_F1-V generated a low avidity anti-F1-V specific IgG antibody.

9.4.4 Use of informatics analysis to identify optimal vaccine formulation components

Principal component analysis (PCA) was used to analyze the anti-F1-V and anti-LcrV titers and avidity indices data and to draw inferences about the contribution of each vaccine formulation component to the generated immune response. PCA enabled the simultaneous investigation of the relationship between the multiple variables of the vaccine regimens used in this study, including αGal modification of the F1-V, use of 50:50 CPTEG:CPH nanoparticles as adjuvants, and amount of soluble versus encapsulated protein.
**Figure 9.4A** depicts the vaccine formulations assessed with PCA. In this figure, the distance along the red line between the perpendicular blue line and the control describes similarity. Soluble formulations and combination of soluble plus encapsulated antigen are clustered together far away from the control, which indicate that those regimens induced higher antibody titers with high avidity. The fully encapsulated antigen in polyanhydride nanoparticle groups were located closer to the control, corroborating previous observations affirming the need for a combination between soluble and encapsulated antigen. Among the clustered groups, the three groups containing αGal-F1-V in a soluble form (S$_{αGal-F1-V}$, S$_{αGal-F1-V}$ + E$_{F1-V}$, S$_{αGal-F1-V}$ + E$_{αGal-F1-V}$) were the regimens that showed the largest difference compared to the unmodified F1-V soluble group (represented by the green square and circle that are highlighted by a blue circle, and the gray diamond, which is closer to these two groups).

**Figure 9.4B** shows the different variables that were grouped in the axis of the previous PCA biplot (**Figure 9.4A**). When related with the distribution of the various vaccine regimens in the two-dimensional space of the PCA plot, it was demonstrated that having the αGal-modification of the F1-V at the front end of the vaccine formulation stimulates a primary immune response, resulting in an enhanced initial (i.e., before administration of the antigen boosting dose) production of high F1-V-specific IgG antibody titers with high avidity.
Figure 9.4 Informatics analysis to identify optimal vaccine formulations. (A) PCA biplot of various vaccine regimens. The plot maps out high dimensional correlations permitting one to track the relative influences of varying the vaccine formulation. Distance along red line between perpendicular blue and the control describe similarity. (B) Variables that define the axis values of the previous PCA biplot. Proximity between points defines similarity.

9.4.5 Epitope Mapping Using Peptide Arrays

To further characterize the binding of the anti-sera obtained after immunization with the various vaccine regimens to the F1 and V antigens respectively, we examined the
binding of the generated antibodies to two separate panels of overlapping peptides. One panel covered the full-length of the F1 antigen (27 peptides) and the other panel covered the full length of the V antigen (53 peptides). The peptide sequences used for this study are presented in Figure 9.5A. To facilitate visualization of the peptide array data, a colored map of the data matrix was generated and is presented in Figure 9.5B. Peptides showing higher immune responses are represented as red, yellow, or light blue. From a visual analysis of the peptide map, two responsive peptides were identified for the F1-antigen (i.e., F1-1 and F1-18), while four responsive regions were observed for the V antigen (V-1, V-2, V-14, V-19, V-20, V-27, and V-44).

Figure 9.5 Differential epitope-recognition by antibodies elicited during immunization with the various vaccine formulations. α1,3GT KO mice were immunized with vaccine regimes presented in Table 1 and boosted 37 days post-vaccination. Sera collected five days after boosting was assayed for epitope recognition using a peptide array for both
the F1- and V-antigens. (A) depicts the sequence of the 80 individual peptides that were evaluated by ELISA. Peptides that showed higher response are boxed. (B) Heat map of the responses of the 80 peptides is shown, beginning at the top with the amino-terminal peptide and then moving down sequentially through the F1-V protein. Peptides which show higher immune response for specific immunization groups are presented with red, yellow, or light blue color and vaccination groups are represented by numbers identifying each of the columns for the heat map. Data are the average measurements for three pulled serum samples per vaccination group (samples from three or four mice were pulled).

Implementation of clustering analysis (i.e., hierarchical clustering) allows for grouping of similar peptides under the different treatment conditions, and rapid identification of the more responsive peptides. Two main peptide clusters were observed from this analysis (Figure 9.6). Peptides that were shown to be immunodominant segments are F1-1, F1-18, V-1, V-2, V-14, V-19, V-20, V-27, and V-44. Similarly, in the PC space, the same peptides were identified as outliers (Supplemental Figure 9.1). From Figure 9.6, a comparison of the immunization groups based on the clustering among different treatments in terms of their proximity in the data space was performed. From visual analysis, the vaccine formulation group containing soluble αGal-F1-V together with encapsulated F1-V ($S_{αGal-F1-V} + E_{F1-V}$) showed recognition of a diverse number of peptides mainly from the V antigen. From clustering analysis of the immunization groups, the vaccine regimen $S_{αGal-F1-V} + E_{αGal-F1-V}$ was shown to be closer to the $S_{αGal-F1-V} + E_{F1-V}$ group. The soluble unmodified F1-V dose ($S_{F1-V}$) was in close proximity to the control (i.e., saline) groups with a limited number of low intensity epitopes.
Figure 9.6 Peptide with significant response were identified by hierarchical clustering analysis. Implementation of clustering analysis allows for grouping of similar peptides under the different treatment conditions, and the peptides which show relatively higher response than others. The two main peptide clusters identified are circled in red. Similarity between vaccinations regimes in terms of the proximity in the data space are highlighted in a red box, similar groups are connected by blue brackets.

9.5 Discussion

The efficacy of vaccines to induce protective immune responses relies on their ability to mimic the immune pathways initiated by natural infections, including signaling to the innate immune system\textsuperscript{20,28}. Directing antigen internalization and promoting immunogenic responses by APCs, as well as preventing rapid degradation of antigen are key steps to
augment and extend immune stimulation\textsuperscript{20,37}. The data presented herein demonstrated the success of using transdisciplinary approaches to rationally design efficacious vaccines against \textit{Y. pestis} by combining two platforms, αGal-modification of the vaccine antigen (i.e., F1-V) and polyanhydride nanoparticles as vaccine delivery vehicles, which results in the induction of a robust immune response.

Characterization by SDS-PAGE and western blot analysis demonstrated successful attachment of αGal-epitopes to the F1-V antigen (\textbf{Figure 9.1}); moreover, the different release profile obtained for the αGal-F1-V in comparison with non-modified F1-V (\textbf{Figure 9.2}) is also indicative of this modification. The release profile observed for the αGal-F1-V antigen may be attributed to a non-uniform protein distribution within the nanoparticles caused by a change in the protein hydrophobicity with the addition of the αGal epitopes. Addition of carbohydrate epitopes may reduce protein hydrophobicity, distributing the antigen closer to the particle surface and resulting in a higher initial burst.

The presence of high-titer anti-αGal antibodies provides an endogenous adjuvant for induction of robust immune responses to αGal-expressing pathogens\textsuperscript{13,16}. This triggers several mechanisms that facilitate antigen uptake and presentation by APCs, including complement activation and FcγR-mediated endocytosis\textsuperscript{17,18,37}. Subsequent antigen processing and presentation by APCs may lead to the induction of a potent and specific immune response. Experimental testing of this hypothesis showed that vaccine regimens containing αGal-F1-V induced higher antibody production as indicated by antibody titers prior to boosting (\textbf{Figure 9.2A}).

Polyanhydride nanoparticles activate DCs in a chemistry-dependent manner \textit{in vitro}\textsuperscript{22,27,28} and identification of the polymer properties that direct particle interaction with
DCs has been well studied in our laboratories. These studies identified the molecular attributes that confer “pathogen-mimicking” properties to the amphiphilic 50:50 CPTEG:CPH nanoparticles\textsuperscript{28,38}. In the present study, vaccine formulations containing encapsulated antigen in polyanhydride nanoparticles were able to induce equal or higher F1-V-specific antibody titers with the exception of 100% antigen-encapsulated nanoparticles. The adjuvant properties of polyanhydride nanoparticles observed \textit{in vitro} may translate \textit{in vivo} allowing for DC migration to the draining lymph node where they can facilitate the induction of an adaptive immune response\textsuperscript{36,37}. However, the presence of soluble antigen is important during the initiation of a primary immune response, which may explain the efficacy of vaccines that combine soluble and encapsulated antigens in comparison to those formulations that contain 100% encapsulated antigen. The efficacy of vaccine regimes that include both soluble and encapsulated F1-V to induce protection after intranasal administration has been previously demonstrated\textsuperscript{32}. Higher production of antibodies with high avidity was especially achieved when soluble αGal-F1-V was delivered together with encapsulated F1-V or αGal-F1-V (Figures 9.3A and 9.3C). These observations demonstrate the efficacy of having the αGal-modified antigen at the “front end” of the vaccine formulation, which can be related to an enhanced initial antigen uptake by APCs mediated via FcγRs and induction of a primary immune response that is αGal-dependent followed by an antigen-specific response provided by the continuous antigen exposure controlled by nanoparticle degradation.

The production of antigen-specific antibodies with high avidity is also important for the induction of a robust immune response that may lead to protection\textsuperscript{32,39}. High antibody avidity is usually achieved by continuous antigen exposure and is associated
with affinity maturation of B cells\textsuperscript{39}. Antibodies with higher avidity were produced after immunization with vaccine formulations containing soluble plus encapsulated antigen (Figure 9.3C). Previous work from our laboratories has shown that the amphiphilic 50:50 CPTEG:CPH nanoparticles are internalized by APCs at a slower rate than hydrophobic nanoparticles\textsuperscript{28,38}. Moreover, the amphiphilic nanoparticles persist within the body after subcutaneous administration acting as a depot (data not shown), which when combined with the slow antigen release dictated by nanoparticle degradation, allows for extended presence of antigen resulting in more avid antibodies. The avidity of an antibody for its antigen is dependent on the affinity of individual antigen-binding sites for the epitopes on the antigen\textsuperscript{40}. Differentiation of B cells with specificity for diverse epitopes on the same antigen into plasma cells induce secretion of antibodies with binding capacities for the same multiple epitopes that elicit proliferation in the first place\textsuperscript{40}. In the data presented herein, the use of hierarchical clustering analysis allowed for identification of vaccine regimes that are similar in the data space and that were able to induce the production of antibodies that recognize several epitopes from both the F1 and V antigens (Figure 9.6). Tellingly, these vaccine formulations (i.e., \(S_{\alpha\text{Gal}-F1-V} + E_{F1-V}\), \(S_{\alpha\text{Gal}-F1-V} + E_{\alpha\text{Gal}-F1-V}\), \(S_{F1-V} + E_{F1-V}\), \(S_{\alpha\text{Gal}-F1-V}\), and \(S_{F1-V} + E_{\alpha\text{Gal}-F1-V}\)) elicited production of antibodies with higher avidity (Figure 9.3C).

Conservation of antigen epitopes during vaccine delivery is an important aspect for protection. In this regard, the amphiphilic 50:50 CPTEG:CPH polyanhydride particles are effective delivery vehicles that have shown to stabilize the F1-V antigen, maintaining its primary, secondary, and tertiary structure \textit{in vitro}\textsuperscript{21}, which may translate into conservation of linear and conformational epitopes \textit{in vivo}. Peptide arrays with the
application of informatics analysis tools (i.e. PCA analysis, and clustering analysis) were used in this study to identify immunodominant segments. In the case of the F1 antigen, the F1-1 and F1-18 peptides were identified as immunodominant. An amino acid region in the F1 antigen that has been related with protection in passive immunotherapy is located at the amine-terminal end of the F1 antigen\(^{41}\). The F1-1 peptide that was recognized by the antibodies elicited by most of the immunization groups in this study may be part of this protective sequence identified at the N-terminus of the F1 antigen. Other immunogenic F1 segments have been identified as linear B and T cell epitopes in the F1 antigen. The sections encoded by amino acids 145-162 and 123-137 have been recognize as B and T cells epitopes, respectively\(^{42,43}\). However, from our peptide array data, the immunization groups did not elicit antibodies against those specific epitopes, with the exception of the S\(\alpha\)Gal-F1-V + E\(_F1\)-V group that showed a weak response to these regions (Figures 9.5 and 9.6).

It is well known that the V antigen is an effector protein and some of its epitopes have been identified as responsible for the contact-dependent Type III secretion system during infection\(^{6,35,44-47}\). Therefore, designing vaccine formulations capable of inducing IgG antibodies capable of recognize the V epitopes responsible for infection is of great importance. In this sense, visualization of the peptide array map allows for identification of vaccine formulations that induce production of epitope-specific IgG antibodies\(^{45-47}\).

Even though the distribution of linear and conformational epitopes for F1-V is not completely known, the presence of a linear section in the F1 segment and a section dominated by \(\alpha\)-helix conformations in the V antigen suggests that conformational epitopes are mainly present in the V region\(^{44}\). Specifically, V-1, V-2, V-14, V-19, V-20, V-
27, and V-44 were identified as immonodominant sequences in our analysis (Figures 9.5 and 9.6). Two sequences in the V antigen have been previously identified to contain one or multiple protective epitopes - the main sequence is encoded by amino acids 168 to 275\textsuperscript{45,48} and the peptides V-27 and V-44, which were identified as immunodominant in this study, are located in this protective region. These peptides were mainly recognized by antibodies elicited after immunization with vaccine regimens that combined the αGal-F1-V and polyanhydride nanoparticle platforms. A second protective region has been identified at the N-terminus of the V antigen (2 to 135)\textsuperscript{45,46,48,49}, which is partially encoded by amino acids within the peptides V-1, V-2, V-14, V-19, and V-20. These peptides were also shown to be immunodominant by the same vaccine regimens. Some of those epitopes were related to recognition by CD4 T cells (i.e., 71-86, 101-116, and 166-178), which are partially encoded by the V-14, V-19, and V-20 peptides\textsuperscript{46,49}.

Identification of protective B and T cell determinants is necessary in order to develop such sequences as vaccine immunogens or to design appropriate antigen delivery vehicles that can protect specific epitopes during the delivery process. Collectively, these results indicate that the combination of αGal-modification and amphiphilic polyanhydride nanoparticles in a vaccine regimen against Y. pestis enhanced the production of F1-V-specific IgG antibodies with high avidity, with recognition of protective epitopes, which suggests its potential to induce protection. Using peptide arrays in combination with informatics tools, we have developed a new toolbox in understanding antigen-antibody interactions, generating information that allows for the rational selection of antigen (i.e., αGal-modification) and adjuvant (i.e., amphiphilic
polyanhydride nanoparticles) based platforms for the design of efficacious single dose vaccines.

### 9.6 Conclusions

A transdisciplinary approach that combined concepts from material science, protein chemistry, immunology, and computational analysis, was applied to rationally design a vaccine for *Y. pestis* capable of inducing a robust immune response. This vaccine formulation combines two platforms, αGal-modification of the F1-V antigen and amphiphilic polyanhydride nanoparticles as vaccine delivery vehicles and adjuvants, in order to direct and enhance the immune response previously obtained by vaccines based on the F1-V antigen and other adjuvants (e.g., liposomes, flagellin, cytokines). High antibody titers with high avidity and with the ability to recognize protective epitopes were elicited by vaccine regimes containing soluble αGal-F1-V and encapsulated αGal-F1-V or F1-V. The αGal-modification of antigen and amphiphilic polyanhydride nanoparticles technologies can function in tandem to design efficacious vaccine platforms for a wide range of pathogens.

### 9.7 Acknowledgments

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9.8 References


9.9 Supplemental data

Supplemental Figure 9.1 Principal component analysis (PCA) of peptide arrays corroborates as outliers the same set of peptides identified by clustering analysis. The plot maps out high dimensional correlations permitting identification of significance responder from the peptide array data.
CHAPTER 10

Conclusions

Innovative and safe vaccine platforms are needed to design efficacious prophylactic and therapeutic vaccines to protect humans from new and re-emerging infectious diseases. Ideally, new vaccine regimens would provide protection from disease following a single immunization. Therefore, the need to design novel drug and vaccine delivery methods is urgent and it requires the use of complementary strategies to stimulate both the innate and adaptive immune systems. This work has focused on the design of targeted drug and vaccine platforms based on amphiphilic polyanhydride particles that can result in the generation of robust immune responses. A transdisciplinary approach that combines concepts from biomaterials, nanotechnology, carbohydrate and protein chemistry, molecular biology, immunology and computational analysis was applied to rationally design and engineer novel vaccine platforms that encompass passive and active targeting strategies. Yersinia pestis, the causative agent of bubonic and pneumonic plague, was the disease of interest in this project; however, the platforms developed can be applied to different diseases.

Passive targeting strategies for the design of delivery vehicles based on polyanhydride particles were explored in the chapters 4 and 5 of this thesis. Polymer chemistry was selected as the main variable to control antigen stabilization and release. The importance of selecting the appropriate polymer chemistry to maintain structural integrity resulting in a biologically stable protein (e.g., antigen and antibodies) was
demonstrated in Chapters 4 and 5. Particles based on amphiphilic 50:50 CPTEG:CPH preserved antigenicity of recombinant proteins and the biological function of antibodies, suggesting that these polymers are promising carriers for *Y. pestis* antigens as well as for the delivery of functional antibodies for passive immunotherapy.

The surface chemistry of the particle may also dictate interactions of the particles with soluble and cellular components of the immune system. The exposure of these particles to serum generally results in the adsorption of specific proteins to their surfaces and alters their recognition by immune cells and their biodistribution. In Chapter 6, the variables guiding protein adsorption phenomena were identified by studying the *in vitro* adsorption of model plasma proteins. These studies demonstrated a direct correlation between polymer hydrophobicity and amount of protein adsorbed. This analysis was expanded upon in Chapter 7 when the effect of the chemistry-dependent adsorption of serum proteins on the uptake and activation of antigen presenting cells (i.e., dendritic cells or DCs) was evaluated. This work revealed changes in particle uptake and activation phenotype of DCs as a consequence of surface adsorption of opsonins (i.e., immunoglobulin G and complement component C3) and provided important insights into the mechanisms mediating polyanhydride particle uptake (i.e., complement receptor-mediated uptake).

The insights obtained with respect to how particle surface chemistry may be important in their interactions with immune cells led to the design and development of active targeting strategies to improve and direct the adjuvant properties of polyanhydride particles. In Chapter 8, a novel approach to targeted antigen delivery was developed by functionalizing the surface of polyanhydride nanoparticles with specific carbohydrates to
provide “pathogen-like” properties that ensure nanoparticle recognition by C-type lectin receptors on DCs. The ability of these novel particles to induce and enhance DC activation by specific interactions with the mannose receptor was demonstrated. The receptor-mediated endocytosis induced by the direct interaction of mannose residues with the mannose receptor may be exploited to design efficacious nanovaccines.

Continuing with the development of innovative vaccine platforms based on active targeting strategies, a novel approach was adopted by combining amphiphilic polyanhydride particles as delivery vehicles with the αGal-modification of vaccine antigens trying to target the endogenous pathways (i.e., FcγR-mediated endocytosis) activated by the αGal motifs (Chapter 8). *Yersinia pestis* was used as a target pathogen to test the proposed vaccine regimes in vivo. The amphiphilic 50:50 CPTEG:CPH nanoparticles were used to deliver the *Y. pestis* antigens based on results from Chapter 4 that showed its ability to stabilize these antigens. Results showed that high antibody titers with high avidity and with the ability to recognize protective epitopes were elicited by vaccine regimens containing soluble αGal-F1-V and encapsulated αGal-F1-V or F1-V. The data presented herein demonstrated the success of using transdisciplinary approaches to rationally design efficacious vaccines against *Y. pestis* by combining two platforms, which results in the induction of robust immune responses.

Taken together, these studies demonstrated the promise of amphiphilic polyanhydride particles as drug delivery devices and vaccine adjuvants, and provided key insights into the rational design of targeted platforms to enhance the induction of antigen-specific immune responses and to facilitate the design of protective targeted therapeutic treatments and vaccines.
CHAPTER 11

Ongoing and Future Work

The research described in this thesis has demonstrated the versatility and promise of biodegradable polyanhydride particles as a platform for targeted vaccine delivery. The ability to incorporate passive and active targeting strategies into these vehicles and combine them with other platforms (i.e., antigen modification) to improve vaccine efficacy provides a basis for future research. While polymer chemistry was the main variable investigated for passive targeting, showing its potential to provide controlled release and antigen stabilization and serum protein adsorption and interactions with antigen presenting cells (APCs), the investigation of other variables like particle size and shape and their effect on interactions with APCs may help design vaccine delivery vehicles that mimic pathogens. On the other hand, the active targeting strategies described herein can be expanded by incorporating different ligands (i.e., peptides, antibodies, haptens) to target other receptors on APCs (i.e., Toll-like receptors, TLRs). A pictorial summary representing the passive and active strategies utilized in this research and proposed as future work is shown in Figure 11.1. While the studies described in Chapter 6 and the proposed incorporation of other ligands provide key insights on the targeting capabilities of the functionalized particles, the downstream mechanisms of antigen processing and presentation initiated by these targeted vaccine adjuvants need to be studied and described. This can be performed by utilizing receptor knockout mice and a combination of individual cell-based and cell population-based methods. It is also
important to relate the results obtained by the mechanistic *in vitro* experiments to the evaluation of the *in vivo* targeting capabilities of the functionalized polyanhydride particles. Applying these studies to the target disease utilized in the present work (i.e., *Yersinia pestis*) could result in optimization of the vaccine formulation with dose sparing and immunity against knockout strains of bacteria designed to function as bioterrorism weapons. Finally, future work combining the broad database of knowledge and platforms gathered through these investigations could be utilized to design single-dose, targeted vaccine strategies for other diseases such as influenza and HIV.

**Figure 11.1** Pictorial summary of passive and active strategies discussed in this work. Particle size and shape may direct particle internalization through different uptake mechanisms (i.e., phagocytosis, endocytosis, or micropinocytosis). Particle chemistry and incorporation of TLR ligands target initiation of molecular danger signals that may enhance APC activation. Finally, incorporation of other ligands (i.e., carbohydrates) on the particle surface can target particle internalization through receptor-mediated endocytosis, which can direct the generated immune response.
11.1 Evaluation of Polyanhydride Particle Size and Shape Effects on the Internalization and Activation of APCs

While the work described in Chapters 4 and 5 demonstrated that tailoring polyanhydride chemistry can be used as a passive targeting strategy for the stability and controlled release of vaccine antigens (i.e., *Y. pestis* antigens and antibodies for passive immunotherapy)\(^1\), chemistry-dependent serum protein adsorption patterns were also identified\(^3\) and their influence on particle uptake by APCs (i.e., DCs) and activation of these immune cells was demonstrated (in Chapters 6 and 7)\(^4\). These findings provided adequate information for the transition from passive targeting to the incorporation of molecules for active targeting; however, there are other physical characteristics of polymeric particles such as size and shape that have the potential of targeting specific mechanisms of particle internalization by APCs\(^5\)\(^-\)\(^18\). The mechanism by which DCs uptake and internalize particles will determine the subsequent processing and presentation pathway (i.e., MHC class I, MHC class II, or cross-presentation pathways)\(^15\)\(^,\)\(^18\)\(^-\)\(^20\). Particle size appears to be a key determinant for the uptake and internalization of particles. Particles having a size range between 0.5 to 10 µm (i.e., microparticles and large nanoparticles) are optimally taken up by phagocytosis, particles <500 nm but > 200 nm are internalized through endocytosis and are trafficked to early endosomal compartments\(^15\)\(^,\)\(^21\) and macropinocytosis is the uptake mechanism that DCs use for small nanoparticles (<50 nm)\(^16\)\(^,\)\(^17\)\(^,\)\(^20\). Particle size is also an option to target a specific and desirable delivery route; for example, for mucosal delivery it is known that particles between 100 and 500 nm in size are preferred because they can pass through the nasal cavity into the lungs\(^22\). Additionally, internalization of both opsonized and non-
opsonized PS and PLGA devices with different shapes exhibited a strong dependence on local particle shape from the perspective of the phagocyte\textsuperscript{11,12,14}. This work demonstrated similarities between bacteria and particles with the same shape\textsuperscript{14}.

Previously developed methods to fabricate monodisperse particles\textsuperscript{23} and particles of different shapes\textsuperscript{11,12,14} can be applied to the copolymer systems used in this research (i.e., CPH:SA and CPTEG:CPH). We have performed preliminary studies to determine the effect of particle shape on the activation of DCs and initial results showed that DCs stimulated with PS and PLGA rods show differential expression of co-stimulatory molecules (i.e., CD40) and secretion of cytokines (i.e., IL12-p40) compared with spheres from these chemistries (\textbf{Figure 11.2}). Based on this work, controlling polyanhydride particle size and shape together with the already generated information on the effect of polymer chemistry upon immune activation may be of great advantage for the rational design of targeted vaccine platforms.

\textbf{Figure 11.2} Dendritic cells stimulated with PS and PLGA rods show different expression of co-stimulatory molecule CD40 (A) and secretion of cytokine IL12-p40 (B) compared with spheres from these chemistries. After stimulation with either spheres or rods made of PS or PLGA for 48 h, supernatants were harvested and assayed for cytokine
secretion and then DCs were harvested and analyzed by flow cytometry for surface expression. Data is presented in mean fluorescence intensity (MFI) for cell surface marker expression and concentration (pg/mL) for cytokine production, and is representative of one experiment performed in triplicate.

11.2 Functionalized Polyanhydride Particles to Target TLRs on APCs

The ability of carbohydrate-functionalized polyanhydride particles to be internalized by DCs and enhancement of their activation mediated by the mannose receptor was demonstrated in Chapter 8. The design of protocols to fabricate these carbohydrate-functionalized particles ensures the targeting of C-type lectin receptors (CLR), which have the potential to efficiently deliver antigens to DCs for processing and presentation to T cells. However, it is known that engagement of other pattern recognition receptors (PRR) on APCs may lead to the initiation of different signaling events and moreover, that signaling through a combination of PRRs may also benefit the generation of robust immune responses. The most well studied PPRs have been TLRs and eleven of these receptors have been identified in humans. TLR signaling plays an important role in determining the quality of the T cell response. For example, the use of lipopolysaccharide (LPS), which is a TLR4 ligand, leads to a preferential cell-mediated response (TH1). The role of TLRs in the initiation of innate immune responses makes them potential targets for vaccines by using them as vaccine adjuvants combined with the polyanhydride particle platform.

We performed preliminary studies on the targeting of TLRs by translating the protocols developed to attach carbohydrate residues to modify the surface of polyanhydride nanoparticles with poly I:C, a TLR3 ligand. Encapsulation of this ligand...
was also tested and the activation of bone marrow derived macrophages with these functionalized particles was evaluated. Initial results (Figure 11.3) demonstrated that pathogen-associated molecular patterns (PAMPs), i.e., poly I:C, embedded within the particle through encapsulation or attached to the particle surface mimic the structure of pathogens leading to increased secretion of inflammatory cytokine. Initiation of inflammatory cytokines by inclusion of PAMPs could help drive more robust adaptive immune responses towards antigens loaded within the nanoparticles. Therefore, using TLRs is an active targeting approach with great potential and it should continue to be an area of investigation to improve the adjuvant properties of polyanhydride particles.

**Figure 11.3** Maximum stimulation of macrophages occurs when nanoparticles encapsulating Poly I:C are phagocytosed. 20 µM cytochalasin D in PBS or PBS alone were administered to the cells for 15 min prior to administration of the nanoparticle treatments. Cell free supernatant samples were collected at 24 h to measure IL-1β, IL-6, IL-10, IL-12 p40 and TNF-α using Luminex® Multiplex assay. Significant differences
between treatment groups are identified by different letters above the histograms (p < 0.05, n=6) as determined by one-way ANOVA with Tukey post-test. * indicates statistical differences of cytochalasin D treatment from PBS via Mann-Whitney U test (p < 0.05, n=6).

11.3 In Vitro and In Vivo Mechanistic Evaluation of Functionalized Polyanhydride Particles

As mentioned before, the in vitro targeting properties of the carbohydrate-functionalized particles were demonstrated by the increased expression of CD206 and CD209 with an accompanying increased expression of MHC II and CD86 on DCs (Chapter 6)\(^6\). However, more studies are needed in order to better comprehend the adjuvanticity and targeting potential of these materials. The next step should be to evaluate the effect that sugar modification and the subsequent targeting of specific receptors on APCs will have on the mechanisms of particle internalization and processing. To accomplish this, the utilization of CLR knockout (KO) mice is proposed to determine the role of CLR engagement on the internalization of functionalized particles by APCs and their activation profile. To further elucidate the mechanisms which nanoparticles exploit to propagate antigen presentation a combination of cell-based and cell population-based methods (i.e., fluorescence microscopy methods and Image Stream, and flow cytometry and Bioplex® analysis, respectively) can be used\(^4,6-9,33\). Preliminary studies using DCs derived from the bone marrow of mannose receptor (MMR)-KO mice and combining ImageStream and flow cytometric analysis corroborated the role of engagement of this receptor by di-mannose-functionalized particles on the activation of DCs. From Figure 11.4, it can be observed that while MMR-KO DCs
internalized functionalized particles at similar rates than wild type (WT) DCs, the levels of expression of major histocompatibility complex II (MHC II) and co-stimulatory molecules (i.e., CD40 and CD84) were lower for MMR-KO DCs, indicating that engagement of the mannose receptor is necessary for the activation of DCs. The methods used with these KO mice can be applied to other mice models (i.e., SIGNR3 KO mice) to expand the evaluation of the interactions of sugars with specific CLRs.

**Figure 11.4** (A) MMR-KO DCs internalized functionalized-particles at similar rates than wild type (WT) DCs, and (B) expression of MHC II and co-stimulatory molecules CD40 and CD84 after stimulation with mannose-functionalized 50:50 CPTEG:CPH nanoparticles was lower for MMR-KO DCs, indicating that engagement of the mannose
receptor is necessary for the activation of DCs. Particle internalization was determined using ImageStream at 30 min, 2 and 48 h, and data is presented as percent of particle positive cells and is the mean of three independent experiments. Flow cytometric analysis was used to determine expression of surface molecules after stimulation with either non-functionalized or functionalized 50:50 CPTEG:CPH nanoparticles. Data is presented as mean fluorescence intensity (MFI) and is the average of three independent experiments performed in triplicate. Error bars represent standard error.

Furthermore, it is necessary to evaluate the in vivo performance of carbohydrate-modified particles. In order to do this, the design and performance of in vivo studies in animal models is proposed to evaluate the adjuvant properties of these materials. Initially, the in vivo distribution of these particles following either intranasal (IN) or subcutaneous (SC) administration to mice can be studied by using a Kodak In Vivo Multispectral Imaging System. The ability to assess particle distribution in real time combined with mechanistic internalization studies will provide insights into how the adjuvant properties of functionalized polyanhydride particles can be used to target the vaccine to the desired tissues to enhance and modulate the subsequent immune response in the presence of antigen.

11.4 Optimization of Yersinia pestis Vaccine by Use of Functionalized Polyanhydride Particles

While single administration of a vaccine formulation of soluble F1-V co-delivered with F1-V loaded 50:50 CPTEG:CPH nanoparticles provided complete protection against lethal Y. pestis challenge and induced a long-lived, high-titer, high-avidity antibody response34 and the αGal-modification of the F1-V antigen studied in Chapter 9 is an
option to improve the efficacy of a *Y. pestis* vaccine, optimization of different parameters is still necessary. Vaccine formulations combining soluble F1-V together with encapsulated F1-V showed superior performance when delivered intranasally or subcutaneously when compared with 100% of encapsulated antigen or 100% soluble antigen. However, only two ratios of soluble to encapsulated antigens were studied (by using different immunization routes) - 80:20 (i.e., intranasally) and 50:50 (i.e., subcutaneously). There may be an optimal ratio of soluble to encapsulated antigen that may convey protection with robust antibody responses. Determination of this optimal total antigen dose will allow for utilization of the lowest antigenic dose thereby reducing cost. A three step experimental design is proposed to achieve the fabrication of a vaccine with dose sparing capabilities. The first two steps involve the reduction of the total antigen dose, while the third step includes the utilization of functionalized polyanhydride particles to enhance and extend the generated immune response. In the first experiment, the encapsulated antigen dose could be maintained at 10 µg of F1-V (same encapsulated dose as that utilized in the studies that resulted in protection), while the soluble antigen dose could be reduced until getting to a final dose eight times lower than that used in the intranasal studies. Figure 11.5 shows F1-V-specific IgG1 antibody titers through 6 weeks post vaccination, and the data indicate that the vaccine regimens are comparable with a formulation containing MPLA and even when this adjuvant was able to elicit higher antibody titers during the initial weeks of the study, at week six these titers start falling which is consistent with previous data. In the first six weeks of the study, it was not possible to observe clear differences between the immunization groups combining different doses of soluble antigen with encapsulated F1-V; this study is still
ongoing. After completing this first experiment, the next step will be to reduce the encapsulated dose in order to find an optimal dose that will result in similar antibody titers to those elicited by the total antigen dose that have previously provided protection.

**Figure 11.5** Kinetics of IgG1 antibody titer for 6 weeks post-vaccination. Soluble antigen dose (S) was reduced from 50 µg following two-fold dilutions to 5 µg of F1-V, while the encapsulated dose of antigen in 50:50 CPTEG:CPH nanoparticles was kept constant (10 µg of F1-V).

Current research has analyzed the vaccine adjuvant and delivery capacity of particles composed of a specific polymer chemistry without the inclusion of any targeting agent on the surface of these carriers. A logical next step, after their success in *in vitro* studies, is to investigate the efficacy of carbohydrate-functionalized polyanhydride particles in generating a robust immune response that compensates for the reduction of antigen dose and that can result in protection. It may be possible for the optimal vaccine formulation to be a cocktail vaccine in which a combination of non-
functionalized and functionalized (with different ligands) particles, and particles with
different sizes and administration through different routes may provide protection.

11.5 Functionalized-Polyanhydride Particles for the Single-Dose, Targeted
Vaccination against Other Infectious Diseases

The transdisciplinary approach proposed in this research for the rational design of
efficacious targeted vaccines showed success in the design of a single-dose, dose
sparing targeted vaccine for *Yersinia pestis*; however, this platform approach can be
applied to other respiratory and non-respiratory diseases that may find an advantage in
using the passive and active targeting strategies designed in this work. A first step may
be to translate this approach for the design of targeted vaccines for other respiratory
diseases such as influenza. It is hypothesized that the development of a single-dose
targeted vaccine based on the use of carbohydrate-functionalized polyanhydride
nanoparticles administered intranasally may result in the induction of robust immune
responses due to the fact that both the administration route and the targeted receptor-
mediated endocytosis pathway mimic the way the influenza virus enters the body and
how it is recognized by the innate immune system (i.e., macrophages)\(^{35}\). It is known that
the influenza A virus uses the mannose receptor on macrophages (i.e., alveolar
macrophages) for viral entry\(^{35}\); therefore the use of mannose-functionalized particles to
deliver influenza antigens may target the same entrance pathway of the pathogen
increasing the possibilities of this platform for success. However, other CLRs (i.e.,
galactose-type lectin receptor) have been also identified as an entry receptor for this
virus, which is targeted by the sugar galactose. There is a need for the development of
rapid strategies for the fabrication of these novel particles. Currently in our laboratories in combination with our collaborators from the ISU Chemistry Department (Dr. Pohl’s research group) whose expertise is in the synthesis of different sugars for particle-attachment, a high-throughput system for the fabrication and evaluation of carbohydrate-functionalized particles has been designed. Preliminary studies demonstrate the ability of this high-throughput set up to attach three different sugars on polyanhydride particles within the same experiment. These particles were evaluated for their capacity of activate alveolar macrophages *in vitro* and preliminary results demonstrated that both mannose- and galactose-functionalized particles enhanced the activation of macrophages (**Figure 11.6**). Although these results demonstrated the potential of the high-throughput approach to accelerate and improve the design of functionalized particles, there are still several variables that should be tested (e.g., particle size, particle chemistry, sugar quantity) to maximize the value from this technique.

![Figure 11.6](image)

**Figure 11.6** Alveolar macrophages stimulated with di-mannose- or galactose-functionalized 50:50 CPTEG:CPH nanoparticles enhanced the expression of the co-
stimulatory molecule CD86 and increased secretion of pro-inflammatory cytokines (i.e., TNF-α). Cell free supernatant samples were collected after 48 h to measure cytokine secretion using Luminex® Multiplex assay. For cell surface marker expression (presented as mean fluorescence intensity, MFI), cells were harvested 48 h after stimulation, stained and analyzed by flow cytometry. Data is the mean of at least three independent experiments performed in triplicate and error bars represent standard deviation. Groups identified with different letters are statistically significant.

The design of an effective HIV vaccine may also benefit from the carbohydrate-functionalized particles and new strategies for their rapid fabrication and optimization. The C-type lectin receptor DC-SIGN has been identified to play a critical role in the pathogenesis of HIV and current studies are looking for interactions between the HIV virus and other CLRs (i.e., mannose receptor). The ability of mannose-functionalized polyanhydride particles to target this receptor was demonstrated in Chapter 8; however it has been also suggested that high-mannose structures may target DC-SIGN more efficiently. Therefore, synthesis and attachment of higher functionality sugars to identify the sugar chemistry that can target this receptor efficiently may benefit immune response enhancement against HIV. Recent work in our laboratories has demonstrated the capability of 50:50 CPTEG:CPH nanoparticles to release and stabilize the gp41 antigen (Figure 11.7), which is a candidate antigen for the design of an efficacious HIV vaccine. These initial studies show the promise of using polyanhydride particles as HIV antigens carriers and their adjuvant properties may be enhanced by functionalizing their surfaces with CLR ligands.
Figure 11.7 50:50 CPTEG:CPH nanoparticles release and stabilize the gp41 antigen, which is a candidate antigen for the design of an efficacious HIV vaccine. (A) Release kinetics of gp41 from 50:50 CPTEG:CPH nanoparticles for 30 days. Protein concentration was determined by micro-BCA assay. (B) Antigenicity of released protein was maintained upon encapsulation and release from polyanhydride nanoparticles. Antigenicity was determined by specific-ELISA against three important HIV neutralizing antibodies (2F5, 4E10, and Ze13).
11.6 References


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