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Development and Characterization of Microencapsulated Nanoparticle Systems for Oral Vaccination by Protein-Antigens

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Development and Characterization of Microencapsulated Nanoparticle Systems for Oral Vaccination by Protein-Antigens

by

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Dedication

It takes a village. This work is dedicated to my friends, family, and fiancé, for the unyielding support, encouragement, and love.

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Development and Characterization of Microencapsulated Nanoparticle Systems for Oral Vaccination by Protein-Antigens

Lindsey Anne Sharpe, Ph.D.

The University of Texas at Austin, 2017

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A composite platform strategy for oral vaccination with subunit antigens was developed to improve i) ease of administration and distribution; and ii) induction of mucosal immunity. The platform is referred to as Polyanhdyride-Releasing MicroParticle Technology, or PROMPT. In its core, polyanhydride nanoparticles based on 1,6-bis-(*p*-carboxyphenoxy)hexane (CPH) and sebacic acid (SA) served simultaneously as adjuvant and delivery vehicle of subunit antigens, while microencapsulation by pH-responsive polymers based on poly(ethylene glycol) (PEG) and poly(methacrylic acid) (PMAA) enabled targeted intestinal delivery of the nanoparticle payload. PROMPT formulations were synthesized by pH-mediated self-assembly to encapsulate nanoparticles. The reversible pH-responsive transition of these formulations coincided with the pH transition experienced during intestinal delivery, such that particles dissociated to release nanoparticles above pH 5. The physicochemical characteristics of the composite microgels were evaluated by Fourier transform infrared spectroscopy, electron microscopy, and confocal microscopy. PROMPT formulations demonstrated pH-dependent burst release of the encapsulated model antigen, ovalbumin, and then sustained release thereafter in both neutral pH and simulated gastrointestinal conditions. The biocompatibility and immunostimulatory capabilities of PROMPT formulations were

evaluated in relevant cell lines to identify lead candidates for *in vivo* immunization experiments. PROMPT composite formulations demonstrated greater than 85% viability at microgel concentrations less than 1mg/mL, as indicated by cellular proliferation and membrane integrity. PROMPT microgels also demonstrated the ability to activate bone marrow-derived dendritic cells *in vitro* by stimulating cell surface marker expression and cytokine secretion. Finally, the ability of lead formulations to elicit immune responses was assessed *in vivo* by administering PROMPT formulations to BALB/c mice by oral gavage. PROMPT formulations induced measurable ovalbumin-specific IgA and IgG in mucosal fluids and blood serum, respectively, while soluble antigen and nanoparticles alone did not. This work shows that microencapsulation of nanoparticles for oral vaccine administration is a promising platform for developing safe, effective subunit-based vaccines.

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Chapter 1: Introduction[1]

Vaccines have reduced substantially the burden of infectious disease, second only to clean drinking water in reducing mortality worldwide (1). Immunization is a cost effective strategy that protects not only the vaccinated individuals, but can indirectly protect the surrounding community through the generation of herd immunity (2).

Development of vaccines against a variety of diseases, including diphtheria, tetanus, polio, measles, mumps, rubella, hepatitis B, and meningitis, have reduced the associated mortality by 97-99% (3). However, even with multiple successful vaccination campaigns, infectious diseases remain the second leading cause of death worldwide, disproportionately affecting children under the age of 5 and people in low income countries (4). In fact, five of the top ten leading causes of death in low income countries are caused by infectious agents: lower respiratory infections (e.g. pneumonia), HIV/AIDS, diarrheal disease, malaria, and tuberculosis. While some of these pathogens currently lack a vaccine necessary for disease control, an estimated 20% of these deaths result from vaccine-preventable diseases, indicating the need for substantial improvement in vaccine technology and administration (4-6).

The majority of infections occurs after crossing one of the body's numerous protective mucosal barriers (5, 7, 8). For example, potentially fatal diarrheal diseases are often caused by enteropathogens crossing the mucosal barrier of the GI tract after ingestion of contaminated water (9). The formation of an immunologically strong mucosal barrier would be an effective strategy to prevent infection at the point of contact between microbes and the host. However, the current standards of vaccine technology

[1] J. Vela Ramirez, L. Sharpe, and N.A. Peppas. *Current State and Challenges in Developing Oral Vaccines*. Adv. Drug Deliv. Rev. 2017, In Press. Vela Ramirez and Sharpe contributed equally to the review article and Peppas oversaw the project.

typically only address pathogens that have already bypassed a mucosal barrier. The majority of licensed vaccines are administered either by subcutaneous or intramuscular injection. The resulting immune response is generally limited to systemic humoral immunity (*e.g.* antibody production) against the pathogen or toxin, with limited cellular immunity (*e.g*. T cell-mediated), and only weak protection generated at the mucosal surfaces (10, 11). In contrast, vaccination at mucosal surfaces successfully induces mucosal antibodies (IgA) and cell-mediated immune responses, while still producing a systemic antibody response (IgG) (12-15).

The largest mucosal surface in the body, the GI tract, is readily accessible via oral administration. The oral delivery of therapeutic drugs represents the current gold standard of administration due to the opportunity for self-administration, improved patient compliance, and the ease of distribution compared to injection-based therapies (16-19). Additionally, vaccine efficacy is highly correlated to its regional coverage, which is affected by the accessibility, stability, and distribution of the formulation (2, 20). Consideration of these parameters is important in the development of next-generation vaccines.

Unfortunately, despite the numerous immunological and practical advantages associated with oral delivery, only a limited number of oral vaccines are available (21, 22). The oral route poses obstacles, including degradation of fragile antigens through the harsh environment in the stomach, requirement of higher doses to generate immunity instead of tolerance, and surpassing physicochemical defense mechanisms of the mucosa to stimulate immune response (21, 23). Each challenge within the GI tract poses a unique engineering problem that requires careful consideration to achieve efficacious vaccine design.

The use of biodegradable polymeric particles for antigen delivery has gained attention as a strategy to prevent degradation of immunogens in the GI tract (24, 25). Polymeric materials can be manipulated based on chemical characteristics in order to tailor release kinetics of the encapsulated payload, direct responsiveness to the local environment (e.g. pH), and provide immune stimulation (23, 26, 27). Specifically, polyanhydrides are a family of biodegradable polymers with desirable properties for novel drug and antigen delivery platforms (25, 28, 29). Polyanhydrides are hydrophobic materials that undergo surface-erosion, and have been demonstrated to stabilize various types of antigens (i.e. proteins, peptides), sustain antigen release, and degrade into biocompatible products (25, 28-30). Furthermore, polyanhydrides possess adjuvant properties and can stimulate both humoral and cellular immune responses (31-33).

The encapsulation of nanoparticle in microspheres has been explored for oral delivery applications, which require cellular uptake for efficacy (34-38). A polymeric coating can improve nanoparticle stability in contact with physiological fluids in the gastrointestinal tract until reaching the absorptive window in the intestinal epithelium and then enable depot delivery of the nanoparticle cargo. A class of pH-responsive complexation hydrogels, based on methacrylic acid (MAA) has been designed to facilitate transmucosal administration of sensitive therapeutics (39-44). MAA is readily polymerized and has a pKa that aligns with the natural pH transition in the GI tract, such that systems remain collapsed in acidic pH (i.e. gastric conditions) and swell in the neutral environment of the upper small intestine for targeted release.

Based on the characteristics of these systems, the main goal of this research was to develop a composite platform as an oral vaccine strategy. The microencapsulation of polyanhydride nanoparticles could enable protection through the harsh environment of

the GI tract and target delivery proximal to the antigen sampling cells within the small intestine. These nanoparticles, in turn, possess "pathogen-mimicking" characteristics and sustain antigen release that facilitates cellular uptake, antigen presentation, and immune stimulation. Herein, the development of microencapsulated polyanhydride nanoparticles as a strategy for subunit oral vaccine delivery is evaluated. The evaluation of these composite microparticles is presented, including characterization of polymer composition, behavior, and ability to release model protein antigen (Chapter 4); safety and immunostimulatory behavior in *in vitro* models (Chapter 5); and ability to induce systemic and mucosal antibody protection *in vivo* (Chapter 6).

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Chapter 2: Background[2,3]

2. 1 INTRODUCTION

Development of vaccines administered orally is desirable for numerous reasons including improved safety and compliance, and easier manufacturing and administration. Additionally, the oral route enables stimulation of humoral and cellular immune responses at both systemic and mucosal sites to establish broader and long-lasting protection. However, oral delivery is challenging, requiring formulations to overcome the harsh gastrointestinal (GI) environment and avoid tolerance induction to achieve effective protection.

This chapter presents a systematic analysis of the barriers associated with the gastrointestinal delivery of vaccines and design strategies for novel delivery vehicles and next-generation oral vaccine development. Section 2.2 discusses the motivation to develop effective strategies for subunit vaccine delivery, and the physiological and immunological criteria to inform vaccine design. Section 2.3 reviews particle systems designed for oral administration of subunit vaccines. Section 2.4 discusses the advantages of the materials used in this work, specifically responsive hydrogels for oral delivery applications and polyanhydride materials for drug and vaccine delivery.

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[3] J. Vela Ramirez, L. Sharpe, and N.A. Peppas. *Current State and Challenges in Developing Oral Vaccines*. Adv. Drug Deliv. Rev. 2017, In Press. Vela Ramirez and Sharpe contributed equally to the review article and Peppas oversaw the project.

2.2 ORAL VACCINES

2.2.1 Types of Vaccines

The history of vaccine development has largely followed Pasteur's guiding principles of "*isolate, inactivate, and inject*" the causative microorganism, with the earliest successes resulting from trial and error (1). However, advances in genetic engineering have enabled improvements in the design of vaccine technology and diversification of formulation types, thus expanding the number of diseases that can be prevented.

The earliest vaccines were live attenuated, meaning they contained a version of the living microbe that had been weakened or altered in the lab so as not to cause severe infection. Live-attenuated formulations most closely mimic natural infections, eliciting strong cellular and antibody responses that are likely to confer long-lived protective immunity (2). Unfortunately, live, weakened vaccines can also pose risks such as inflammation, uncontrolled replication, and disease, particularly in immuno-compromised patients. Additionally, though extremely rare, attenuated pathogens have the potential to revert to a pathogenic form and cause the disease. For example, the live oral poliovirus vaccine (OPV) has not be administered in the United States since 2000 due to the risk of vaccine-associate paralytic poliomyelitis and the availability of a safer alternative in the form of an injected inactivated vaccine (3). Advancements in genetic engineering have reduced the unpredictability of experimental attenuation and improved the safety associated with live attenuated viruses in a variety of ways, including manipulation or elimination of genes required for replication (4).

A safer alternative to live-attenuated vaccines is killed whole-cell vaccines, which consist of the disease-causing microbe inactivated by chemicals, heat, or radiation.

Inactivated vaccines can still prompt an immune response, but cannot replicate. Consequently, these vaccines are safer and more stable options than live vaccines, but stimulate a weaker immune response, generally requiring additional doses or booster shots to maintain protection (5, 6).

While vaccine development has traditionally focused on either live or killed whole organism vaccines, next-generation vaccine development has begun to focus on even safer and more cost-effective vaccine candidates: subunit vaccines. Subunit vaccines are considered the safest alternative as they do not contain any live components of the pathogen. They can be divided into four main categories: protein-based, polysaccharides, conjugates, and toxoids.

Protein-based subunit vaccines use a specific and isolated protein that is presented as an antigen to the immune system. These molecules can be harvested and purified from the cultured microbe or manufactured using recombinant DNA technology(7). However, proteins are fragile structures and are easily denatured and degraded by changes in pH or presence of proteolytic enzymes(8, 9). Polysaccharide vaccines mimic the polysaccharide capsules associated with infectious bacteria, thereby eliciting an immune response. Similar to protein subunit vaccines, they are not very immunogenic, and, therefore, are associated with short-term immunological responses, not long-term memory.

Conjugate vaccines also create a response against the pathogen's protective polysaccharide capsule; however, they include a carrier protein in addition to the polysaccharides to improve generation of long-term protective immunity. Some of the commonly used protein carriers include diphtheria and tetanus toxoids and are, therefore, generally used against bacterial infections. Lastly, toxoid vaccines are used against pathogens in which bacterial toxin is the primary cause of illness, such as diphtheria and

tetanus (7). They are inactivated versions of the toxins and, therefore, are both safe and stable. However, most toxoid vaccines require the use of adjuvant, such as aluminum or calcium salts, for an effective immune response.

All subunit vaccines differ from inactivated immunizations by containing select antigenic parts of a pathogen that are required to elicit a protective immune response. These formulations provide excellent stability and safety profiles, but the process to find the appropriate combination of the aforementioned antigenic components in order to produce an effective immune response is extremely time-consuming(2, 10). Furthermore, subunit vaccines tend to be less immunogenic than their whole-cell counterparts. Current research has focused on the addition of adjuvants in order to enhance the immune response through inclusion of immunostimulatory molecules or design of antigen-delivery systems (2).

2.2.2 Oral Administration

Oral delivery is the most desirable and patient-accepted route of administration, with over 60% of commercialized small molecule drug products using the oral route (11, 12). Despite this, only a small fraction of currently licensed vaccines are oral formulations due to the inherent obstacles presented by the gastrointestinal system. The induction of a robust protective immune response by oral immunization requires: (i) successful delivery of the intact and active antigen to the intestine, (ii) transport across the mucosal barrier, and (iii) subsequent activation of antigen-presenting cells (11, 13, 14). However, the GI tract poses difficulties to each step, including degradation of fragile antigens through the harsh environment in the stomach and requirement of higher doses to generate immunity instead of tolerance (15, 16). Each challenge within the GI tract

poses a unique engineering problem that requires careful consideration to achieve efficacious vaccine design.

2.2.2.1 Advantages of Oral Administration

Vaccine efficacy is dependent on both the degree of protection conferred to individuals as well as the total coverage, accessibility, and costs associated with administering the formulation (17). Vaccine distribution represents one of the main limiting factors in the impact of these prophylactic systems, particularly in developing nations with limited resources (18, 19). Oral formulations allow for self-administration, which is ideal for the widespread and rapid distribution of vaccines as it minimizes the need for trained healthcare personnel (20-22). This could further reduce cost of vaccine programs, since training and mobilization of health care workers can account for up to 25% of the cost of introducing a new vaccine (23). Additionally, needle-free administration would eliminate occupational needle-stick injuries, which occur in approximately 5% of health-care workers each year, exposing them to blood-borne infectious diseases such as HIV/AIDS and Hepatitis (24).

From a regulation standpoint, oral vaccines could enable more cost-effective production since they do not require the extensive purification necessary for injected formulations. Parenteral injections require a) aseptic technique during synthesis and manufacturing, b) equipment and training of the healthcare personnel for optimal delivery, and c) appropriate use of sterile needles (25). Moreover, use of these traditional techniques generates a huge amount of biohazardous waste (26), which the majority of developing countries simply do not have the infrastructure to handle properly. All of these factors increase the cost of immunizations, which can significantly affect their access in emergent regions.

Oral immunization has the potential to improve vaccine efficacy simply by increasing accessibility and coverage, however the oral route also provides the additional advantage of stimulating mucosal immunity. The mucosal epithelium covers the largest surface area in the body and constitutes the first line of defense against external pathogens (16, 27, 28). These mucosal surfaces involve physicochemical and biological barriers working in unison to regulate entrance of nutrients and mount responses to foreign materials (29-31). Eliciting prophylactic immunity in the infection entry site can help prevent infectious diseases. However, the same defense mechanisms designed to exclude pathogens must also be circumvented to develop efficacious oral vaccines.

2.2.2.2 Challenges of Oral Administration

In order to prompt a robust immune response, the oral delivery of antigens needs to overcome multiple physicochemical and biological barriers in the GI tract. Among them is the biological barrier of the intestinal epithelium and its mucus secreting layers which serve to digest consumed material for nutrient absorption and to protect the body from the invasion of pathogenic threats (30, 31). To accomplish these tasks, the GI tract includes a highly acidic environment in the stomach, a significant pH range along the length of the GI tract, and the presence of proteolytic enzymes responsible for protein degradation. These characteristics can interfere with the delivery of fragile biomolecules, such as antigenic proteins or peptides, which are highly susceptible to degradation and denaturation (11). Furthermore, there is a temporal limitation for the absorption of these formulations due to the residence time in the small intestine (3-4 h), where the majority of absorption processes occur (32).

Another major hurdle in the development of oral vaccines is that a higher dose of antigen is needed to induce an immune response when compared to traditional parenteral

immunizations (16). This characteristic limits the possible formulations used as carriers as they must be able to successfully carry the required antigen dosage. Larger doses also increase the risk of inducing tolerance instead of stimulating a protective response (20, 33, 34). The GI tract is constantly exposed to a variety of pathogens. If a vaccine does not induce the appropriate danger signals, the body can recognize it as non-pathogenic and avoid triggering an immune response, resulting in immune tolerance instead of protection (35, 36). Thus, it is critical in the design of oral vaccine carriers to include potent adjuvants in order to sufficiently stimulate the immune system.

2.2.3 The Oral Route: Physiology and Immunology

The gastrointestinal tract is designed for the digestion and uptake of water, nutrients, and small molecules, however, it also performs preemptive and surveillance activities to protect the integrity of the system (11, 37). Thus, upon oral administration, vaccine formulations encounter a variety of biological and physicochemical mechanisms designed to prevent the entrance of foreign material to the body and mount immune responses towards them, if necessary. In order to develop next-generation vaccines that can overcome the aforementioned challenges in the generation of immunity (and avoidance of tolerance) pertinent to the oral route, it is necessary to consider the conditions within the gastrointestinal tract.

2.2.3.1 Organization of the GI Tract

The GI tract is approximately 20 feet long and it consists of heterogeneous surfaces, mucosal thicknesses, pH levels, enzymatic conditions, residence times and cellular components (8, 11, 29, 38). As shown in **Figure 2.1**, the GI tract is divided in two broad segments: The upper GI tract includes the oral cavity, pharynx, esophagus, and

the stomach; while the lower GI tract involves the small intestine (with three sections: duodenum, jejunum, and ileum), the large intestine (also with three divisions: cecum, colon, and rectum), and anus (11). Each one of these segments has different purposes and carries specific processes designed to absorb nutrients using either passive or active mechanisms.

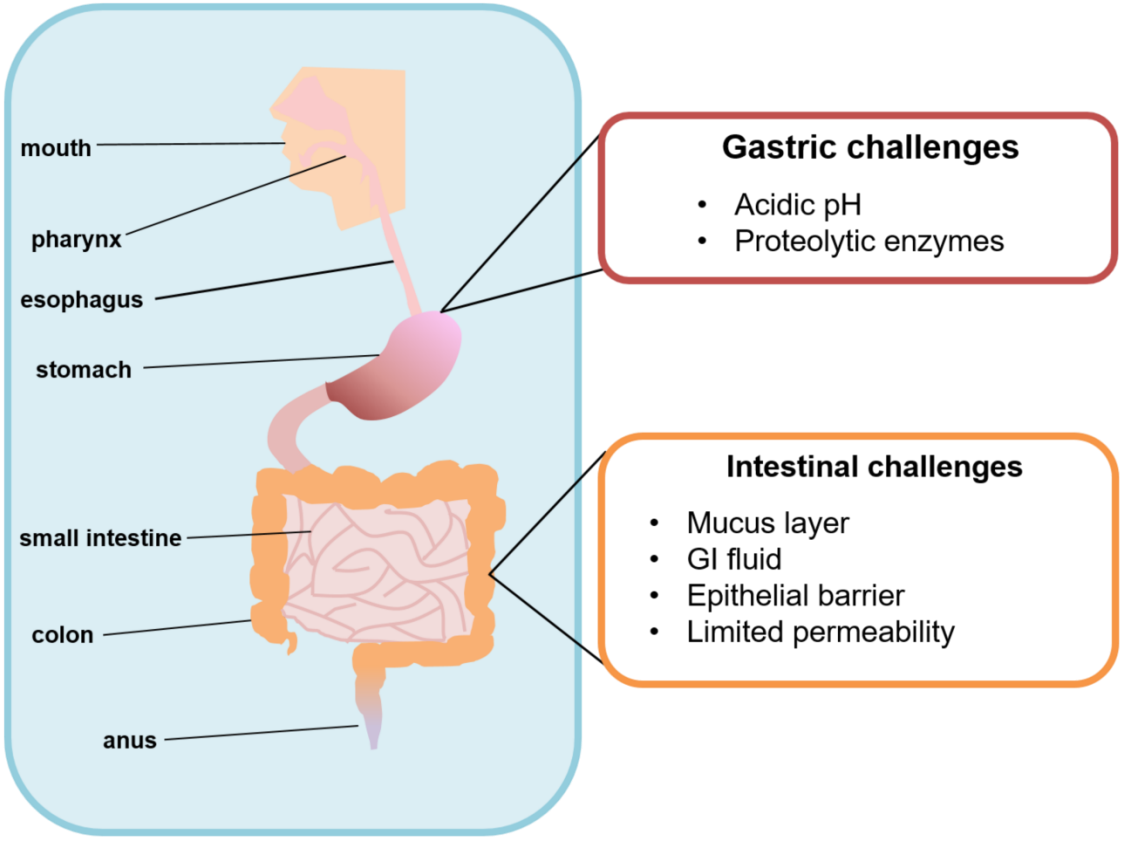

Figure 2.1 Physiology of the gastrointestinal system and the challenges it presents for oral vaccines.

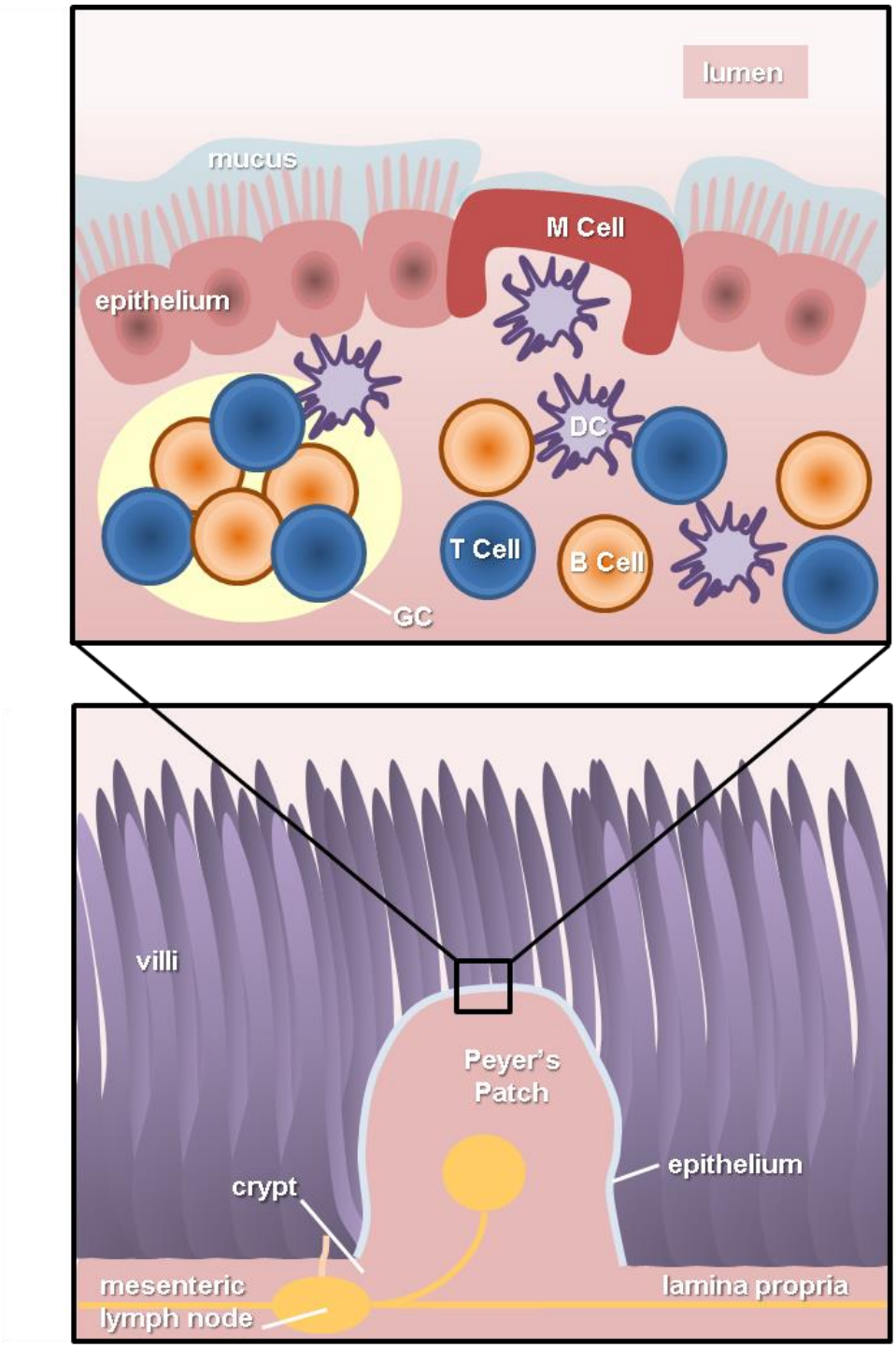

Figure 2.2 Anatomy of the gastrointestinal immune system.

The surface of the GI tract consists of a physical and chemical barrier formed by an impermeable layer of epithelial cells. This barrier functionality is critical as the GI tract is the initial point of contact between the body and the external environment and its first line of defense against pathogens (31, 39). The lining of the GI tract is composed of a heterogeneous population of cells with diverse roles based upon their location (39, 40). An overview of this organization is presented in **Figure 2.2**.

The most populous cells in the GI tract are the enterocytes, which provide the primary barrier functionality of the intestine due to the formation of tight junctions between the cells. Additional intestinal cells include goblet cells, Paneth cells, microfold or M cells, intestinal epithelial stem cells (IESCs), and enteroendocrine cells (11, 30, 39).

In order to understand and select appropriate cellular targets for vaccine delivery a brief overview of their characteristics is presented in **Table 2.1**. Based on their cellular functions, enterocytes, goblet cells, and M cells are some of the key players involved in gut protection, transport, and immunity (36, 41). Specifically, M cells have become one of the targets for delivery vehicles to bypass the barriers from the GI system more efficiently, and trigger immune responses towards their antigens (42-45).

In addition to the cellular organization, there is also a critical difference in the subjacent layers that constitute the physicochemical barrier of the GI tract. There are four layers that form this barrier: mucosa, submucosa, muscularis externa, and serosa (or adventitia) (23, 48). Each one of them has different characteristics and cellular composition depending on their corresponding roles, as described next.

a) Mucosa: This is the surface layer of the GI tract and also responsible for mucus secretion. It is divided into three different sections: the epithelium, lamina propria, and muscularis mucosa. It is the primary point for absorption of food and drug molecules,

therefore its cells secrete enzymes (e.g. pepsinogen) and chemical substances (e.g. hydrochloric acid) to process nutrients.

b) Submucosa: This region is where the circulatory, nervous and lymphatic systems interact with the gut tissues, specifically with the outer layers.

c) Muscularis externa: This is an important muscular region, comprised by longitudinal and circular fibers that guide the food bolus through the GI tract.

d) Serosa: The outermost layer of the intestine and consists of multiple epithelial sections

Besides this layered organization of the epithelia, there are also regional differences on the thickness and mucosal activity (48). The characteristics of mucus and other physicochemical barriers of the GI tract also need to be taken into account when developing novel vaccine delivery vehicles to efficiently deliver across them into immune cells (38, 48).

Table 2.1 Characteristics and functions of intestinal cells.

Cell type	Characteristics	Function	References
Enterocytes	-Most abundant cells in the small intestine -Column-like shape -Have an apical membrane domain covered by microvilli and a carbohydrate glycocalyx	-Nutrient digestion and absorption -Ion uptake from lumen to enterocyte cytoplasm -Important for innate immunity	(8, 30, 39, 46)
Goblet cells	-Mucus-secreting (especially MUC 2)	-Maintenance of the mucus protective layer -Regulation of intestinal wall	(8, 30, 47-49)
Paneth cells	-Located in the deepest parts of the crypts of Lieberkühn (formed by the folding of the intestine)	-Shielding the epithelial wall by generation of antimicrobial proteins (AMPs) that disrupt pathogen integrity	(39, 50-52)
M cells	- <1% of total cells in the intestinal lumen -Cover lymphatic bodies, including lymphoid follicles and Peyer's patches -Short microvilli and thin mucus layer -Heavily invaginated -Have a protruding glycocalyx	-Efficient transcytosis activity -Antigen sampling -Actively transportation of pathogens -Receptor-mediated and non-specific antigen uptake -Delivery of these microbial parts to sub epithelial dendritic cells	(16, 37, 39, 45, 49, 53)
Intestinal epithelial stem cells (IESCs)	-Located at the base of the crypts in the colon -Continuously migrate and mature to their final shedding into the lumen	-Maintenance of healthy cellular populations in the intestine	(50, 54, 55)
Enteroendocrine cells	-Located in the mucosa -Placed between other epithelial cells	-Secretion of hormones important for digestive functions -Mediation interactions between central and enteric endocrine systems	(30, 56)

2.2.3.2 Biological and Physicochemical Barriers

Epithelial cells form the physical and biological barrier that prevents the permeation of pathogenic material into the human body, though there are also additional physicochemical barriers involved in the mucosae (64). One of the most critical barriers is the presence of a robust mucus lining. Mucus is a hydrogel (>95% water) consisting of

a mixture of proteins, carbohydrates, lipids, salts, and antibodies (38, 54, 55). This complex fluid is primarily generated by mucins secreted by goblet cells. There are over twenty different mucin molecules in this family, of which the most abundant are MUC2, MUC5AC, and MUC6 (38). These mucin molecules act as monomers, which are subsequently linked by disulfide bonds to synthesize larger molecules that can reach sizes of 0.5-40 MDa (38, 64). The resulting mucus lining is a tridimensional crosslinked network of the aforementioned mucins (2-5% w/w), which forms a viscoelastic gel with shear-reducing properties that can vary depending on the composition, site, and physiological conditions (38, 55).

The lubricating and shielding functions of mucus are essential in maintaining a healthy homeostasis (65). The mucus lining contains both a firmly and a loosely adherent layer, the thicknesses of which are dependent upon their location along the GI tract. The presence of both the firmly and loosely adherent layers creates a slippage plane, which aids in the transport of undigested food and is essential for the protection and integrity of the GI tract (38, 64). Firm layers are formed by cell-bound mucins, glycolipids and glycoproteins that constitute the glycocalyx (38, 54). There are five mucins that are known to be present in the GI tract, MUC1, MUC3, MUC4, MUC12, and MUC13 (37). The thickness and composition of the loose layers have shown to be dependent on the diet of the subject (66). Since this mucus layer is focused on coating and lubricating undigested material, it protects the firmly bound layer through the peristaltic motion in the GI tract (55, 64). This structural composition is one of the reasons that mucus has such an important role maintaining the integrity of the GI system. Penetration of the mucus layer is one of the most important characteristics that orally administered vaccines need to have in order to reach the immunological sites within the gut.

One of the most formidable challenges that vaccine delivery vehicles need to overcome, specifically for protein antigens, is the composition of the gastrointestinal fluid (39, 67). This complex mixture is composed of water, bile salts, and enzymes (e.g. pepsin) and its hydrogen ion concentration changes its overall pH depending on its location (39, 68). Enzymatic degradation, in particular, poses one of the most significant threats to the stability of protein molecules delivered orally (39, 45). These enzymes are multiple proteases such as pepsin, trypsin, and lipase.

Pepsin, which is present in the stomach, is a proteinase that hydrolyses protein amide bonds. The activity of this enzyme is enhanced under acidic pH (<2.5), and it loses its activity at pH higher than 8 (50, 69). This characteristic allows it to perform digestive functions at the low pH environment in the stomach. Another enzymatic protein present in the GI tract is trypsin. This protease is secreted by the pancreas and, together with carboxypeptidase and chymotrypsin, is present in the duodenum in large quantities (>1 g). The action of these three enzymes is responsible for 20% of the degradation of ingested proteins (50, 70). The remaining degradation is completed by the actions of the aforementioned elements of the gastrointestinal fluid.

Another physicochemical barrier that has a very critical role in is the variation of pH throughout the GI tract. Most proteins are sensitive to their pH environment, with their stability at risk in acidic conditions due to the possible denaturation of their structure. The GI fluid is made of a mixture of saliva, ingested food and liquid, and refluxed liquid from the intestine, therefore the pH of each intestinal segment varies depending on the location, and the fasted or fed state of the host (23).

Overall, the pH range of the GI system varies from 1.0-7.0; in the stomach it is between 1.0-3.0, in the duodenum it fluctuates between 6.0 and 6.5; and in the colon it is

5.5-7.0. This gradient in pH is due to changes in the overall concentration of hydrogen ions caused by the presence of hydrochloric acid (HCl). In addition to affecting the stability and activity of delivered biomolecules, the pH directly impacts the dissolution of drugs and proteins (71). It has been shown that the pH of the stomach is between 1.0-2.0 (with 0.01-0.1 M HCl) in the fasted state, and ranges from 3.0-7.0 (10-3-10-7 M HCl) after food ingestion (39). The significant changes in the local pH of the GI tract have a measurable impact on orally delivered proteins and necessitates specially designed antigen delivery carriers to accomplish oral vaccination (39, 68).

2.2.3.3. Gastrointestinal Immunity

The ultimate challenge of any antigenic administration is the elicitation of a robust response towards the immunogen. As previously discussed, there are multiple biological and physicochemical barriers that oral vaccine formulations have to overcome. However, if they succeed, they still need to stimulate the immune system by engaging their activation mechanisms. The intestine is the mucosal site that holds the highest number of immune cells in the body, and it is regulated by the gut-associated lymphoid tissue (GALT), that coordinates effector and inductive sites (21). Inductive sites in the GI tract involve the coordinated action of Peyer's patches, lymphoid follicles and antigen presenting cells (APCs), while effector sites mainly include the lamina propria (LP) and surface epithelium.

Following administration of oral vaccines, antigens travel through the GI tract. Upon entering the small intestine, M cells in the Peyer's patches sample and transport the immunogens across to APCs. These materials are then taken up and processed by DCs that present antigenic fragments on their surface to activate naïve CD4+ T cells (56). These helper cells further interact with antigen-specific B cells that then undergo class

switching to become immunoglobulin-secreting cells. Upon maturation, B cells travel from the Peyer's Patches through the lymphatic system to reach the mesenteric lymph node before entering systemic circulation. When these cells reach distant effector sites, they differentiate and maturate into plasma cells. In parallel, dendritic cells (DCs) migrate to the lymph nodes to activate humoral and cellular responses by interacting with germinal centers. A further analysis of the characteristics of the most relevant immune sites in the GALT is necessary to understand the process of generating gut immunity (21).

Peyer's patches (PP) are believed to be one of the largest lymphoid tissues in the GALT. They are formed by organized immune cells, and generally include B-cell rich follicles protected by a mesh-like formation known as the interfollicular region (IFR) made by T-cells (43). They are slightly elevated lymphatic organs with a dome shaped structure that are located in the ileum within the small intestine. PPs have only efferent lymphatics; therefore they are protected by a follicle-associated epithelium (FAE). This FAE contains the previously described M cells that allow the sampling and transport of antigenic fragments from the intestinal lumen into the PPs (48). These formations represent the main port of entry for antigens in order to elicit immunity in the gut and mucosae. Active targeting mechanisms towards M cells are being explored in order to efficiently deliver antigen into the Peyer's patches (21, 43). Further exploration of these strategies is discussed in detail in a subsequent section.

Some of the most common and important APCs present in the GALT are DCs. They take advantage of their location in the sub epithelial dome (SED) region below the FAE, where they can take up antigens directly from M cells (43, 56, 72). DCs are specialized immune cells that process and present antigenic fragments to mucosal B and

T cells to initiate antigen-specific immunity. There are three different DC subsets present in the GI tract: CD11c+ DCs in the SEDs, CD8α+ DCs in the IFRs, and CD11c-CD8α- DCs in both locations (73). Finally, these cells play a significant role in the homing of activated T and B cells to the lamina propria, because of their processing ability of retinoic acid (43). They express retinal dehydrogenase, an enzyme that can transform ingested vitamin A into retinoic acid (74). This molecule induces gut imprinting molecules including α4β7 integrin and CCR9 (75). Engagement of these APCs is critical in the initiation of local and systemic immunity; hence development of vaccine delivery carriers with targeting mechanisms towards these cell populations is important.

As previously mentioned, recruitment and activation of B and T cells are important in the generation of adaptive and long-lasting immunity towards an antigen. B cells make up the 75% of the cellular population of PPs, and are primarily located in the follicle region (43). It is in these locations that germinal centers form, including during homeostatic conditions (43). Germinal center formation is characteristic of strong thymus-dependent antibody responses, and the generation of GC B cells is an important part of triggering T helper cell responses (76). These antibody-secreting lymphocytes are critical in the generation of serum and mucosal immunoglobulins and the host protection from bacterial and viral infections.

Cellular responses, generally performed by T cells, represent the other fundamental component of immunity. These populations are also involved in the development of robust humoral responses, via the initiation of B cell maturation. Follicular helper CD4+ T cells (TFH) provide essential co-stimulatory signals to B cells in germinal centers (29, 43, 77). Naïve T cells in the GALT are located mainly in the PPs, where they represent 20% of the total cell population (43). However, there are also other

phenotypes (e.g. Th1, Th2 and Treg) present in the gut (78, 79). Upon their activation, they can become tissue-resident memory T cells, or circulating-memory T cells, both of which may be more effective defense mechanisms than antibody-based responses. Memory cells control an infection by the secretion of cytokines and recruitment of other immune cells (77). However, they can also cause tissue damage if there is a prolonged infiltration of such cell populations.

The barriers and challenges for antigen delivery in the GALT discussed in this section underline the need of novel design mechanisms for antigen delivery that can protect the cargo, penetrate the biological and physicochemical barriers, and possess adjuvant capabilities that can elicit robust and balanced immune responses. Optimal vaccination strategies would generate both humoral and cellular immunity with innate and adaptive components.

2.3 Oral Vaccine Strategies: Delivery Systems

As previously mentioned, subunit vaccines require the use of delivery systems and/or immunostimulants to induce immune protection. In order to efficiently deliver stable antigens, it is necessary to: i) design carriers that can protect the payload through these conditions (57), ii) release the vaccine within the small intestine residence time to antigen presenting cells across the epithelial layers, and iii) enhance the immune responses elicited by the vaccine with the adjuvant capabilities of the delivery vehicles (19).

There are a multitude of parameters to be taken into account when designing delivery systems for the oral delivery of subunit vaccines. Among some of their controllable properties include size, geometry, antigen loading and release kinetic capabilities, and finally the ability to include functional molecules to improve their

performance. Tailoring these characteristics can prolong the residence time of immunogens, enable the co-delivery of antigens and adjuvants to boost their immunogenicity and target immune cells (specifically APCs) for efficient transport, uptake and presentation. Furthermore, the material properties of these vehicles have the potential to act as immune-potentiators as well.

This section includes a brief overview of the most commonly explored delivery vehicles for oral vaccination, as shown in **Figure 2.3**, their characteristics, and the responses obtained after their administration.

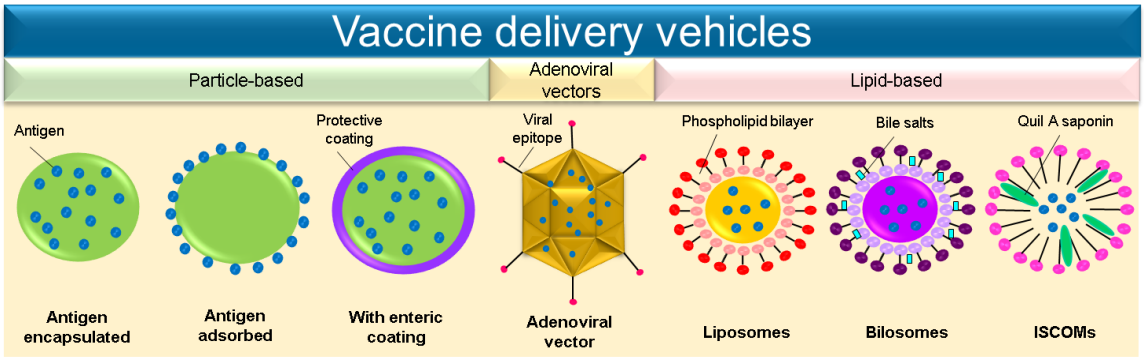

Figure 2.3 Polymeric and particulate oral vaccine delivery vehicles

2.3.1 Polymeric/Particulate Vaccine Design

Polymeric microparticles (MPs) and nanoparticles (NPs) have been extensively explored for the development of subunit-based vaccines. Proteins, DNA, and polysaccharide vaccine components are fragile molecules that could be structurally degraded during transition through the gastrointestinal tract or the mucosal layer, resulting in diminished bioactivity. Entrapment or encapsulation of the antigenic payload

within polymeric particles affords protection, while also preventing antigen dilution over the large surface area of the GI tract. In addition to robust structural stability, particles can provide control over release site and profile for improved delivery of stable antigens. Furthermore, NP carriers have demonstrated the capability to efficiently deliver an antigenic payload directly to phagocytic APCs through passive or active targeting to stimulate cellular and humoral responses(58).

Particulate delivery systems passively provide characteristics of adjuvant behavior to weakly immunogenic subunit vaccines simply by virtue of APC recognition and internalization. However, NPs enable the incorporation of enhanced adjuvant strategies through co-delivery of immunomodulators or by manipulation of surface properties for enhanced or targeted uptake by immune cells, a strategy that is further discussed in section 7 (59). Furthermore, there is a diversity of both synthetic and natural materials with desirable physicochemical properties capable of responding to physiological changes, making polymeric particles a versatile option for rational vaccine design (60).

2.3.1.1. Synthetic Polymers

Polyester nanoparticles, in particular poly(lactic) acid (PLA) and poly(lactic-co-glycolic) acid (PLGA), are the leading synthetic polymers explored in preclinical studies for oral vaccine administration due to their biocompatibility, biodegradability, and controlled sustained release patterns of encapsulated antigens for up to several months(61-63). Additionally, both PLA and PLGA are FDA-approved materials, which can expedite the development and approval of the delivery carriers.

Biodegradable vaccine delivery systems allow for prolonged antigen release and are a viable strategy to achieve single-dose administration, which is particularly desirable in the context of reducing number of repeated administrations required for long-term

protection and the cost implications for mass vaccination campaigns. For several antigens studied, including plasmid DNA and protein antigen payloads, single administration significantly improved long-term IgA and IgG antibody titers in comparison to soluble antigen, attributed to sustained antigen release mediated by particles (64-67).

Antibody responses can be further improved compared to encapsulation of the antigen alone by co-delivery of antigens with immunostimulants, such as the TLR4-adjuvant monophosphoryl lipid a (MPLA) (68), or co-polymerization with PEG as a stabilizer to improve NP stability in gastrointestinal conditions and antigenicity of encapsulated antigen (69). Zhu *et al.* designed a PLGA-based system containing both immunostimulant and GI stabilizing strategies for an effective HIV peptide vaccine. PLGA nanoparticles containing three different TLR ligands and the HIV Env epitope were coated with the methacrylate-based polymer Eudragit FS30D (70). This pH-responsive polymer was chosen to selectively deliver the antigenic payload to the large intestine. While orally administered PLGA nanoparticles induced local responses in the small intestine, the two-part PLGA/Eudragit microparticle formulations induced immunity in the rectal and vaginal mucosa, ultimately protecting against rectal or vaginal viral challenge. The study demonstrates the possibility to target regions of the GI tract in a pH-dependent manner.

While these polyesters provide several advantages for vaccine design, acidification of the microenvironment within the delivery vehicles upon degradation can prove to be unfavorable for the encapsulated agents, causing disruption to tertiary structure and protein degradation (71, 72). Efforts have been made to optimize PLGA particle fabrication methods and add stabilizing agents, which have helped to alleviate deleterious effects of local acidification (65). In a strategy to circumvent the stability

issues associated with encapsulation, PLGA has been evaluated as an adjuvant for particulate delivery of surface-adsorbed antigens (73, 74). This strategy does not afford controlled release of the antigen, but could provide an organic and biodegradable alternative to inorganic adjuvants such as alum.

Additionally, other synthetic pH-responsive materials have been explored for oral vaccination due to their ability to protect antigens from degradation and provide targeted release. Mannan-coated methacrylic acid-based copolymers demonstrated both pH-dependent release of encapsulated antigens as well as uptake and activation of APCs (75), while co-delivery of antigen with the mucosal adjuvant cholera toxin (CT) elicited significantly increased IgG and IgA antibodies as compared to soluble dosages(76).

2.3.1.2 Natural polymers

Natural polymers (e.g. polysaccharides) tend to be non-toxic, biocompatible, and biodegradable as well as possess mild gelation conditions for encapsulation of sensitive macromolecules. They have been extensively explored in drug delivery applications. Additionally, carbohydrates are desirable oral vaccine components given the numerous lectin-receptors expressed by M cells in the intestinal mucosa.

Chitosan is a cationic polysaccharide with advantageous properties for oral delivery including mucoadhesion and an ability to reversibly disrupt epithelial tight junctions(77, 78). However, chitosan particles are limited by their high solubility in acidic conditions, risking the integrity of the sensitive payload. Strategies to overcome chitosan's dissolution in acidic pH include encapsulation of antigen-loaded chitosan particles within liposomes to protect transit through the stomach (79), stabilization by crosslinking with tripolyphosphate and glutaraldehyde (80), and electrostatic coating with the anionic polysaccharide alginate (81, 82). These strategies significantly improve

particle stability and payload retention in acidic environment to protect the antigen, and induced significantly higher antibody titers *in vivo* as compared to unmodified chitosan particles.

2.3.2 Lipid-based Vehicles

Lipid-based vaccine delivery carriers are some of the most commonly used vehicles for oral administration. Among these are liposomes, bilosomes, and immune-stimulating complexes (ISCOMs). They are based on the separate encapsulation of hydrophilic and lipophilic agents using lipid bilayers.

2.3.2.1 Liposomes

Liposomes are spherical vesicles formed by one or more phospholipid bilayers synthesized from cholesterol and other non-toxic lipids. The properties of these systems vary depending on their composition (*i.e*. their size, charge, and protein compatibility), and can be optimized by changing their fabrication parameters. These liposomal systems also offer the ability to deliver multiple active agents with vastly different properties, since they can be located in different compartments of the carrier. Specifically, water-soluble molecules, such as proteins, RNA, carbohydrates, or peptides are encapsulated in the inner layer of these vehicles; meanwhile lipophilic compounds can be included in the external section of the formulation (83, 84).

A variety of liposome-based vaccines for oral administration have been previously synthesized to target a wide range of viral and bacterial diseases. For example, an influenza A viral vaccine was produced using a construct DNA vaccine with a pcDNA 3.1(+) plasmid encapsulated in cationic liposomes. Oral immunization with this formulation induced humoral and cellular immune responses, in addition to increasing

cytokine production (85). Liposomes have also been used to prevent bacterial infections, such as *Salmonella Enteritidis*. A vaccine to prevent this disease was created using a liposome-associated carrier with the recombinant SefA protein by Pang and collaborators. This oral vaccine was able to generate protective immunity in chickens and a significant reduction of intestinal bacterial load was observed after oral challenge with 2×10^6 CFUs of live *Salmonella Enteriditis* (86).

Liposomes have demonstrated their ability to deliver diverse antigens, including DNA, peptides, and proteins. For example, encapsulation of a DNA-based antigen (*Mycobacterium* pcDNA3.1$^+$/Ag85A) in liposomal formulations enhanced its presence in the epithelium, M cells, DCs and PPs within the small intestine of C57BL/6 mice after three oral immunizations. The ability of the system to induce antigen-specific mucosal immunity made this formulation a potential vaccine carrier (87). In a different study, delivery of antigenic peptides and CTL epitopes within liposomes allowed their efficacious transport to APCs and improved the host response towards these antigens (88-90). Additionally, the adjuvant capabilities of these formulations have been tested using model antigens (*e.g.* ovalbumin, bovine serum albumin). These experiments have shown that liposomes can effectively load and release stable protein. They are also able to elicit Th1/Th2 immunity, reflected by the generation of mucosal and systemic antibody responses(15, 83, 91, 92). Finally, these systems can also be decorated with targeting molecules (*e.g.* carbohydrates) to enhance their efficacy. In oral immunization experiments, lectinized liposomes were able to effectively target M cells in the PPs, resulting in elicited mucosal responses with high antibody titers (83, 93). Overall, liposomes have shown promising properties for vaccine delivery applications.

2.3.2.12. Bilosomes

A different lipid-based carrier being explored for oral immunization is bilosomes. These non-ionic surfactant vesicles have adjuvant functionalities and incorporate bile salts in their formulation. Bilosomes are typically synthesized with monopalmitoyl glycerol (MPG), cholesterol (CH), and dicetyl phosphate (DCP); and surfactants such as sodium deoxycholate (SDC), or sorbitan tristearate (STS). Similar to liposomes, bilosomes also have a bilayer with polar and non-polar ends, permitting the integration of vaccine elements with significantly different properties.

Traditional liposomal vesicles can be disrupted by bile salts; however, if vesicles are fabricated in the presence of bile salts, such as bilosomes, they are no longer affected by their action and remain stable. These systems are able to stimulate humoral and cellular immune responses and the inclusion of bile salts allow the protection of the cargo from the harsh environment from the GI tract (94-96). One of the main advantages of bilosomal formulations is the improved stability that they can confer to fragile antigens. In previous studies it has been shown that bilosomes are able to entrap and stabilize a variety of fragile antigens, including tetanus toxoid (TT), A/Panama (influenza A immunogen), diphtheria toxoid, Bac-VP1 (hand, foot and mouth disease vaccine candidate) (94, 96-101).

Additionally, adjuvant and drug release studies with bilosomes have been carried using model antigens such as bovine serum albumin (BSA) and cholera toxin subunit B (95, 102). Their immunogenic abilities have also been explored using various disease models. Previously, mannosylated bilosomes targeting DCs for oral immunization against hepatitis B virus generated both systemic and local immunity, including in the mucosa (98).

The use of these formulations induced production of soluble immunoglobulin A at all local and distal sites of the GI tract. A different set of studies performed using a subunit vaccine against influenza in an orally administered formulation also elicited high antibody titers and cellular responses. Specifically, Th1 and Th2 responses were successfully produced(95, 98). As summarized here, the aforementioned benefits provided to different antigens because of bilosomal entrapment, make this system a feasible vaccine delivery platform for oral immunizations.

2.3.2.3. Immune-stimulating Complexes

Immune-stimulating complexes (ISCOMs) are second-generation liposomes regarded as both a carrier and as an immunostimulant for vaccine delivery. Synthesized using colloidal saponin (often QuilA extracted from the tree Quillaja saponaria), cholesterol and other phospholipids (generally phosphatidylethanolamine or phosphatidylcholine), these nano-sized vectors (~40 nm) with self-adjuvant abilities are organized in open-caged structures (15, 103-105). These vehicles have been used to entrap bacterial and viral envelope proteins to prompt vaccines against such pathogens. Classical ISCOMs are self-assembling systems fabricated in the presence of a non-ionic detergent that is removed post-synthesis (104).

These formulations have been shown to have a great breadth of applications, incorporating antigens to prevent herpes simplex virus 1, hepatitis B, respiratory syncytial virus, *Escherichia coli*, *Brucella abortus*, and *Plasmodium falciparum* infections (104, 106-108). ISCOM-based vaccines have shown to be highly immunogenic, generating balanced humoral and cellular responses in different animal models(104). The properties of this system engage components of both the innate and adaptive immune systems. This characteristic makes this platform a highly desirable

delivery methodology, although their intricate action mechanisms remain to be fully elucidated.

2.3.3 Adenoviral vectors

Traditional vaccines were based on the use of killed or attenuated pathogens, but as previously discussed there are risks in the immunization of vulnerable populations with such platforms due to the potential reversal of their pathogenicity. However, with advances in genetic engineering and molecular virology, there are some alternatives for the use of such microbial structures without their detrimental side effects. Adenoviruses are double-stranded DNA viruses, with a ~40 kb genome, they are species-specific and have different serotypes. While this platform was initially devised for gene delivery, due to its highly immunogenic nature it became less attractive for therapeutic use.

However, based on the advancement and optimization in the synthesis of adenoviral vectors, they have become interesting possibilities as vaccine delivery carriers. The adenoviral genome is well studied and can be readily manipulated, thus allowing the synthesis of non-pathogenic vectors. Another advantage of these systems is that most of these viruses in their original form only induce mild diseases in immunocompetent human adults. These systems can also be modified to nullify their replication mechanism, further reducing their ability to infect a host.

The previously mentioned features have prompted the use of adenoviral vectors as vaccine delivery vehicles for the treatment of viral diseases. Vaccines using adenoviral vectors have targeted a wide range of some of the most challenging diseases, including HIV, influenza, rabies, botulism, dengue, SARS and Ebola (109-116). They are able to generate robust cellular and humoral immune responses. Oral immunization with the most common adenoviral vaccine vectors (AdHu5) have been shown to induce potent

CD8+T cell responses and antibody responses, but not engage CD4+T cell responses (109, 117-119). The multiple isotypes (e.g. IgG2a, IgG1) generated by such vectors indicates the elicitation of a Th1/Th2 response, however it is predominantly skewed towards the first one(109).

Additionally, adenoviral vectors activate innate immunity mechanisms by the expression of pathogen-associated molecular patterns (PAMPs) on their surface, initiating the secretion of pro-inflammatory cytokines, activation of complement, and the differentiation of APCs. One of the important considerations during the development of novel delivery vehicles is their ability to induce strong responses in relevant models for clinical application. These systems have been used for administration of vaccines in multiple animal models including rodents, dogs, non-human primates, and most importantly, they have reached human clinical trials (109). By taking advantage of their immunogenic characteristics, adenoviral vectors represent an alternative to killed or attenuated vaccines, and their further use and optimization remains as a valuable option for pathogen-mimicking delivery vehicles.

2.4 Biomaterials for Composite Oral Vaccine Platform Design

Employing multiple materials in a composite design can address the distinct nature of the challenges associated with i) protecting subunit antigens through the GI tract for targeted delivery, and ii) stimulation of immune cells to generate a protective response. The development of intelligent hydrogels for oral delivery of sensitive therapeutics and polyanhydride materials as nanovaccines with subunit antigens are discussed herein to motivate their selection in this work.

2.4.1 Hydrogels for oral protein delivery

Hydrogels are three-dimensional, polymeric networks consisting of crosslinked hydrophilic components. In certain environmental conditions, hydrogels can imbibe large amounts of water or biological fluids while remaining insoluble. Physical integrity in aqueous media is maintained by physical crosslinks (e.g., entanglements, crystallites) and/or chemical crosslinks (e.g., tie-points, junctions) (120-123). High affinity for water absorption gives hydrogels physical properties resembling living tissues, such as a soft consistency and low interfacial tension with aqueous media. These properties match living tissues more than any other class of synthetic biomaterial, and are therefore highly biocompatible for biological applications (124). Hydrogel use thus extends beyond the drug delivery applications discussed here, to tissue engineering, surface coating, contact lenses, and diagnostics among others (124-131).

Stimuli-responsive hydrogels are of particular interest for oral delivery as they can respond to environmental changes to alter network structure, swelling behavior, permeability, or mechanical strength, as well as control drug release (132). Many different physical and chemical stimuli have been applied to smart hydrogel systems. Physical stimuli include temperature, electric field, and light. Chemical and biochemical stimuli, such as pH, ionic strength and molecular recognition events, are more commonly exploited in oral delivery (121).

The primary barriers to oral delivery of therapeutics, particularly protein and peptide drugs, as discussed in Section 2.2.3 are (i) inactivation in the gastrointestinal (GI) tract due to denaturation by acidic pH or digestive enzymes and (ii) poor permeability through the epithelial membrane into the bloodstream (126). Hydrogels provide a

platform to protect therapeutics through the complex environment of the gastrointestinal tract and achieve site specific delivery utilizing fundamental physiological changes.

2.4.1.2 pH-Responsive Hydrogels

pH-Responsive hydrogels have become popular platforms for the oral delivery of drugs. Such systems can be tailored for drug delivery to specific organs (e.g., small intestine, colon), or intracellular vesicles (e.g., endosomes, lysosomes). For intestinal delivery, particles on the micron-scale (1-1000 μm) offer a larger surface area and are not taken up by M cells (133). Particles on the nanoscale (50-200 nm) are used for intestinal delivery for cellular internalization (134, 135). Particles less than 10 nm are cleared by lymph drainage (134). The two basic strategies for imparting pH-responsive behavior are incorporating (i) ionizable groups with solubility and/or conformational changes in response to environmental pH, and (ii) acid-sensitive bonds that cleave to release molecules anchored into the backbone (136). Ionizable polymeric systems are pH-sensitive due to the basic or acidic pendant groups of the polymer network. Ionization of the pendant groups results in a net charge in the polymer network. Due to the electrostatic repulsions of the charged polymer network, the pores increase in size, allowing for the influx of water and increased swelling.

pH-Responsive hydrogels can be classified as anionic or cationic. Anionic hydrogels are ionized, and thus swollen, at a pH higher than the pKa of the polymer network (123, 137). Intestinal drug delivery systems take advantage of pH-responsive anionic hydrogels to protect drugs from gastric degradation and denaturation at low pH and release drugs in specific locations, such as the upper small intestine and colon, further in the GI tract. Ionic strength of the solution also affects the swelling of pH-

responsive hydrogels (138, 139). At pH below the pKa, there is minimal effect of ionic strength on swelling since the hydrogel is in the collapsed state. Experimental observations found that as the ionic strength increases, the degree of swelling decreases for anionic hydrogels at a pH above the pKa of the polymer network (139, 140). Increasing the ionic strength of the solution leads to ion shielding which diminishes the degree of electrostatic repulsion of the negative carboxylic acid groups (141).

In contrast to anionic hydrogels, cationic hydrogels are ionized at a pH lower than the pKa of the polymer network (142). Cationic hydrogels are suited for drug release in the stomach or intracellular environments. Amino acid groups of cationic polymers impart high water solubility at acidic pH and low water solubility at neutral pH. In an oral delivery system, cationic polymers provide protection of the drug in the oral cavity (pH 5.8 - 7.4) (143), while releasing the drug in the stomach (pH 1 - 3.5) (144). Due to the low solubility at neutral pH suppressing drug release, cationic polymers often serve as taste-masking formulations (144-147).

Our lab has successfully developed complexation, pH-responsive copolymer systems that address the aforementioned barriers with immense potential in oral therapeutic delivery. These systems consist of a family of grafted copolymers that include poly(ethylene glycol) (PEG) grafted on poly(methacrylic acid) (PMAA), designated as P(MAA-g-EG), as well as other polyacids (**Figure 2.4**). These complexation pH-sensitive hydrogels respond to the surrounding environment, protecting drugs from the harsh environment of the stomach, and releasing them in the small intestine. Methacrylic acid (MAA) imparts pH-sensitivity due to the ionizable carboxyl pendant groups. Carboxylic acids begin to deprotonate at pH values above its pKa of 4.8, developing a

negative charge in the network. The ratio of deprotonated to protonated carboxylic acid groups can be determined using the Henderson-Hasselbach equation

$$pH = pKa + log\frac{(A^-)}{(HA)} \qquad \text{(Equation 2.1)}$$

where pH is the environmental pH, pKa is that of the acid group, (A^-) is the concentration of deprotonated acid groups, and (HA) is the concentration of protonated acid groups. Interpolymer complexation occurs when protons of the carboxylic acid groups of PMAA backbone form hydrogen bonds with the etheric oxygen of the PEG tethers (148).

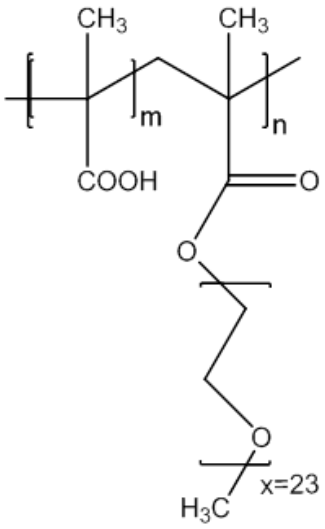

Figure 2.4 Representative structure of one unit of P(MAA-g-EG)

At low pH, P(MAA-g-EG) networks are collapsed due to complexation. As the environmental pH increases above the pKa of 4.8, deprotonation causes disruption of the polymer complexes, as well as ionization and electrostatic repulsion of the acid groups of the MAA backbone. The polymer network swells and the mesh size, ξ, increases due the

effects of deprotonation, as demonstrated in **Figure 2.5**. The mesh size, or the network correlation length, is the end-to-end distance of the polymer chains between junction points. The complexed, or collapsed, network has a mesh size of 70 Å, while that of the decomplexed, or swollen, network is 210 Å (149). In the oral delivery route, this network is collapsed in the low pH environment of the stomach, providing protection of the drugs, and then swells in the increased pH environment of the upper small intestinal allowing for drug release.

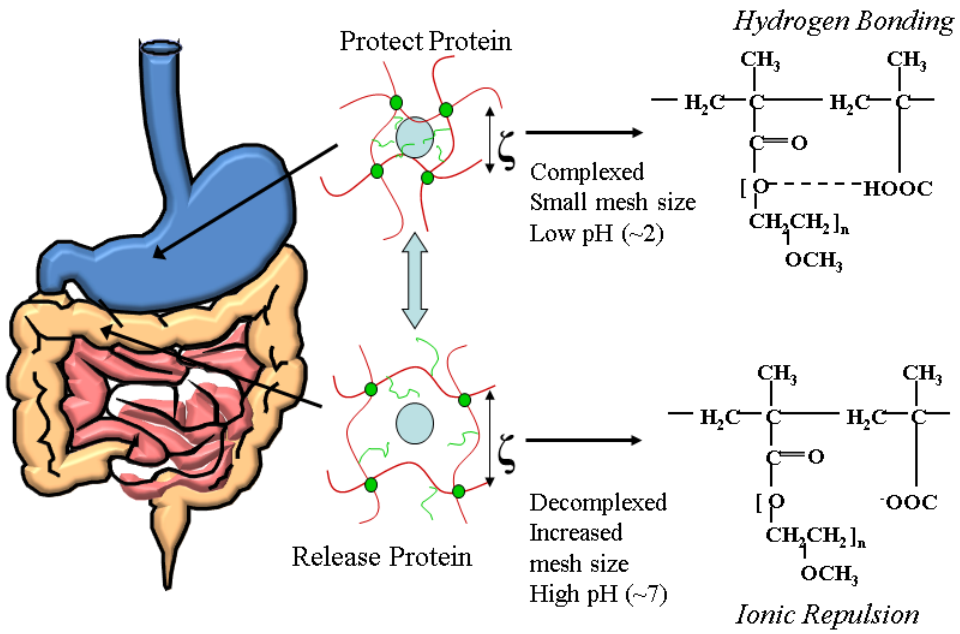

Figure 2.5 Schematic of anionic complexation hydrogels for pH-responsive oral delivery. This class of materials can deliver a therapeutic through the harsh environment of the stomach, protecting it from denaturation by acidic pH or digestive enzymes. Hydrogels swell in the upper small intestine due to decomplexation and subsequent water imbibition that swells the network, increases mesh size and enables targeted release of the entrapped therapeutic in neutral pH conditions.

The pH-responsiveness of P(MAA-g-EG) was first investigated by Klier et al. and Peppas and Klier further studied this polymer network for applications in oral drug delivery systems (150, 151). An evaluation of grafted PEG chain lengths determined that PEG chains with a molecular weight of 1000 exhibited the highest degree of complexation in low pH conditions (152) . Equimolar amounts of carboxylic acid groups of MAA and etheric oxygen molecules of PEG result in the largest amount of complexation (153). Adjusting the amount of carboxylic acid groups or other substituent groups tailors the hydrogel system for a specific pH value and, therefore, the site of drug release. Our laboratory has optimized the pH-dependent swelling behavior of P(MAA-g-EG) hydrogel systems for release targeted in the upper small intestine. Previous studies have demonstrated the successful use of this hydrogel system for the oral delivery of proteins, such as insulin (154, 155), calcitonin (156, 157), and interferon alpha (157). Representative *in vitro* release of insulin from P(MAA-g-EG) microparticles can be found in Figure 3 (158).

Another important feature that takes advantage of the pH-responsive behavior of the P(MAA-g-EG) system is release of PEG tethers. In the decomplexed state the grafted PEG tethers are no longer hydrogen bonding with carboxylic acids of the PMAA backbone and act as mucoadhesion promoters on the surface of the polymer network. Tethered PEG chains interpenetrate the mucus layer of the small intestine, participating in physical entanglement and hydrogen bonding with the polysaccharide components (159). Mucoadhesion increases the residence time of the carrier at the site of absorption, which promotes increased bioavailability (160). It is important to note that pH-sensitivity is designed for targeted release of drugs in the upper small intestine, as well as triggering the PEG tethers to promote mucoadhesion at the target absorption site.

2.4.1.2 Biodegradable Hydrogels

While the predominant strategy for oral delivery employs pH-responsiveness for site specific release in the GI tract, other environmental changes, such as enzymatic population, have been exploited to achieve site-specific drug delivery. As mentioned previously, colon-specific delivery can be desirable for both local and systemic therapeutic treatment. A common strategy to achieve colonic delivery exploits microbial enzymes predominantly found in the colon, such as reductive (e.g., azeoreductases) and hydrolytic (e.g., glycosidases) enzymes (161). Dextran hydrogels have potential in colonic-specific delivery by undergoing degradation by a dextranase, allowing release in the presence of the colon's microbial enzyme. Bronsted, et al. demonstrated the potential of dextran hydrogels for oral delivery by successfully releasing the anti-inflammatory agent, hydrocortisone (162). Dextran hydrogels continue to be explored for delivery of peptide and protein drugs, such the peptide hormone salmon calcitonin (163).

Incorporation of cleavable crosslinking agents is another strategy to trigger site-specific degradation. Azoaromatic bonds as crosslinking agents, which can be degraded by azeoredutases, also targets colon-specific delivery. By synthesizing hydrogels containing both pH-sensitive monomers and azoaromatic crosslinkers, Kopecek, et al. created a system that began to swell in the small intestine, making crosslinks accessible to azeoreductases by the time the particles reach the colon for protein drug release (164-166). More recently, Knipe, et al. demonstrated pH-responsive microgels crosslinked with a trypsin-degradable peptide linker to achieve intestine targeted release of siRNA loaded nanogels for inflammatory bowel diseases (167, 168) .

2.4.2. Polyanhydrides for Drug and Vaccine Delivery

Polyanhydrides are a versatile class of biodegradable materials used in medical and pharmaceutical applications. Degradation is imparted by the hydrolytic reactivity of the anhydride linkage (**Figure 2.6)**, enabling modification of the backbone structure while maintaining biodegradability of the polymer. The degradation kinetics of polyanhydrides can be tailored by modifying the length of the main chain, including aromatic or glycol functionalities to tailor relative hydrophobicity/hydrophilicity, and copolymerizing anhydride monomers in various ratios (169-173). Additionally, anhydride bond cleavage is base catalyzed and, therefore, pH-dependent (174, 175).

Polyanhydrides are prepared by melt condensation (170), in which a dicarboxylic acid is refluxed using an excess of acetic anhydride to obtain a mixed anhydride solution. High molecular weight polyanhydrides are obtained by removing the acetic anhydrides using high temperatures and vacuum. Reaction time, temperature, and the presence of catalysts all have an impact on the resulting polymer chain length, purity and polydispersity index.

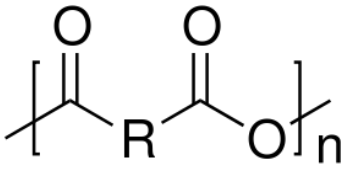

Figure 2.6 Basic chemical structure of polyanhydrides

The combination of the hydrophobic backbone and hydrolytically labile anhydride linkages enables control over hydrolysis rate simply by manipulation of the polymer composition. Examples of monomers for polyanhydrides used as biomaterials include those containing aliphatic groups such as sebacic acid (SA) and those containing

aromatic groups such as 1,3-bis(p-carboxyphenoxy)propane (CPP), 1,6-bis(p-carboxyphenoxy)hexane (CPH), and 1,8-bis(p-carboxyphenoxy)-3,6-dioxaoctane (CPTEG), which is structurally similar to CPH but contains ethylene glycol groups in place of the hexane in the backbone. Inclusion of more hydrophobic anhydrides, such as CPH, into polyanhydride copolymers can drastically slow release of the encapsulated payload, while inclusion of the more amphiphilic anhydrides, such as CPTEG, can accelerate polymer degradation and impart higher burst effect of the payload (171, 176). Degradation rates vary from weeks to several months by simple modification of the molar ratio of the monomers (172, 177, 178)

The ability to tailor degradation kinetics by adjusting the reacting monomers makes polyanhydrides highly desirable materials for drug delivery applications. Accordingly, polyanhydrides have been used to encapsulate a wide range of drugs, ranging from small molecule drugs to delicate protein therapeutics (179-182). The polyanhydride-based Gliadel® wafer, composed of a 20:80 ratio of CPP and SA and loaded with carmustine is FDA-approved as a post-surgical brain tumor implant (183).

More recently, polyanhydrides have been investigated for mucosal vaccine design. In addition to improving the stability of encapsulated antigen as compared to other biodegradable materials, such as polyesters, polyanhydride materials have been shown to modulate immune response without the requirement of supplementary adjuvants (184). Copolymers of methyl vinyl ether (PVM) and maleic anhydride (designated as P(VM-g-MA)) have demonstrated Th1-adjuvant activity. Upon immunization with ovalbumin-loaded and ligand-coated P(VM-g-MA) nanoparticles, a balanced IgG1 (Th2) and IgG2 (Th1) response was generated (185, 186). Th1-adjuvant capacity was mediated by nanoparticles promoting a close interaction between the

antigen and APCs, which can be further enhanced with targeting strategies, and also by acting as an agonist of various TLRs (187). Single dose immunization with P(VM-g-MA) NPs containing outer membrane vesicles (OMVs) derived from *Shigella* induced antibody protection (IgG1, IgG2, and IgA) as well as Th1 cytokines, ultimately protecting mice against lethal challenge (188), demonstrating the potential of polyanhydrides as a platform for protection by subunit antigens.

Extensive research has been done on polyanhydrides based on various copolymers of CPH, SA, and CPTEG (Figure 2.7). These materials have demonstrated biocompatibility, antigen stabilization, tailorable release kinetics, and immunomodulatory effects in both *in vitro* and *in vivo* models (189-195). A diversity of antigenically stable proteins have been released after encapsulation into polyanhydride nanoparticles, including tetanus toxoid, ovalbumin, PspA (pneumococcal surface protein A), viral hemagglutinin (an influenza surface protein), and HIV antigens (172, 176, 196-198). Additionally, *in vivo* studies using murine models have demonstrated the induction of humoral and cellular responses after subcutaneous or intranasal administration, sufficient to protect against lethal challenge (197, 199).

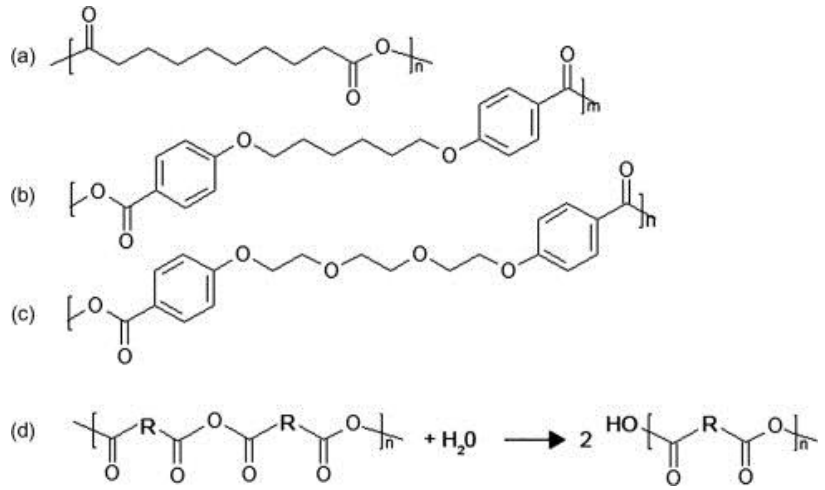

Figure 2.7 Chemical structures of a) sebacic acid, b) 1,6-bis(p-carboxyphenoxy) hexane, c) 1,8-bis(p-carboxyphenoxy)-3,6-dioxaoctane, and d) polyanhydride hydrolysis mechanism (172)

2.5 SUMMARY

The development of successful oral vaccines using subunit antigens requires careful design of delivery vehicles and incorporation of molecules that can potentiate their effect to elicit strong and balanced immune responses. As described, there are several advantages in the use of the oral route to improve vaccination efficacy; however, there are challenges including the protection of these fragile proteins, their release, and the adjuvant ability of their carriers.

There are currently a wide range of materials being explored for design of oral vaccine carriers. However, the potential to combine materials that address distinct aspects of the challenges to oral vaccination is an interesting strategy. Complexation polymers based on methacrylic acid are promising materials for targeted intestinal delivery of fragile therapeutics. Polyanhydride nanoparticles have demonstrated desirable characteristics for antigen delivery as adjuvants and/or delivery vehicles. Therefore,

exploiting the properties of both systems to achieve an oral subunit vaccine platform is a promising approach.

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Chapter 3: Objectives and Specific Aims

The development of oral subunit-based vaccines requires the use of delivery systems to induce immune protection. To elicit protective immunity, these systems must i) protect the payload through gastric conditions; ii) deliver stable antigen within the small intestine residence time proximal to antigen sampling cells; iii) facilitate transport across the epithelial layer to lymphoid tissue; and iv) stimulate antigen presenting cells in a pathogen mimicking manner to induce protective immune responses.

The overall goal of this research was to design a novel composite vaccine delivery vehicle to address the challenges of oral administration and elicitation of immune response. A combination of concepts and techniques from polymer chemistry, drug delivery, and immunology were employed to create a two component oral delivery strategy for nanovaccines containing protein antigen.

This multi-component system was a collaborative effort, comprised of polyanionic microgel matrix encapsulating polyanhydride nanoparticles. Development of the encapsulated nanoparticles have been investigated in great detail by Professor Balaji Narasimhan and his research team at Iowa State University for use as vaccine platforms via subcutaneous or intranasal administration. Nanoparticles based on poly(sebacic acid) (SA), and 1,6-bis(*p*-carboxyphenoxy)hexane (CPH) have demonstrated ability to stabilize encapsulated antigens, sustain protein release, and stimulate humoral and cellular response (1-5).

The focus of the present work is the development of the microgel matrix designed to protect the polyanhydride nanoparticles and their sensitive antigenic payload through gastric conditions and subsequently release nanoparticles upon exposure to intestinal conditions. Inspired by the use of crosslinked hydrogel networks for pH-sensitive protein

delivery (6, 7), polyanionic microgels were synthesized using methacrylic acid (MAA) and poly(ethylene glycol) monomethyl ether monomethacrylate (PEGMMA). Microencapsulation of the nanoparticles was done via a pH-triggered assembly process. Several formulations were synthesized by varying the feed ratios of the polyanhydride nanoparticles and exploring two different polyanhydride compositions: Poly(SA) and 20:80 CPH:SA. Formulations were evaluated for successful synthesis, pH-responsiveness and sustained release of protein antigen, *in vitro* cytocompatibility and immunostimulatory capacity. Finally, the ability of PROMPT composite microgels to stimulate systemic and mucosal protective antibody response was evaluated *in vivo*.

The following specific aims were addressed in this research:

Specific Aim 1: Design, synthesize and characterize pH-responsive microencapsulation systems for the oral delivery of polyanhydride nanoparticles to sustain subunit antigen delivery (Chapter 4).

Specific Aim 2: Evaluate the cytocompatibility and immunostimulatory properties of the composite vaccine carriers *in vitro* (Chapter 5).

Specific Aim 3: Perform an *in vivo* evaluation of microencapsulated nanoparticles to induce antigen-specific systemic and mucosal antibody protection after oral immunization (Chapter 6).

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Chapter 4: Development and Characterization of Microencapsulation Systems for the Oral Delivery of Nanoparticles

4.1 INTRODUCTION

The Global Vaccine Action Plan (GVAP), published in 2012, designated non-syringe delivery mechanisms as a research priority for the development of next-generation vaccines that are "more effective, less expensive and easier to manufacture and deliver" than traditional vaccines (1). Oral delivery is easily the safest and most practical alternative for vaccine administration. Additionally, the oral route enables stimulation of humoral and cellular immune responses at both systemic and mucosal sites to establish broader and long-lasting protection (2-4).

However, with the exception of a few successful live attenuated oral vaccines, administration of bolus oral doses is ineffective at stimulating protective systemic or mucosal responses. The oral route poses a myriad of challenges to overcome, including pH-dependent or enzymatic denaturation of the vaccine in the stomach, achieving adequate uptake of antigen in the small intestine, and delivering an appropriate dosage to elicit protection instead of tolerance (5, 6). Furthermore, while traditional vaccines consist of attenuated or inactivated pathogens, not all organisms can be attenuated and those that can often impose safety risks including inflammation, uncontrolled replication, and disease in immunocompromised patients (7, 8).

Thus, researchers have turned to subunit vaccines, such as protein antigens, to improve vaccine safety. However, especially in the context of the oral route, protein antigens are fragile structures that can be both weakly immunogenic and poorly absorbed, requiring carefully designed delivery systems to provide protection and enhance the resulting immune response (7, 9).

We present here a multi-component strategy to deliver subunit vaccines via the oral route. In their core, polyanhydride nanoparticles (PNPs) based on 1,6-bis-(*p*-carboxyphenoxy)hexane (CPH) and sebacic acid (SA) serve simultaneously as adjuvant and delivery vehicles of protein antigen. Polyanhydride nanoparticles have demonstrated substantial promise as drug and antigen delivery vehicles (10-12). Polyanhydrides are biodegradable polymers that have been shown to stabilize a variety of protein and peptide antigens, have tailorable sustained antigen release kinetics, and possess both excellent biocompatibility and adjuvant properties (13, 14).

To achieve selective delivery of a depot of PNPs to the small intestine, particles are incorporated into pH-responsive microgels synthesized by a self-assembly process. The pH-responsive polymer is composed of poly(methacrylic acid) (PMAA) with poly(ethylene glycol) (PEG) tethers, designated as P(MAA-g-EG). Crosslinked P(MAA-g-EG) hydrogels and other similar complexation hydrogel formulations have been developed for oral delivery of therapeutic proteins, such as insulin (15, 16), interferon β (17), TNF-α (18), and even for the design of oral vaccine formulations (19, 20).

In this work, design of a self-assembled instead of crosslinked hydrogel enables facile inclusion and release of much larger payloads. Polymeric coating of nanoparticles for oral vaccine delivery has been investigated previously for chitosan (21, 22) and PLGA nanoparticles (23). Delivering nanoparticles encapsulating antigen, instead of antigen alone, provides the additional feature of the sustained release of the entrapped antigen. The depot effect is known to be an effective mechanism for conventional adjuvants, such as alum and incomplete Freund adjuvant (24).

The composite platform is referred to as Polyanhydride-Releasing MicroParticle Technology, or PROMPT. Design of PROMPT for oral vaccine delivery is described in

Figure 4.1. In this work, the synthesis and characterization of PROMPT formulations are described. Additionally, pH-dependent dynamic release and sustained release kinetics of the model antigen, ovalbumin (ova), were evaluated from PROMPT microgels synthesized with varying feed ratios of antigen-loaded nanoparticle using two different polymer chemistries, Poly(SA) and 20:80 CPH:SA, to evaluate the composite microgels as an oral vaccine platform.

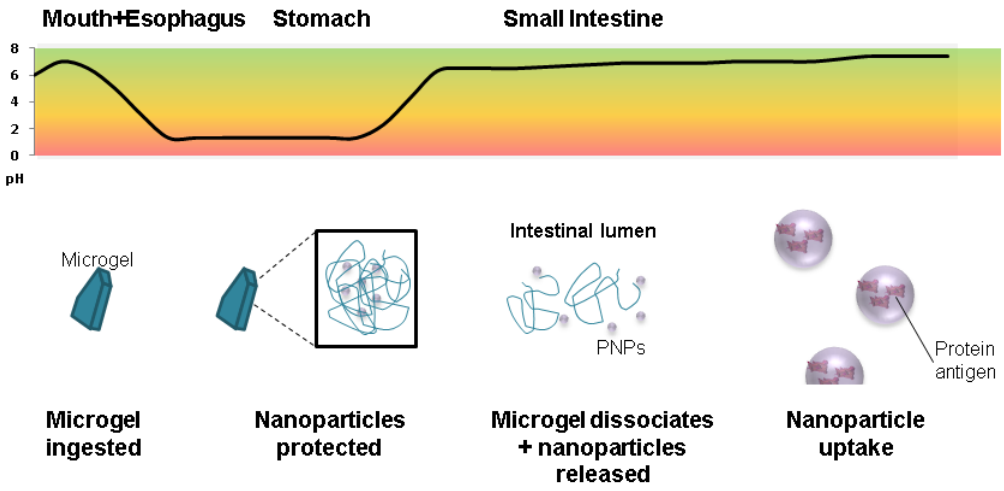

Figure 4.1 Design for oral delivery of vaccines for immunization at the intestinal mucosa. Microencapsulation using a copolymer of poly(methacrylic acid) and poly(ethylene glycol) protects polyanhydride nanoparticles from the harmful effects of the low pH and enzymatic destruction in the stomach and and enables selective release in the upper small intestine.

4.2 EXPERIMENTAL METHODS

4.2.1 Materials

All reagents were used as received. Methacrylic acid (MAA), 1-hydroxycyclohexyl phenyl ketone (Irgacure® 184), Span® 80, CdSeS/ZnS alloyed quantum dots (λ_{em}=525nm) and albumin from chicken egg white were obtained from Sigma-Aldrich (St.Louis, MO). Polyethylene glycol monomethyl ether methacrylate (PEGMMA, ME 1000) was purchased from Polysciences Inc. (Warrington, PA), and TAMRA-cadaverine obtained from Biotium (Fremont, CA). EDC (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride) (EDC), and N-Hydroxysuccinimide (NHS) were obtained from Thermo Fisher Scientific (Waltham, MA). All other solvents and buffers were also obtained from Thermo Fisher Scientific (Waltham, MA). Polyanhydride polymers were synthesized by collaborators at Iowa State University.

4.2.2 Synthesis

4.2.2.1 Synthesis of P(MAA-g-EG)

P(MAA-g-EG) polymer was synthesized by a photoinitiated free-radical polymerization. MAA and PEGMMA were added in a 1:1 molar ratio of hydrogen bonding groups in a 1:1 (w/w) solution of deionized water and ethanol to yield a final 1:5 total monomer to solvent ratio. The photoinitiator Irgacure® 184 was added at a 1 wt % with respect to total monomer content. The mixture was homogenized by sonication and then purged of oxygen, a free-radical scavenger, by nitrogen purging the sealed flask for 20 minutes. Polymerization was initiated using a Dymax® BlueWave 200 UV point source (Dymax, Torrington, CT) at 150 mW/cm^2 intensity, and allowed to proceed for 2 hours while stirring.

Linear polymer was purified from unreacted monomer by inducing polymer-ionomer collapse. The pH of the polymer solution was adjusted to 10 by addition of 50% sodium hydroxide solution. The polymer was collapsed while keeping the pendant groups ionized by lowering the dielectric constant of the suspension, in this case by addition of acetone, which caused immediate flocculation and sedimentation of the polymer. Polymer was collected by centrifugation at 4,000 rcf for 10 min and supernatant containing unreacted monomer removed. The pellet was resuspended in water and the process was repeated three times. The polymer solution was neutralized, dialyzed against deionized water in 3.5 kDA molecular weight cutoff dialysis tubing (Spectrum Labs, Rancho Dominguez, CA) for 10 water changes, and then freeze dried and stored at room temperature with desiccant.

4.2.2.2 Synthesis of Polyanhydride Polymers

Synthesis of diacids of 1,6-bis(*p*-carboxyphenoxy)hexane (CPH), prepolymers of sebacic acid (SA) and CPH, as well as homopolymers of sebacic acid and the copolymer with molar composition 20:80 CPH:SA, were performed by collaborators at Iowa State university, as previously reported (25, 26). A typical reaction to yield each polymer precursors is described briefly. Prepolymers of both SA and CPH were refluxed in an excess of acetic anhydride for 30 and 60 minutes, respectively, under nitrogen. Acetic acid was removed by evaporation at 50^{o}C. SA prepolymer was purified by addition of chloroform, followed by addition of 1:1 mixture of anhydrous ethyl ether and petroleum ether. The solution was stirred for 10 minutes, filtered and dried under vacuum overnight. CPH prepolymer was filtered, washed with ethyl ether and then vacuum dried overnight.

Poly(SA) and 20:80 CPH:SA were synthesized by melt polycondensation of acetylated prepolymers at 180^{o}C under vacuum for 90 minutes. Resulting polymer was

dissolved in methylene chloride and precipitated in dry petroleum ether. Purified polymer was dried under vacuum overnight. The chemical structure of all polymers was verified by ^{1}H NMR and molecular mass determined using gel permeation chromatography prior to use.

4.2.2.3 Polyanhydride Nanoparticle Synthesis

Polyanhydride particles were synthesized by anti-solvent nanoencapsulation as described previously (25, 26). For these studies 20:80 CPH:SA or Poly(SA) (20 mg/mL) was dissolved in methylene chloride with Span® 80 (1% v/v) and the desired encapsulating agent: either ova (5% w/w) or quantum dots (1% v/v). The polymer solution was sonicated at 50 Hz for 30 seconds using a probe sonicator (Fisher Scientific Model 50 Sonic Dismembrator) and then rapidly poured into a pentane bath at a solvent to non-solvent ratio of 1:250. Resulting particles were collected by filtration (Whatman 50, Fisher Scientific) and characterized using scanning electron microscopy (SEM, Zeiss Supra40 Scanning Electron Microscope, Oberkochen, Germany).

4.2.2.4 PROMPT Composite Microgel Synthesis

Antisolvent precipitation was used to produce particle dispersions of polyanhydride nanoparticles stabilized by P(MAA-g-EG). Linear P(MAA-g-EG) polymer was dissolved in deionized water (pH 6) at 20 mg/mL. Polyanhydride nanoparticles were added at varying ratios (10% or 20 wt% relative to polymer content), and sonicated at 50 Hz for 30 seconds to achieve a dispersed solution. HCl, at 0.1N concentration, was used as the anti-solvent, added to the polymer-nanoparticle dispersion at a volumetric ratio of 1:100. Upon addition of solvent into the antisolvent phase, particles rapidly formed and began to flocculate.

Flocculated particles were collected by centrifugation (4500 rcf, 5 min), and then freeze-dried. The final particles were acquired by crushing the particle pellet with a mortar and pestle to obtain a fine powder. Synthesized microgel formulations are described in Table 4.1

Table 4.1 Summary of PROMPT composite microgel formulations synthesized

Notation	Polyanhydride Nanoparticle Composition	Nanoparticle Feed Ratio (wt% of polymer)
10% Poly(SA)	Poly(SA)	10
20% Poly(SA)	Poly(SA)	20
10% 20:80 CPH:SA	20:80 CPH:SA	10
20% 20:80 CPH:SA	20:80 CPH:SA	20

4.2.3 Characterization

4.2.3.1 Copolymer Characterization

Copolymer composition was verified by ^{13}C-NMR using a Varian DirectDrive 600MHz nuclear magnetic resonance spectrometer (Palto Alto, CA). A minimum of 50 mg of dried polymer was dissolved in 700 μL of D_2O. All NMR Spectra were analyzed using MestReNova Software.

Polymer molecular weight was determined by gel permeation chromatography (GPC) using a Malvern Viscotek TDAmax Triple Detection SEC System (Malvern Instruments, Malvern, Worcestershire, UK), equipped with an A6000M column. Samples were dissolved in 0.1M Na_2HPO_4 to a final concentration of ~4 mg/mL for 1 hour prior to analysis at 30^{o}C. For all samples, an injection volume of 100 ul and a flow rate of 1.0 mL/min were used. Molecular weight data was calculated using OmniSEC software.

4.2.3.2 Scanning Electron Microscopy

Scanning electron microscopy (SEM) was used to examine the surface morphology, particle shape, and size of microparticle formulations in the dried state. SEM samples were prepared by dusting carbon-tape covered aluminum stubs with lyophilized and crushed microgels. Samples were coated with 12 nm of platinum/palladium using a Cressington 208HR sputter coater (Watford, England, UK). Coated samples were imaged with a Zeiss Supra 40VP Scanning Electron Microscope (Oberkochen, Germany).

4.2.3.3 Confocal Microscopy

Confocal microscopy was used to verify nanoparticle incorporation during PROMPT Synthesis. P(MAA-g-EG) was labeled with TAMRA-cadaverine prior to PROMPT synthesis by an EDC-NHS reaction. To accomplish this reaction, 200 mg purified P(MAA-g-EG) was mixed with 50 mg EDC and 80 mg NHS in DI water for 2 minutes, prior to the addition of 52 μL of a 10 mg/mL TAMRA-cadaverine solution. The reaction solution was then incubated for two hours at room temperature. The labeled P(MAA-g-EG) polymer was washed three times using the ionomer collapse procedure and then freeze-dried.

Nanoparticle formulations were synthesized as previously described to encapsulate 1%(w/w) 525 nm quantum dots. The fluorescent P(MAA-g-EG) and nanoparticle formulations were used in the PROMPT synthesis procedure previously described. Fluorescent microgels were sprinkled onto a coverslip and imaged by an FV10i-DOC inverted laser-scanning confocal microscope (Olympus, Tokyo, Japan) using a built-in UPLSAPO 60x phase contrast oil immersion objective (NA=1.35). All settings and image adjustments were maintained for all images obtained.

4.2.3.4 Fourier Transform Infrared Spectroscopy

Fourier transform infrared spectroscopy (FTIR) spectra was used to investigate the molecular structure of the polymer starting materials, P(MAA-g-EG) and polyanhydride polymers, as well as the PROMPT composite microgels. All spectra were collected from 2500-650 cm^{-1} as the average of 64 scans on a Nicolet iS10 FT-IR Spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) outfitted with Smart iTX accessory for measurement in ATR mode.

4.2.3.5 pH-dependent dissociation

Dissociation of the composite microgels was verified by microscopy using an Olympus IX73 inverted microscope (Olympus, Tokyo, Japan). Microgels containing quantum-dot loaded nanoparticles were hydrated in 0.1M HCl, and then 50μL of sodium hydroxide was added to adjust the pH to 6.5. Bright field and fluorescence images of the dissociation were captured using the 10x objective.

4.2.4 Protein Release Kinetics

4.2.4.1 Ovalbumin release kinetics from polyanhydride nanoparticles

In vitro release kinetics of ovalbumin from 20:80 CPH:SA and Poly(SA) formulations was performed using a micro bicinchoninic acid (BCA) assay (Thermo Fisher Scientific). Nanoparticles were suspended at 10 mg/mL in phosphate buffered saline (PBS, pH 7.4) with 0.01% sodium azide and maintained at 37^{o}C under constant agitation for the duration of the study. Release samples were collected at 1 hour, and on days 1, 2, 3, 5, 10, 15, 20 and 30 to evaluate sustained release of ova from the nanoparticles. To collect samples, nanoparticles were centrifuged at 10,000 rcf for 10 minutes, and supernatant collected and replenished with fresh buffer. Samples were

stored at 4°C until analysis. After 30 days, the remaining encapsulated protein was extracted by addition of sodium hydroxide to a final concentration of 50 mM. Experiments were performed in triplicate.

4.2.4.2 Ovalbumin release kinetics from PROMPT composite microgels

In vitro release kinetics of ovalbumin from PROMPT formulations were performed using a micro bicinchoninic acid (BCA) assay (Thermo Fisher Scientific). PROMPT formulations were suspended at 1mg/mL in phosphate buffered saline (PBS, pH 7.4) with 0.01% sodium azide and maintained at 37°C under constant agitation for the duration of the study. Release samples were collected at 1 hour, and on days 1, 2, 3, 5, 10, 15, 20 and 30 to evaluate sustained release of ova. At each time point, suspensions were centrifuged at 4000 rcf for 10 min, and a 2 mL aliquot collected and replaced with fresh PBS. Samples were stored at 4°C until analysis. After 30 days the remaining encapsulated protein was extracted by addition of sodium hydroxide to a final concentration of 50 mM. Experiments were performed in triplicate.

4.2.5 Dynamic Release of Ovalbumin from PROMPT formulations

4.2.5.1 Experimental setup

The pH-dependent release of ovalbumin from PROMPT composite microgels was evaluated using a two-stage dissolution protocol to simulate the transition from gastric to intestinal conditions. A Distek 2100 B Dissolution System (Distek, Inc., North Brunswick, NJ) with paddle apparatus was used according to United States Pharmacopeia (USP) 29 Chapter 711 Apparatus II guidelines. Small volume dissolution vessels (100

mL) were coated with Sigmacote siliconizing reagent (Sigma-Aldrich) to prevent protein adsorption and covered over the course of the experiment to minimize buffer evaporation.

Fasted state simulated gastric fluid (FaSSGF) and fasted state simulated intestinal fluid (FaSSIF) were prepared according to the manufacturer's instructions (Biorelevant.com, London, England). For 500 mL of FaSSGF buffer, 1.0g of NaCL was dissolved in 450 mL of DI water. The pH was adjusted to 1.6 by addition of hydrochloric acid and then total volume made up to 500 mL. Half of the volume was used to dissolve 0.03 g the FaSSIF/FeSSIF/FaSSGF biorelevant powder, and then the volume completed to 500 mL with the remaining HCl/NaCL solution. FaSSIF was prepared at 2x concentration by dissolving 0.420 g sodium hydroxide (NaOH; Fisher Scientific, Waltham, MA), 3.438 g sodium phosphate anhydrous (NaH_2PO_4; Sigma Aldrich, St. Louis, MO), and 6.816 g sodium chloride (NaCl; Fisher Scientific, Waltham, MA) in 450 mL DI water. The pH was adjusted to 6.9 by addition of sodium hydroxide. Subsequently, 250mL of the NaOH/NaH2PO4/NaCl solution was used to dissolve 2.240g of the FaSSIF/FeSSIF/FaSSGF biorelevant powder. The pH of the FaSSIF buffer was adjusted by addition of sodium hydroxide such that a 1:1 ratio of the FaSSGF and FaSSIF solutions (0.5 mL of each) reached a final pH of 6.5, before the volume of FaSSIF was brought up to 500 mL by addition of the remaining NaOH/ NaH2PO4/NaCl solution. Both buffers were stored at room temperature and used within the recommended 48 hour window.

4.2.5.2 Dynamic Release Protocol

Dynamic release was evaluated from 10 mg of either 10% Poly(SA), 20% Poly(SA), 10% 20:80 CPH:SA or 20% 20:80 CPH:SA carefully added into dissolution vials with 30 mL of FaSSGF buffer. At 10, 20, and 20 minutes, 1mL aliquots were

removed with a sampling needle fit with 10 μm filters and replaced with FaSSGF to maintain constant volume. After 30 minutes in gastric conditions, 30 mL of FaSSIF solution was added to the dissolution vessel, and samples taken in the same manner at 10, 20, 30, 60, 120, 180, and 240 minutes with volume replacement by a 1:1 mixture of FaSSGF:FaSSIF. Impellers were set to 100 rpm to maintain constant agitation, and dissolution apparatus water bath maintained at 37^{o}C for the duration of the experiment. Experiments were performed in triplicate.

4.2.5.3 Ovalbumin Specific Enzyme-Linked Immunosorbent Assay (ELISA)

Release samples were analyzed with an ovalbumin sandwich ELISA. High-binding 96-well plates (Costar 3590) were coated with 100 μL monoclonal anti-chicken egg albumin (Sigma Aldrich, A6075 clone OVA-14) diluted 1:2000 in PBS overnight at 4^{o}C. Plates were washed three times with a washing buffer comprised of PBS with 0.05% tween20 (PBST), blocked for 2 hours at room temperature with a 2% BSA solution in PBST, and then washed three times with PBST again. 100 μL of each release sample was added in triplicate and incubated overnight (18 hours) at 4^{o}C. Serial dilutions of chicken egg white ovalbumin (Sigma Aldrich, A5503) were included in each ELISA plate to establish a standard curve. Following incubation, plates were washed three times with PBST. Rabbit anti-chicken egg albumin polyclonal antibody (Sigma Aldrich, C6534) at 1:10,000 dilutions in PBST was added to each well and incubated for 1 hour at 37^{o}C. Plates were washed three times with PBST and alkaline phosphatase-conjugated goat anti-rabbit IgG detecting antibody (Sigma Aldrich, A3687) was used at 1:10,000 dilution, 100 μL per well, and incubated for 1 hour at 37^{o}C. Plates were washed three times with PBST. Color development was achieved by addition of 100 μL p-nitrophenyl phosphate

(PNPP) substrate solution (Thermo Fisher Scientific). Absorbance was measured at 405 nm on a Biotek Cytation 3 Cell Imaging Multi-Mode Reader (Winoosky, VT, USA).

4.3 Results and Discussion

4.3.1 Polymer Characterization

As discussed previously, polymer samples were synthesized by free radical polymerization and the ensuing materials were tested by a variety of techniques. The composition of the P(MAA-g-EG) copolymer (**Figure 4.2**) was verified by ^{13}C NMR spectroscopy (**Figure 4.3**). The final polymer composition is based upon both the molar feed ratio and reactivity ratio of the monomer components. Relative molar composition of polyethylene glycol and methacrylic acid were calculated according to **Table 4.2**. Methyl groups in the polymer backbone from both PEG and MAA groups correspond to the peak centered at 17.3 ppm, while terminal methyl groups on the PEG tethers appear at 58 ppm. Peak assignment was confirmed using 2D HSQC NMR. The molar percent of PEG chain was calculated as the ratio of terminal PEG methyl groups over the total backbone methyl groups from both MAA and PEG groups. The MAA molar fraction of the copolymer was determined as (1 – molar fraction of PEG). The resulting P(MAA-g-EG) copolymers were determined to have MAA content between 91.4% and 94.3%, or 95.6-98.6% of the MAA added in the feed ratio, as reported in **Table 4.3**.

The molecular weight of P(MAA-g-EG) copolymer was determined using GPC and is summarized in **Table 4.4**. The resulting polymer had an average molecular weight range between 55-57 kDa with polydispersity indices of 2.1-2.6, which are consistent with the reported literature for free radical polymerization (27, 28).

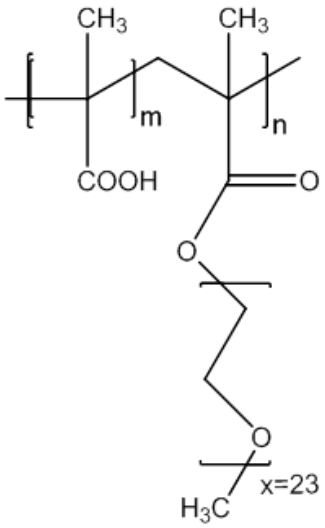

Figure 4.2 Representative structure of one unit of P(MAA-g-EG)

Table 4.2 Representative calculations to determine relative molar composition of P(MAA-g-EG) copolymer using ^{13}C NMR spectra analysis. Relative molar calculations refer to the integrals of the peaks labeled in Figure 4.1: a: 58 ppm, PEG terminal methyl groups; f: 17.3 ppm backbone methyl groups.

	Peak Assignment (ppm)	Integral	Relative moles calculation	Molar % in Feed	Calculated Molar %
MAA	17.3 (f)	15.29	$\frac{\int f - \int a}{\int f}$	95.6%	93.5%
PEGMMA	58 (a)	1.0	$\frac{\int a}{\int f}$	4.4%	6.5%

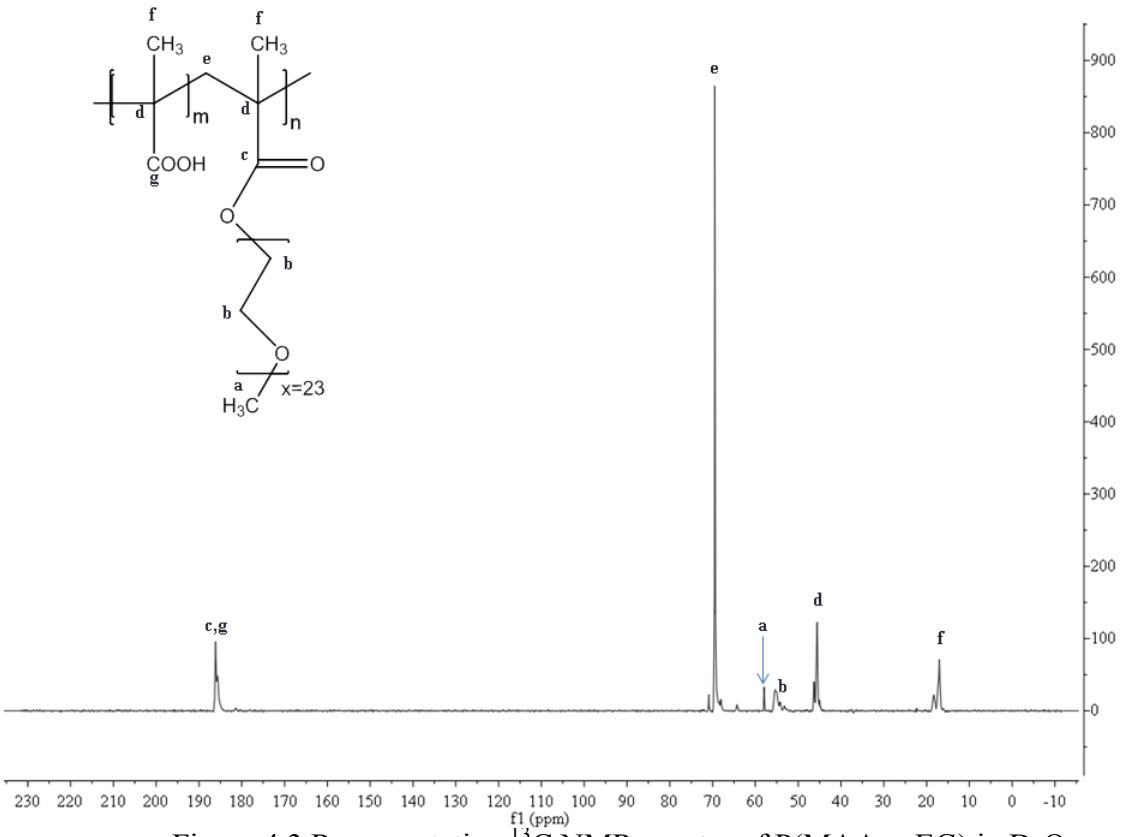

Figure 4.3 Representative ^{13}C NMR spectra of P(MAA-g-EG) in D_2O

Table 4.3 Copolymer composition of P(MAA-g-EG) synthesized by free-radical polymerization as determined by ^{13}C NMR

	Batch 1	**Batch 2**	**Batch 3**
%MAA	93.4	94.3	91.4
%PEGMMA	6.5	5.7	8.6

Table 4.4 Molecular weight of P(MAA-g-EG) determined by GPC

Sample	$\mathbf{M_z}$	$\mathbf{M_w}$	$\mathbf{M_n}$	$\mathbf{M_w/M_n}$	$\mathbf{R_h}$ **(nm)**
1	121,699	55,638	25,943	2.145	5.79
2	126,670	55,297	21,152	2.616	5.64
3	126,829	57,159	23,528	2.429	5.79

4.3.2 Microgel Morphology

SEM was used to confirm and evaluate the self-assembled composite systems, synthesized using two different polyanhydride nanoparticles, Poly(SA) and 20:80 CPH:SA, at two different feed ratios, 10 and 20%. The self-assembly process resulted in disperse particle flocs that were subsequently collected by centrifugation and dried by lyophilization. The dried powder was robust and able to be crushed into a fine powder with mortar and pestle. All four formulations exhibit similarly irregular morphology resulting from the preparation process, as shown in **Figure 4.4**. SEM images at 5000x indicated unentrapped nanoparticles associated with the microgel surface, with both 20% formulations appearing to have more surface-association than microgels with 10% nanoparticles.

Confocal microscopy was used to confirm that nanoparticles were incorporated throughout the microgels during the synthesis process. Both the P(MAA-g-EG) and nanoparticles were modified with fluorescent agents prior to microgel synthesis, prepared in the same manner as unmodified microgels, and corresponding z stack images obtained.

The xy and yz orthogonal projections from a z-plane in the middle of the particles, shown in **Figure 4.5**, confirm that the nanoparticles are incorporated throughout the self-assembled microgels for both 10% and 20% nanoparticle feed ratios.

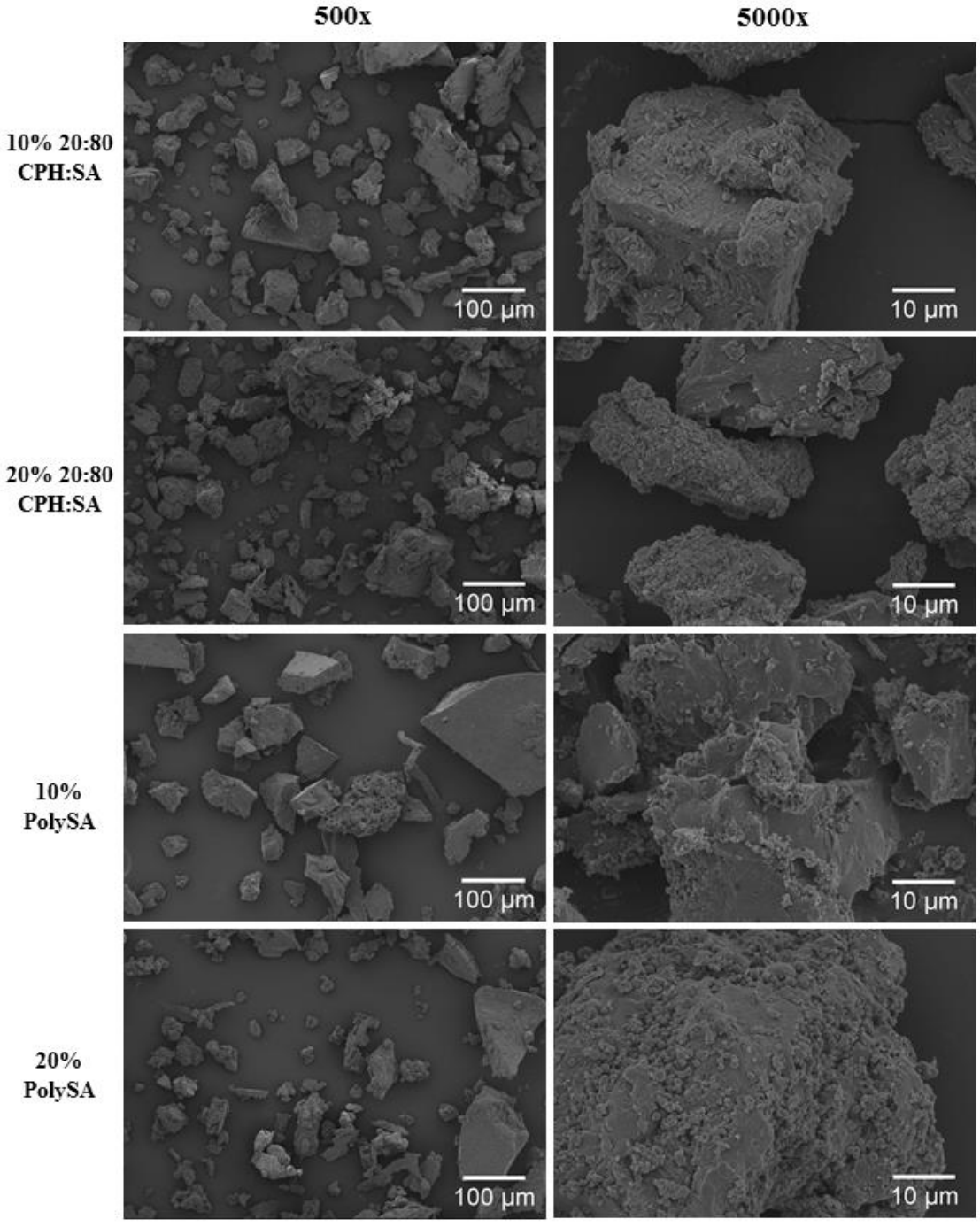

Figure 4.4 Representative SEM micrographs of PROMPT composite microgels synthesized with 10 and 20% feed ratios of Poly(SA) and 20:80 CPH:SA nanoparticles.

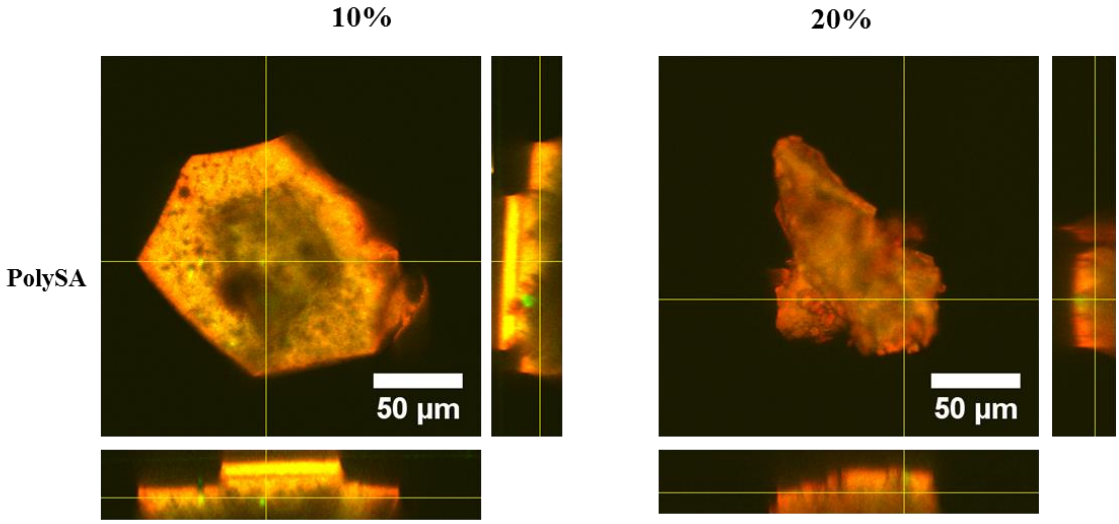

Figure 4.5 Representative images from z-stack scans of PROMPT composite microgels synthesized with 10 and 20% feed ratios of Poly(SA) nanoparticles. The xy and yz orthogonal projections confirm nanoparticle incorporation throughout self-assembled microgels.

4.3.4 Fourier Transform Infrared Spectroscopy

Fourier Transform Infrared spectroscopy (FTIR) spectra were obtained for linear P(MAA-g-EG), poly(SA), 20:80 CPH:SA, as well as the four PROMPT composite microgel formulations (**Figure 4.6)**. FTIR spectra of P(MAA-g-EG) dissolved in acidic and neutral pH conditions prior to freeze drying confirm the macromolecular changes the polymer undergoes in response to changes in environmental pH – namely the ionization of the carboxylic acid groups of the MAA.

Characteristic functional groups were consistent with previously reported spectra (29-31) and are summarized in **Table 4.4.** The region between 1750-1500 cm^{-1} generated the most information about the pH-dependent macromolecular changes. At pH 2, the carbonyl of the carboxylic acid appeared at 1717 cm^{-1}. Ionization of the carboxylic acid

of the MAA group at neutral pH shifted the carbonyl peak towards 1640 cm^{-1} and resulted in the appearance of a strong C(=O)-O^- stretching peak.

The FTIR spectra for the polyanhydrides appear in **Figure 4.6B**. The characteristic peak reported for poly(SA) is the asymmetric carboxyl stretching of the aliphatic anhydride bond at 1810 cm^{-1} and appeared in both the Poly (SA) and 20:80 CPH:SA spectra. Additionally, the 20:80 spectra contain the characteristic CPH peak at 1605 cm^{-1}, indicative of the aromatic ring structure (32, 33).

The FTIR spectra of the PROMPT formulations in **Figure 4.6C** contain peaks from both spectra, providing molecular evidence of the surface-adsorbed nanoparticles observed by SEM. All four formulations are similar, with the primary difference being the presence of the characteristic CPH peak at 1605 cm^{-1} in the 20:80 CPH:SA or its absence in the Poly(SA) based formulations. The presence of the P(MAA-g-EG) carboxylic acid peak at in the 1720-1700 cm^{-1} confirms the hydrogen bonding, or complexation of the polymer and nanoparticles, that drives the self-assembly process in acidic conditions.

Table 4.5 Comparison of characteristic functional groups from FTIR spectra of P(MAA-g-EG) hydrated at pH 2 and 7 prior to lyophilization

Functional Group	**Reported Wavenumber Range (cm^{-1})**	**pH 2.0**	**pH 7.0**
C=O stretching vibrations	1725-1650	1719	1654
C(=O)-O^- asymmetric stretching	1650-1550	-	1560
O-CH_2 symmetric deformation	1475-1445	1466	1447
OH in plane bending + C-O stretching	1440-1395	1388	1398
O-H deformation	960-875	961	948

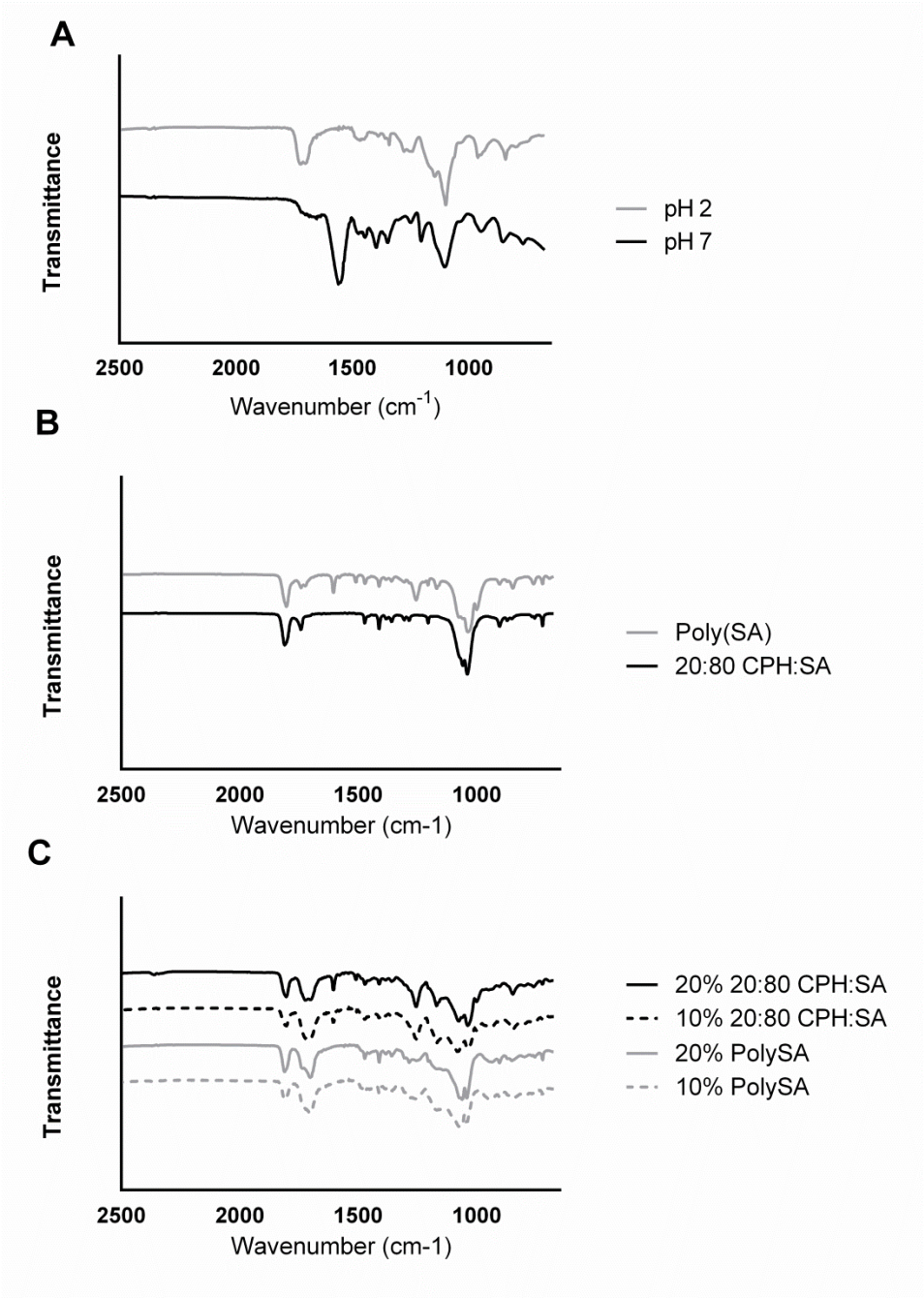

Figure 4.6 ATR-FTIR spectra of A) P(MAA-g-EG) hydrated in pH2 and pH 7 aqueous conditions prior to lyophilization, B) polyanhydride polymer formulations, and C) PROMPT composite microgels synthesized by pH-triggered self-assembly of P(MAA-g-EG) and nanoparticle dispersions.

4.3.5 pH-Dependent Dissociation

The pH-dependent dissociation of microgels to release nanoparticles was observed after suspending composite particles in aqueous solution at pH 1.2 and then adjusting the pH to 6.5. Representative images of 20% Poly(SA) microgels demonstrate that particles remained intact in acidic conditions without observable release of the fluorescent payload encapsulated by the nanoparticles (**Figure 4.7 A,C**). Within one minute of addition of the sodium hydroxide to adjust the pH, microgels were mostly dissolved to release the nanoparticle payload (**Figure 4.7 B,D**).

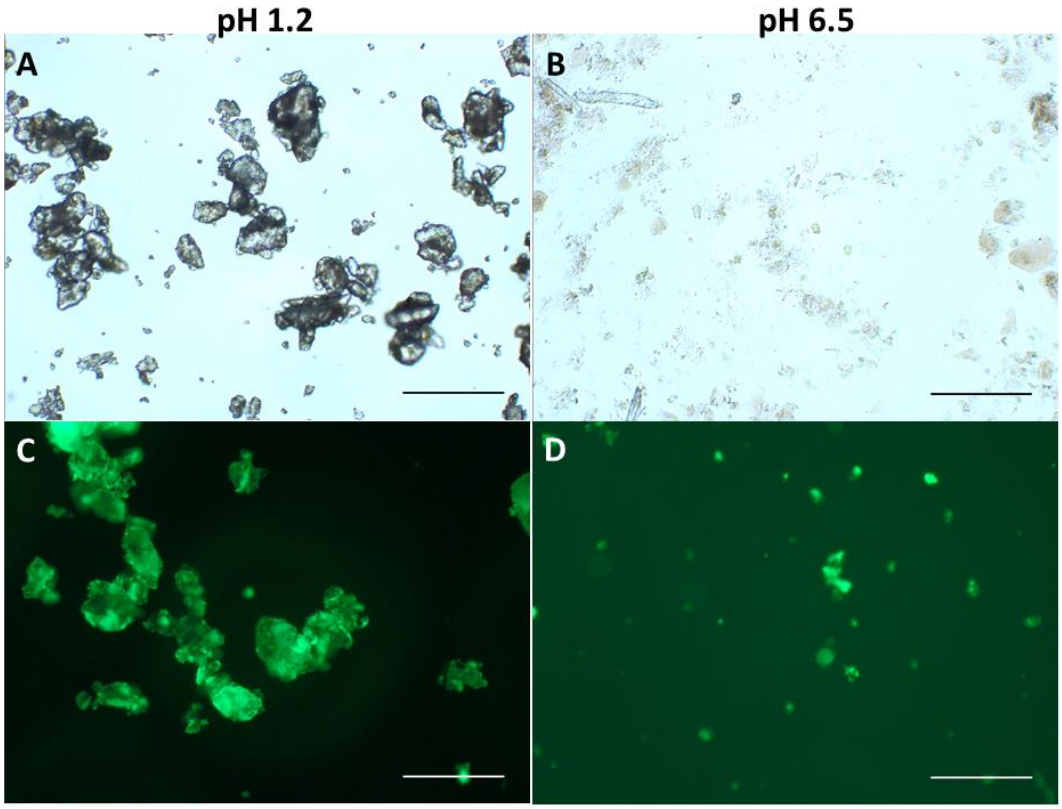

Figure 4.7 Representative microgel dissociation in intestinal pH conditions. Microgels remain intact at acidic pH (A,C) and dissociate to release the nanoparticle cargo in neutral pH conditions (B,D). Scale bar: 200 μm

4.3.6 Ovalbumin Release Kinetics from Polyanhydride Nanoparticles

The protein release kinetics from carrier systems is important in the design and optimization of drug and vaccine delivery vehicles. Release kinetics of ovalbumin from Poly(SA) and 20:80 CPH:SA nanoparticle was quantified using a microBCA assay, and are shown in **Figure 4.8**. Both formulations had very similar release kinetics with high initial burst releases, associated with the relatively high loading content (34). The burst release of the 20:80 CPH:SA was slightly lower, which correlated with previously reported trends that increased nanoparticle hydrophobicity slowed surface erosion and, therefore, decreased the release rate of encapsulated protein. After the initial burst, sustained release of ovalbumin continued with >95% of the encapsulated payload released from both formulations by day 30.

The initial burst release of antigen can more rapidly induce a primary immune response, which could be advantageous for vaccine delivery (35). However, the antigen dose, particularly via the oral route, must fall within a narrow range, as either too low or too high a dose could result in tolerance instead of a protective response (6, 36). The controlled and sustained release of the antigen has been demonstrated to induce a stronger and more lasting humoral immune response, due to its persistence at the target location (37-39).

The loading efficiency of the two nanoparticle chemistries was calculated by comparing the total mass of ovalbumin loaded into the nanoparticles, quantified after extraction, to the theoretical loaded amount introduced during nanoparticle synthesis. Encapsulation of Poly(SA) and 20:80 CPH:SA nanoparticles were approximately 85% and 68%, respectively, accounting for the difference in overall mass of ova (μg) released per mass of nanoparticles (mg), shown in **Figure 4.8B.**

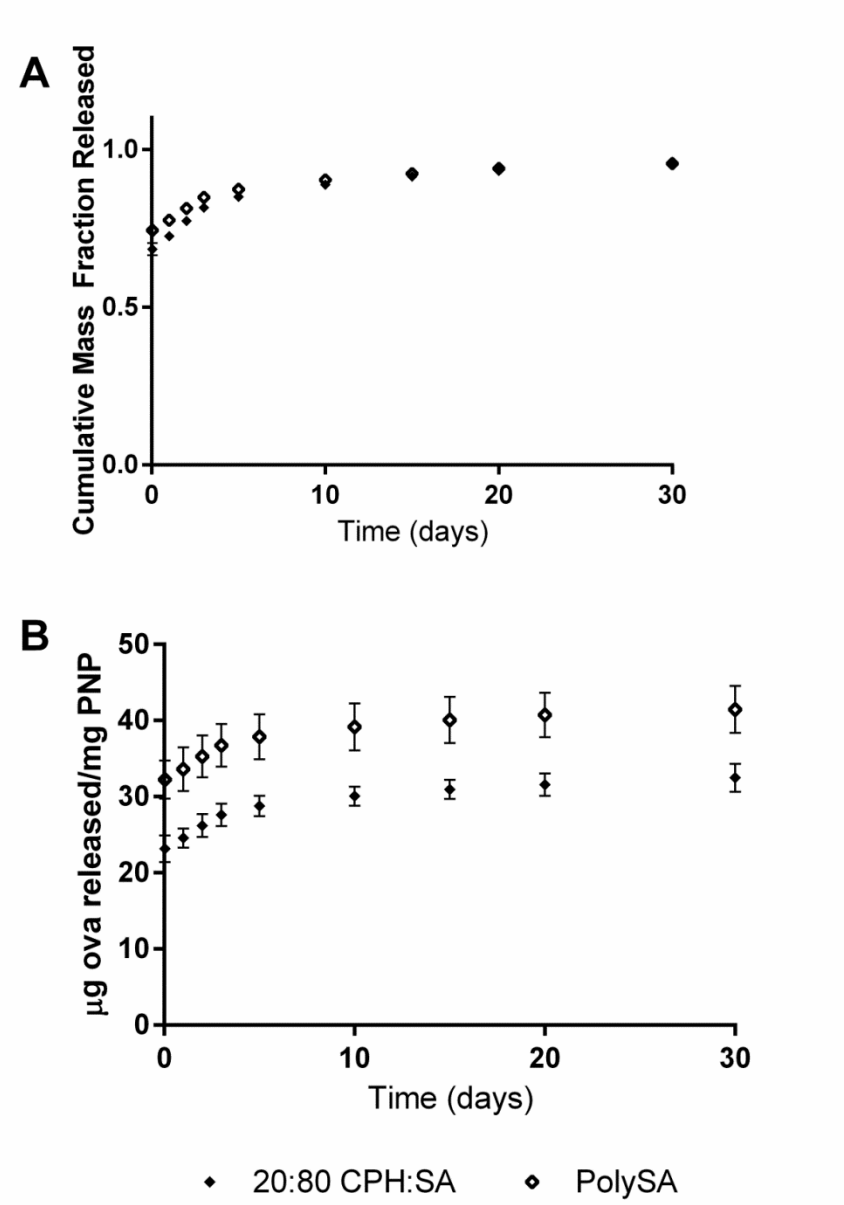

Figure 4.8 Ovalbumin release kientics from polyanhydride nanoaprticles. A) Cumulative mass fraction of ovalbumin released from Poly(SA) and 20:80 CPH:SA nanoparticle formulations over the course of 1 month at pH 7.4 as detected by microBCA assay. B) Mass ovalbumin released normalized to total mass of nanoparticle (n=3 ± S.E.M.)

4.3.7 Ovalbumin release kinetics from PROMPT microgels

Release of ova from PROMPT microgels was evaluated to determine i) the efficiency of the microencapsulation process and ii) the effect of microencapsulation on the ova release kinetics. Nanoparticle encapsulation efficiency from the microgel self-assembly process was quantified by comparing the total protein released from PROMPT formulations, determined after extraction, to the total protein loaded into the respective nanoparticle formulation, as described in section 4.3.6. Encapsulation efficiency of PROMPT composite microgels synthesized with either 10% or 20% feed ratio of the Poly(SA) or 20:80 CPH:SA nanoparticles are shown in **Figure 4.9**.

All of the formulations had roughly 40-50% encapsulation efficiency of the nanoparticles introduced during microgel self- assembly, except for 20% PolySA, which had an encapsulation efficiency of approximately 30% (**Figure 4.9A**). Interestingly, the increase in nanoparticle feed ratio significantly increased the incorporation of 20:80 CPH:SA nanoparticles, but not the Poly(SA) nanoparticles. The increased efficiency of 20:80 CPH:SA nanoparticle inclusion is attributed to the more hydrophobic nature of the polymer composition, which would favor the hydrophobic environment created during microgel self-assembly. Conversely, the more hydrophilic PolySA nanoparticles would less favorably reside within that same hydrophobic environment. This less favorable interaction does not appear to affect encapsulation efficiency at a 10% nanoparticle feed ratio, however, does reduce the encapsulation efficiency at the higher 20% feed ratio.

PROMPT microgels exhibited a similar release trend of ova as the non-encapsulated nanoparticles, undergoing an initial burst release and a subsequent sustained delivery (**Figure 4.10**). However, the burst release of all PROMPT microgel formulations was lower than that observed for the nanoparticle formulations alone, ~50% in microgels

compared to ~60-70% for nanoparticles. The P(MAA-g-EG) protection shows potential to slow the solvent diffusion to nanoparticles, thereby mitigating the degree of burst release and enabling a more sustained delivery of antigen. However, it should be noted that these experiments do not match the physiological environment, where there are larger volumes and higher rates of fluid exchange, which can certainly affect the initial protein release kinetics (40).

While nanoparticle chemistry primarily affected the encapsulation efficiency, it was the nanoparticle feed ratio that affected the burst release from the composite formulations. Microgels synthesized with 10% nanoparticle feed ratio exhibited higher fractional burst release compared to the 20% feed ratio. Dissociation is driven by the hydrophilic nature of the charged groups on P(MAA-g-EG) in neutral pH conditions. Accordingly, microgels synthesized with the lower nanoparticle feed ratio have higher P(MAA-g-EG) content per mass, facilitating a more rapid dissociation of the microgel and exposure of nanoparticle surface area.

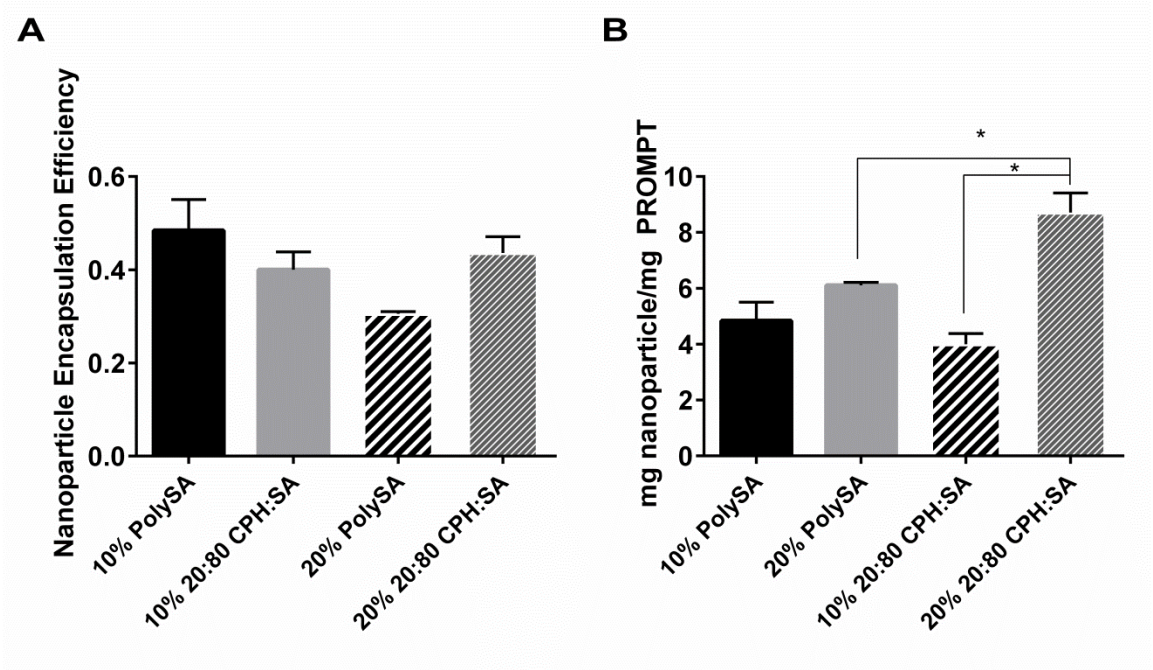

Figure 4.9 Encapsulation of nanoparticles into PROMPT microgels during self-assembly. A) Mass of nanoparticles normalized to mass of PROMPT microgels. B) Encapsulation efficiencies of PROMPT microgels based on nanoparticle feed ratios of 10% and 20% during self-assembly process. (n=3 ± S.E.M., $p \leq 0.05$)

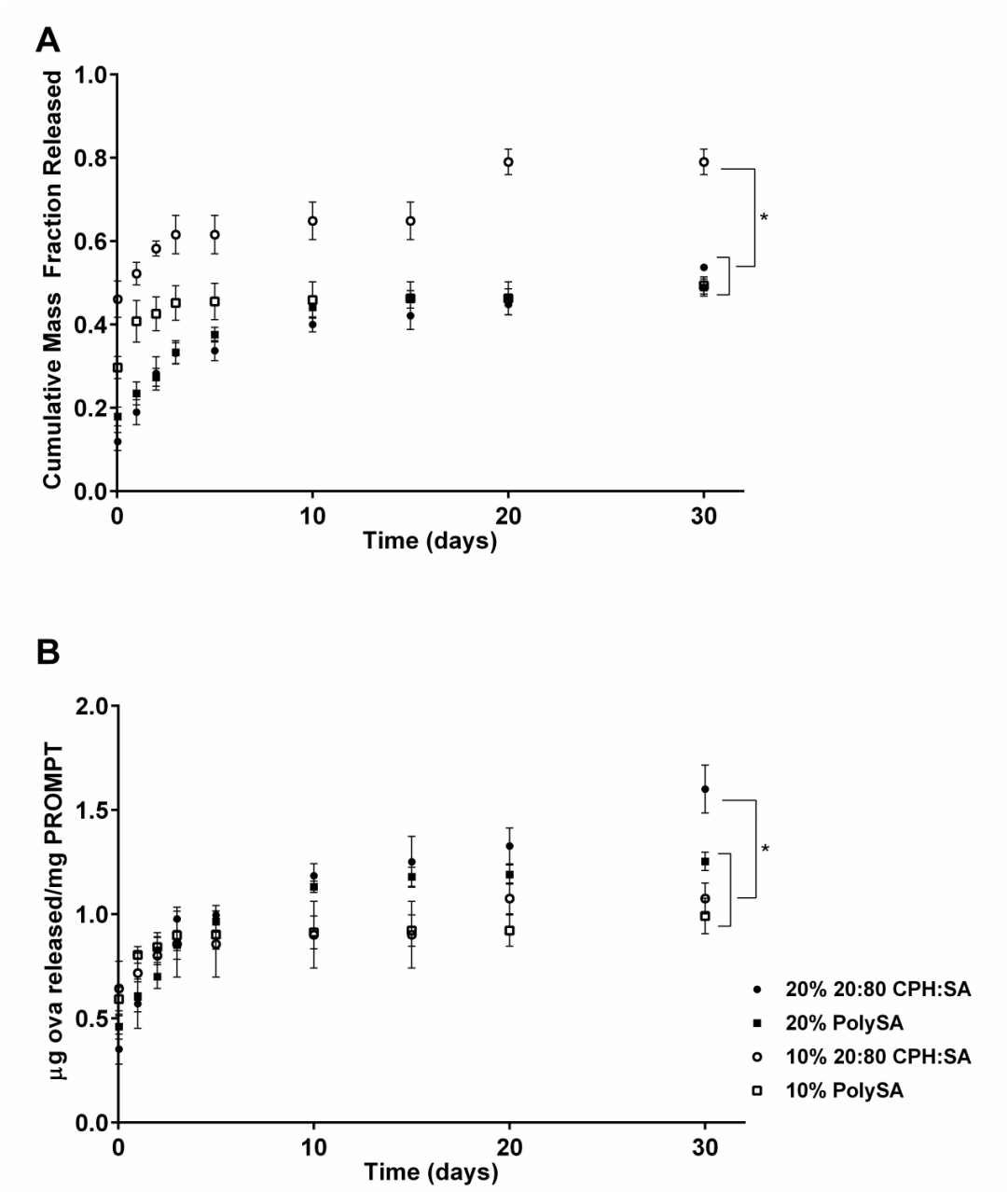

Figure 4.10 Ovalbumin release kinetics from PROMPT composite microgels. A) Cumulative mass fraction of ovalbumin released from PROMPT formulations synthesized with 10 and 20% feed ratios of either Poly(SA) or CPH:SA nanoparticle formulations over the course of 1 month at pH 7.4 as detected by microBCA assay. B) Mass ovalbumin released normalized to total mass of composite microgels (n=3 ± S.E.M., $p \leq 0.05$)

4.3.8 Dynamic Release of Ovalbumin from PROMPT formulations

To evaluate the potential of PROMPT formulations for oral delivery, a two-stage dissolution experiment was used to mimic the transit of the microgel through the acidic environment of the stomach into the more neutral environment of the upper small intestine using a USP Class II dissolution apparatus. **Figure 4.11** demonstrates ova released from PROMPT formulations as A) fraction ovalbumin released and B) total μg protein release per mg of PROMPT. Negligible ova release was detected in the gastric conditions (0-30 minutes) for all formulations, due to that fact that i) polyanhydride hydrolysis occurs much more slowly in acidic conditions than neutral or basic environments (41), and ii) microgel complexation in acidic conditions minimize polyanhydride surface in contact with gastric environment. This result is promising as any premature release of the payload in the stomach would be degraded by acidic conditions and proteolytic enzymes and, consequently, be unable to exert any immunological/therapeutic effect.

Immediately upon shifting to intestinal mimicking conditions, ova release was detected. Similar to experiments in PBS alone, microgels exhibited sustained release kinetics after the initial burst of protein. Microgels synthesized with 20% feed ratio of nanoparticles released more protein than the 10% microgels. Additionally, 20:80 CPH:SA containing formulations released more ova than Poly(SA) microgels at the same feed ratio. Therefore, the increased incorporation efficiency for 20:80 CPH:SA into microgels offset both the lower encapsulation efficiency of ova into the nanoparticles and the slower release kinetics of the more hydrophobic 20:80 CPH:SA chemistry.

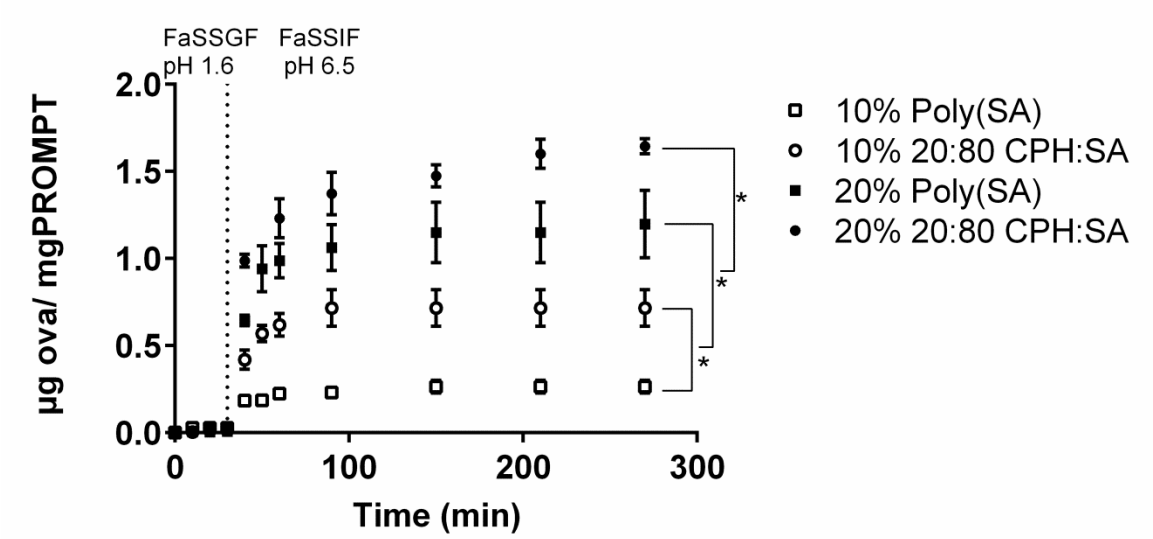

Figure 4.11 Ovalbumin release from PROMPT composite microgels after a two-stage dissolution protocol in simulated gastric conditions (0-30 minutes) and simulated intestinal conditions (30-270 minutes). (n=3 ± S.E.M, *p≤0.05)

4.4 CONCLUSIONS

A novel self-assembled microgel was developed for depot delivery of polyanhydride nanoparticles. P(MAA-g-EG) polymer composition and molecular weight were verified by ^{13}C NMR and GPC, respectively, and are consistent with previous literature. The P(MAA-g-EG) self-assembly process occured in a reversible pH-dependent fashion to encapsulate polyanhydride nanoparticles in acidic conditions and selective release in neutral pH conditions. SEM micrographs demonstrate a polydisperse population of microgels with nanoparticles adsorbed to the surface, while confocal microscopy confirmed nanoparticles are also incorporated throughout the microgel. FTIR confirmed macromolecular structures of both polyanhydride chemistries, pH-dependent

macromolecular changes in the P(MAA-g-EG) polymer, and incorporation of both materials into the self-assembled microgels.

The composite PROMPT microgels released the model antigen, ova, in a pH-controlled manner for gels synthesized at two feed ratios and with two nanoparticle chemistries: Poly(SA) and 20:80 CPH:SA. No ova release was detected from any formulation in gastric conditions. Upon transition to simulated intestinal conditions, microgels exhibited sustained ova release after the initial burst of protein. Increasing the feed ratio of nanoparticles into the microgels increased the total amount of ova delivered, as well as decreasing the observed burst release, likely due to the comparatively lower P(MAA-g-EG) content resulting in slower dissociation of the microgels.

Overall, these results suggest that pH-dependent microencapsulation is a viable platform to achieve targeted intestinal delivery of polyanhydride nanoparticles. Furthermore, tailoring the feed ratio and composition of the nanoparticles allows for manipulation of protein release kinetics to potentially tailor a desirable balance between antigen burst release and subsequent persistence from nanoparticles to achieve an effective oral immunization strategy.

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Chapter 5: *In Vitro* Evaluation of Microencapsulated Polyanhydride Nanoparticles: Cytocompatibility and Dendritic Cell Stimulation

5.1 INTRODUCTION

Evaluation of carriers in relevant *in vitro* models is an important step in the design vaccine and drug delivery platforms. Oral vaccine formulations will encounter a variety of cell populations, including epithelial cells of the small intestine, and if successfully transported, the diversity of immune cells in the Peyer's patches. Establishing safety of the materials with relevant cell lines is important when considering translation of the materials to *in vivo studies.* Furthermore, *in vitro* assays can be used to evaluate the immunostimulatory potential of vaccine formulations.

Dendritic cells (DCs) are among the most common and important antigen presenting cells in the gut-associated lymphoid tissue (GALT). DCs are principally responsible for inducing a primary immune response and enhancing secondary immune responses, processing and presenting antigenic fragments to B and T cells to initiate antigen-specific immunity. CD11c+ DCs are one of the subsets that reside in the sub-epithelial dome region directly below the follicle associate epithelium, facilitating antigen uptake directly from M cells (1, 2).

Antigen processing and presentation are activated by cellular engagement of pathogen-associated molecular patterns (PAMPs) with pattern recognition receptors on host cells (3, 4). Cell surface markers, such as MHC II, MHC I, and co-stimulatory markers such as CD80, CD86, CD40 are critical for antigen presentation by DCs to activate T cells and stimulate clonal expansion. Additionally, activated DCs may produce cytokines to mediate immune responses (3). Engagement of these APCs is critical in the

initiation of local and systemic immunity. Therefore, development of vaccine delivery carriers with immunostimulatory capabilities is important.

In this work, the cytocompatibility of microencapsulated nanoparticle formulations, designated as Polyanhydride-Releasing Oral MicroParticle Technology (PROMPT), was evaluated in two representative cell lines: Caco-2 and RAW264.7. Caco-2 cells are a human derived colon carcinoma cell line widely used to evaluate drug absorption across the intestinal epithelium (5, 6). RAW264.7 cells are murine derived macrophages. Cytocompatibility studies were used to indicate the maximum tolerable concentration without disruption to metabolic activity and cell membrane integrity. The stimulatory abilities of PROMPT formulations were evaluated by expression of cell surface markers and cytokine secretion in bone-marrow derived murine dendritic cells.

5.2 Experimental Methods

5.2.1 Materials

Chemicals for composite microgel synthesis include methacrylic acid (MAA), 1-hydroxycyclohexyl phenyl ketone (Irgacure® 184), and Span® 80 received from Sigma Aldrich (St. Louis, MO), and polyethylene glycol monomethyl ether methacrylate (PEGMMA, ME 1000) purchased from Polysciences Inc. (Warrington, PA All other solvents and buffers were also obtained from Thermo Fisher Scientific (Waltham, MA). Polyanhydride polymers were synthesized by collaborators at Iowa State University as previously described (7, 8).

The materials required for cell culture include: RPMI 1640 from American Type Culture Collection (Manassas, VA), Dulbecco's Modified Eagle Medium (DMEM), penicillin—streptomycin, and fetal bovine serum from Thermo Fisher Scientific

(Waltham, MA), L-glutamine from Sigma-Aldrich (St. Louis, MO) and granulocyte macrophage colony stimulating factor (GMCSF), purchased from PeproTech (Rocky Hill, NJ). Materials needed for flow cytometry included sodium azide and bovine serum albumin from Sigma Aldrich (St. Louis, MO), and unlabeled hamster IgG, antimouse CD16/CD32 antibody, PE-Cyanine7 CD11c Monoclonal Antibody (N418), phycoerythrin (PE) antimouse MHC II (I-Ek, clone 14-4-4S), fluorescein isothiocyanate (FITC)- conjugated antimouse CD80 (B7–1, clone 16–10A1), and respective isotypes PE-Cyanine7 Armenian Hamster IgG Isotype Control, (PE) rat IgG2a κ Isotype Control, and FITC-conjugated Armenian Hamster IgG from eBioscience (San Diego, CA).

5.2.2 Synthesis

5.2.2.1 Polyanhydride Nanoparticle Synthesis

Polyanhydride particles were synthesized by anti-solvent nanoencapsulation as described previously (7, 8). Briefly, either 20:80 CPH:SA or Poly(SA) (20 mg/mL) were dissolved in methylene chloride with Span80 ® (1% v/v). The polymer solution was sonicated at 50 Hz for 30 seconds using a probe sonicator (Fisher Scientific Model 50 Sonic Dismembrator), then rapidly poured into a pentane bath at a solvent to non-solvent ratio of 1:250. Particles were collected by vacuum filtration (Whatman 50, Fisher Scientific), and characterized using scanning electron microscopy (SEM, Zeiss Supra40 Scanning Electron Microscope, Oberkochen, Germany).

5.2.2.2 PROMPT Composite Microgel Synthesis

Antisolvent precipitation was used to produce particle dispersions of polyanhydride nanoparticles stabilized by P(MAA-g-EG). P(MAA-g-EG) dissolved in

deionized water (pH 6) at 20 mg/mL. Polyanhydride nanoparticles were added at 10 or 20 wt% relative to P(MAA-g-EG), and sonicated at 50 Hz for 30 seconds to achieve a dispersed solution. The anti-solvent (0.1N HCl) was used at a volumetric ratio of 1:100. Upon addition of solvent into the antisolvent phase, particles rapidly form and begin to flocculate.

Flocs were collected by centrifugation (4500 rcf, 5 min), then freeze-dried. The final particles were obtained by crushing the particle pellet with a mortar and pestle to achieve a fine powder. PROMPT formulations are denoted in this work as either PROMPT 20:80 CPH:SA or PROMPT Poly(SA) based on the nanoparticle cargo.

5.2.3 Cytocompatibility

Cytocompatibility of formulations was evaluated in Caco-2 colon adenocarcinoma cells and RAW 264.7 murine macrophages, obtained from American Type Culture Collection (ATCC, Rockwell, MD). Both cell lines were cultured in Dulbecco's Modified Eagle's medium, supplemented with 10% (v/v) FBS, 4mM L-glutamine, and 1% (v/v) of an antibiotic solution (100 U/mL penicillin and 100 μg/mL streptomycin). Cells were sub-cultured in T-75 flasks at 37^{o}C in a 5% CO_2 environment. Media was refreshed every 72 hours and cells passaged at 80-90% confluence.

For cytotoxicity studies, cell culture-treated 96-well polystyrene plates were coated with a 1:100 DPBS:fibronectin solution overnight at 4^{o}C. The fibronectin solution was aspirated and wells rinsed once with DPBS prior to plating cells. Caco-2 and RAW 264.7 cells were seeded at 25,000 and 20,000 cells per well, respectively, in completed DMEM without phenol red. Cells were maintained in the 96-well plates for 48 hours prior to undergoing any cytocompatibility experiments.

Solutions of microgels (PROMPT) and linear polymer (P(MAA-g-EG)), were prepared in DMEM without phenol red (+10 %FBS, 4mM L-glutamine, 1% P-S) at concentrations ranging from 0.25-1 mg/mL. Solutions of polyanhydride nanoparticles were also prepared in completed DMEM without phenol red at concentrations ranging from 50-200 μg/mL for nanoparticle formulations, corresponding to theoretical mass of nanoparticles encapsulated in the various microgel formulations. Media was carefully aspirated from all wells and replaced with either 100 μL of a formulation, unmodified growth media as a positive control, or water as a negative control and allowed to incubate for 4 hours with Caco-2 cells and 24 hours with RAW 264.7 macrophages at 37°C and 5% CO_2.

Proliferation of cells after formulation exposure was used as an indicator of cell health, as measured by the CellTiter 96 ® AQueous One Solution Cell Proliferation MTS Assay (Promega, Madison, WI). Briefly, 20 μL of MTS assay solution was added to the treated wells and incubated for 90 minutes. After incubation, absorbance measurements were made at 490 nm and 690 nm, using a Biotek Cytation 3 Cell Imaging Multi-Mode Reader (Winoosky, VT, USA). Cytocompatibility is reported as relative cell proliferation, calculated by subtracting the background absorbance and normalizing to the average absorbance value of the positive control well, using the following equation:

$$Relative\ proliferation = \frac{A_{490} - A_{690}}{A_{positive\ control}} \qquad \text{(Eq 5.1)}$$

The integrity of the cellular membranes was evaluated using the Promega CytoTox One™ Homogenous Membrane Integrity Assay (Promega, Madison, WI). Twenty minutes prior to the end of the designated incubation period, plates were removed

from the incubator to equilibrate to room temperature. The experimental supernatant (50 µL) was transferred into a fresh black-walled 96-well plate and mixed gently with 50 µL of CytoTox One™ reagent for 30 seconds to ensure complete mixing of reagent and supernatant. The samples were incubated for an additional 10 minutes at room temperature and then 25 µL of assay stop solution was added to each well. Fluorescence measurements were made with an excitation wavelength of 560 nm and emission wavelength of 590 nm. Percent cytotoxicity was calculated by subtracting the culture medium background (media only, no cells) from all fluorescence values of experimental wells, and then normalizing the corrected experimental values relative to the negative control:

$$\%\ Cytotoxicity = 100\ x\ \frac{(Experimental - Culture\ Medium\ Background)}{(Maximum\ LDH\ Release - Culture\ Medium\ Background)} \quad \text{(Eq 5.2)}$$

Results are reported as relative percent viability, calculated as 100% - % cytotoxicity. ANOVA analysis was used to compare multiple groups and a post-hoc Dunnett's test was performed to confirm statistical significance of the experimental groups against the media only.

5.2.4 Dendritic Cell Stimulation

5.2.4.1 Dendritic Cell Culture and Stimulation

Bone marrow derived dendritic cells were prepared from bone marrow cells isolated from femurs of Balb/c mice as described previously (9). Following mouse euthanasia, bones were excised, cleaned of muscle and tissue and cut at both ends. The marrow was flushed out in complete 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)) until bones appeared transparent. Cells were collected by centrifugation and suspended in complete medium supplemented with 10 ng/mL GMCSF. The cells were then placed in T-75 cell culture flasks in 10 mL of DC medium containing 10 ng/mL GMCSF and incubated at 37 °C under 5% CO2 atmosphere. On day 3, 10 mL of fresh DC medium with 10 ng/mL GMCSF was added. On day 6, 10 mL of the culture medium was removed, cells collected by centrifugation and resuspended into 10mL fresh DC medium containing 10ng/mL GMCSF, then added back to the original flask. On day 8, DCs were removed from the flasks, counted, resuspended in fresh DC medium with 10 mg/mL GMSF, and transferred to 24-well plates (1×10^6 cells/well) for 24 hours prior to stimulation.

DCs were stimulated with either 750 μg/mL of PROMPT formulations synthesized with either 10% or 20% feed ratio of QD-loaded Poly(SA) or 20:80 CPH:SA nanoparticles. Non-stimulated (NS) DCs and 200 ng/mL lipopolysaccharide (LPS) were used as negative and positive controls, respectively. DC cultures were incubated with stimulants for an additional 48 h (37 °C, 5% CO2), at which time the supernatants and DCs were harvested for cytokine production and cell surface marker expression, respectively. Experiments were performed in triplicate.

5.2.4.2 Flow Cytometry Evaluation of Cell Surface Markers

The expression of cell surface markers CD11c, CD80, and MHC II were assessed after a 48 h incubation period of the DCs with the stimulation treatments. The adherent DCs were harvested from the culture dishes by scraping, placed in polystyrene tubes (BD FALCON™, Franklin Lakes, NJ), centrifuged (250 rcf, 10 min), and resuspended in Fc blocking solution consisting of PBS buffer with 0.1% anti-CD16/CD32, 0.1% unlabeled

hamster IgG, 0.1% sodium azide, and 0.1% bovine serum albumin. After blocking, the DCs were stained and fixed for evaluation of cell surface markers using monoclonal antibodies against CD11c, CD80, CD40, and MHC II. The samples were analyzed using a Becton-Dickinson LSRFortessa SORP Flow Cytometer (San Jose, CA) and FlowJo (TreeStar Inc, Ashland, OR).

5.2.4.3 Cytokine Release

After stimulation for 48 h, cell-free supernatants were assayed using a Bio-Plex Pro Mouse Cytokine Th1 Panel (IL-1β, IL- 2, IL-6, IL-10, IL-12p70, IFN-γ, and TNF-α; Bio-Rad, Hercules, CA) and a BioPlex200® Multiplex System.

5.3 RESULTS AND DISCUSSION

5.3.1 *In Vitro* Cytocompatibility

5.3.1.1 Caco-2 Cytocompatibility

Caco-2 cells were exposed to the microgel starting materials, P(MAA-g-EG) and polyanhydride nanoparticles, as well as the PROMPT composite microgels at various concentrations to determine the maximum concentration that could be tolerated without deleterious effects. The incubation time of four hours corresponds with the average intestinal residence time (10).

The relative cellular proliferation of Caco-2 cells incubated with PROMPT microgel formulations, as compared to cell treated with media alone, ranged from 84%-99% (**Figure 5.1A**). Interestingly, the only statistically significance effects on cellular proliferation were observed for the 10% Poly(SA) and 10% 20:80 CPH:SA microgel formulations, which have a higher content by mass of P(MAA-g-EG) than the 20% formulations. Studies have reported reduced viability in presence of ionizable

methacrylic acid groups, which can simultaneously acidify the pH of the cell culture media and chelate the positively charged calcium ions necessary for cellular function (11, 12). These observations occurred only at 0.25 mg/mL for the 10% Poly(SA) formulations, but across all concentrations for 10% 20:80 CPH:SA. The mild cytotoxicity in 10% 20:80 CPH:SA formulations could be associated with the slightly increased hydrophobicity of the CPH:SA chemistry, which has been correlated with increased cytotoxicity (13). However, it is likely this effect is an artifact of the cell culture environment that would not transfer to the much more dynamic physiological environment of the small intestine, in which concentrations of the microgel formulations in contact with cells would not only be much lower, but also subject to constant liquid replacement, i.e. sink conditions. Nevertheless, a minimum of 80% relative proliferation was observed for all formulations, which has been set as an acceptable threshold to indicate cellular compatibility with biomaterials (12, 14, 15).

Additionally, PROMPT formulations had negligible effects on cell membrane integrity as measured by lactose dehydrogenase (LDH) membrane integrity assay (**Figure 5.1B**). The assay detects release of LDH from disrupted membranes into the culture media by addition of resazurin, which is converted by leached LDH into the detectable fluorescent agent resofurin. Furthermore, P(MAA-g-EG) polymer and polyanhydride nanoparticles introduced at concentrations correlating to those evaluated for intact PROMPT microgels demonstrated no statistically significant effects on either cellular proliferation (**Figure 5.1A,C**) or membrane integrity (**Figure 5.1C,D**). This is a promising result as cells are more likely to encounter the dissociated components of the delivered PROMPT microgels in a physiological setting than the intact particles.

5.3.1.2 RAW 264.7 Cytocompatibility

Similarly, RAW264.7 cells were exposed to composite microgel formulations of concentrations ranging from 0.25 – 1.0 mg/mL, as well as the individual components, linear P(MAA-g-EG) and nanoparticle formulations at their corresponding concentration ranges, but for an extended incubation time of 24 hours (**Figure 5.2**). Incubation time was extended for the macrophage cell line to indicate which concentrations and formulations would be viable for future internalization and stimulation experiments, which typically have incubations times of 24-48 hours (16, 17).

Cellular proliferation relative to cells treated with cell culture media dropped as low as 60% for the highest concentration of PROMPT microgels. This was attributed to the static nature of the cell culture conditions, in which microgels resting on top of the cells can prevent nutrient exchange. However, there were no statistically significant effects at the lower concentrations and no deleterious effects on cell membrane integrity for the extended incubation period.

The concentration-dependent effect on cellular proliferation is not concerning given that macrophage cells are not likely to encounter intact particles, especially at high concentrations, but instead the dissociated components, e.g. polyanhydride nanoparticles or P(MAA-g-EG) polymer chains. Both the nanoparticles and P(MAA-g-EG) polymer, at all tested concentrations, had no negative effects on proliferation of the macrophage cell line. There was, however, a reduction of cell viability as measured by membrane integrity for both nanoparticles formulations in a concentration-dependent fashion (**Figure 5.2D**), though cells still maintained well above the minimum 80% threshold.

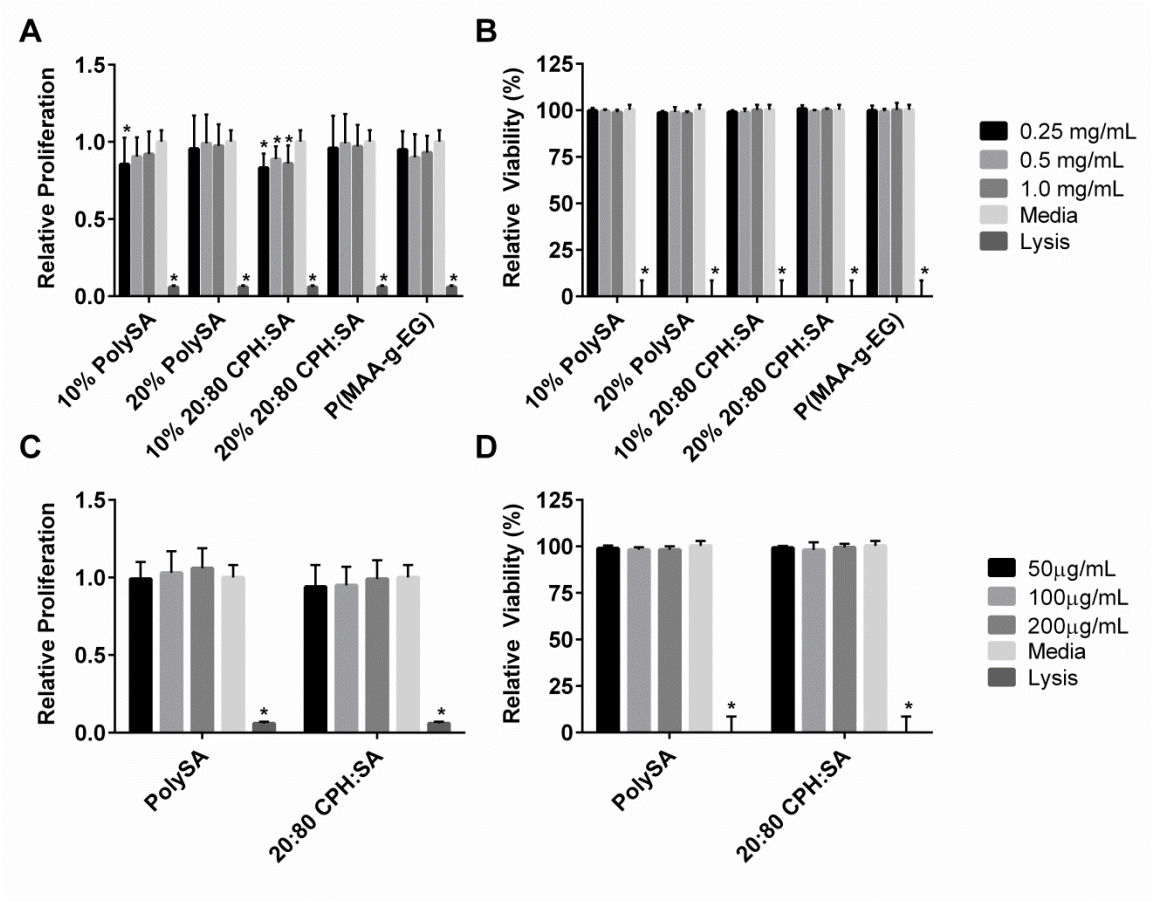

Figure 5.1 Evaluation of *in vitro* cytocompatibility of Caco-2 colon adenocarcinoma cells after 4 hour incubations with PROMPT composite microgels, and the individual components P(MAA-g-EG) and PNP nanoparticles at varying concentrations. Cytocompatibility was determined by relative cellular proliferation, as measured by MTS proliferation assay (A,C), and membrane integrity, as measured by LDH release using Promega CytoTox One™ Homogenous Membrane Integrity Assay (B,D). (n = 15 ± SD; * $p<0.05$)

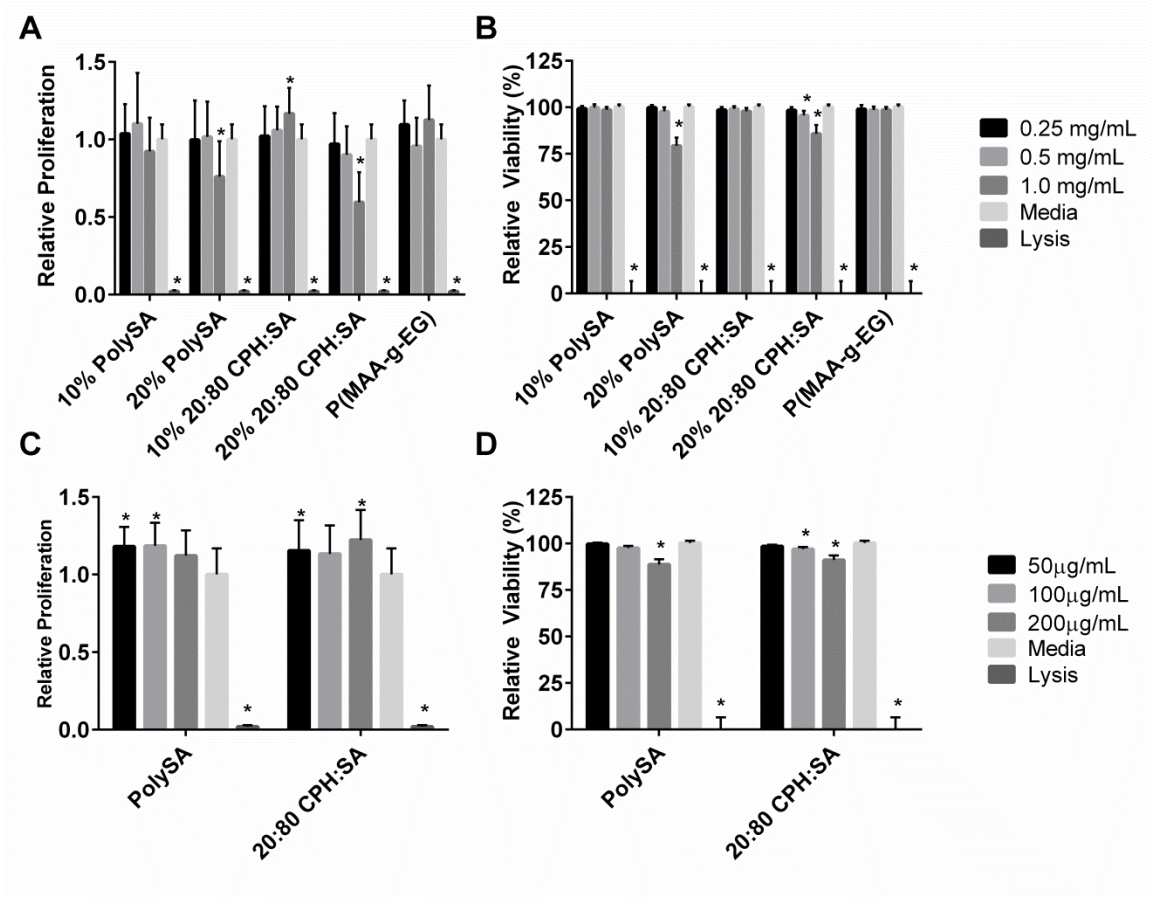

Figure 5.2 Evaluation of *in vitro* Cytocompatibility of RAW264.7 murine macrophages after 24 hour incubations with PROMPT composite microgels, and the individual components P(MAA-g-EG) and PNP nanoparticles at varying concentrations. Cytocompatibility was determined by relative cellular proliferation, as measured by MTS proliferation assay (A,C), and membrane integrity, as measured by LDH release using Promega CytoTox One™ Homogenous Membrane Integrity Assay (B,D). (n = 15 ± SD; * $p<0.05$)

5.3.2 Dendritic Cell Stimulation

To evaluate activation of CD11c+ DCs by PROMPT formulations, flow cytometry was used to measure cell surface expression of co-stimulatory molecules CD80 and MHC II after 48 hours of stimulation. Additionally, supernatants were collected and analyzed for cytokine production. Non-stimulated groups with medium only acted as a negative control and LPS was used as a positive control. Prior to stimulation assays, the BMDC cultures were >80% positive for CD11c. Data are from three independent experiments, performed in triplicate.

5.3.2.1 Cell surface marker expression

Previous studies have demonstrated the chemistry-dependent internalization and activation of DCs by polyanhydride particles (16, 17). To assess immunostimulatory capabilities of composite PROMPT formulations, flow cytometry was used to measure nanoparticle internalization, as well as levels of cell surface expression of MHC II and co-stimulatory molecule CD80.

DCs demonstrated significantly higher nanoparticle internalization after incubation with PROMPT formulations synthesized with a higher feed ratio of nanoparticles, demonstrating efficient dissociation of the microgels to release nanoparticles for uptake even in the static cell culture conditions (**Figure 5.3**). Formulations synthesized with Poly(SA) nanoparticles demonstrated the ability to better promote cell surface marker expression compared to the more hydrophobic 20:80 CPH:SA containing formulations, consistent with previous observations (9, 17). As demonstrated in **Figure 5.4**, only the 20% Poly(SA) formulation significantly increased MHC II expression. However, both 20% Poly(SA) and 20% 20:80 CPH:SA formulations stimulated CD80 expression compared to non-stimulated DCs. Formulations synthesized

with 10% feed ratio of nanoparticles did not induce substantial cell surface marker expression compared to nonstimulated DCs, indicating that both composition and sufficiently high dose of nanoparticles are important considerations for DC stimulation.

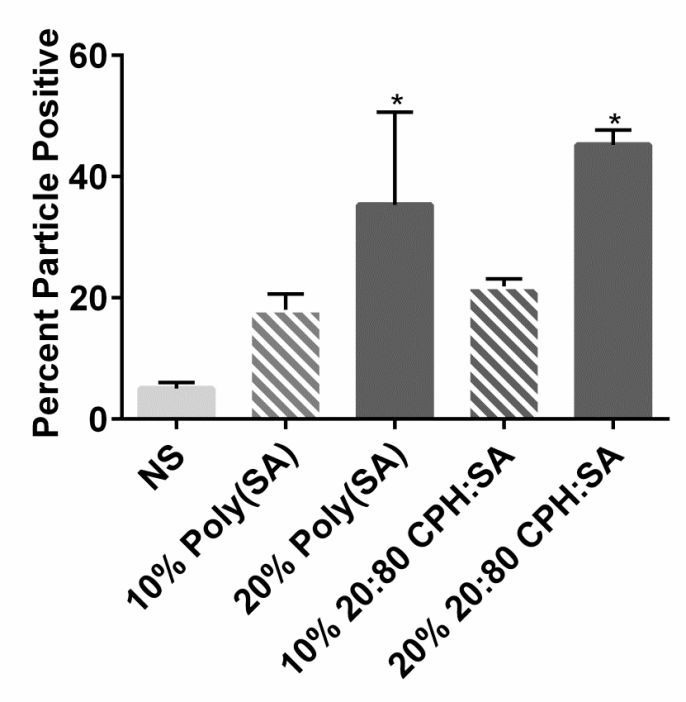

Figure 5.3 Analysis of nanoparticle internalization by bone marrow-derived dendritic cell after stimulation with PROMPT formulations (n = 3 ± SD *p≤0.05)

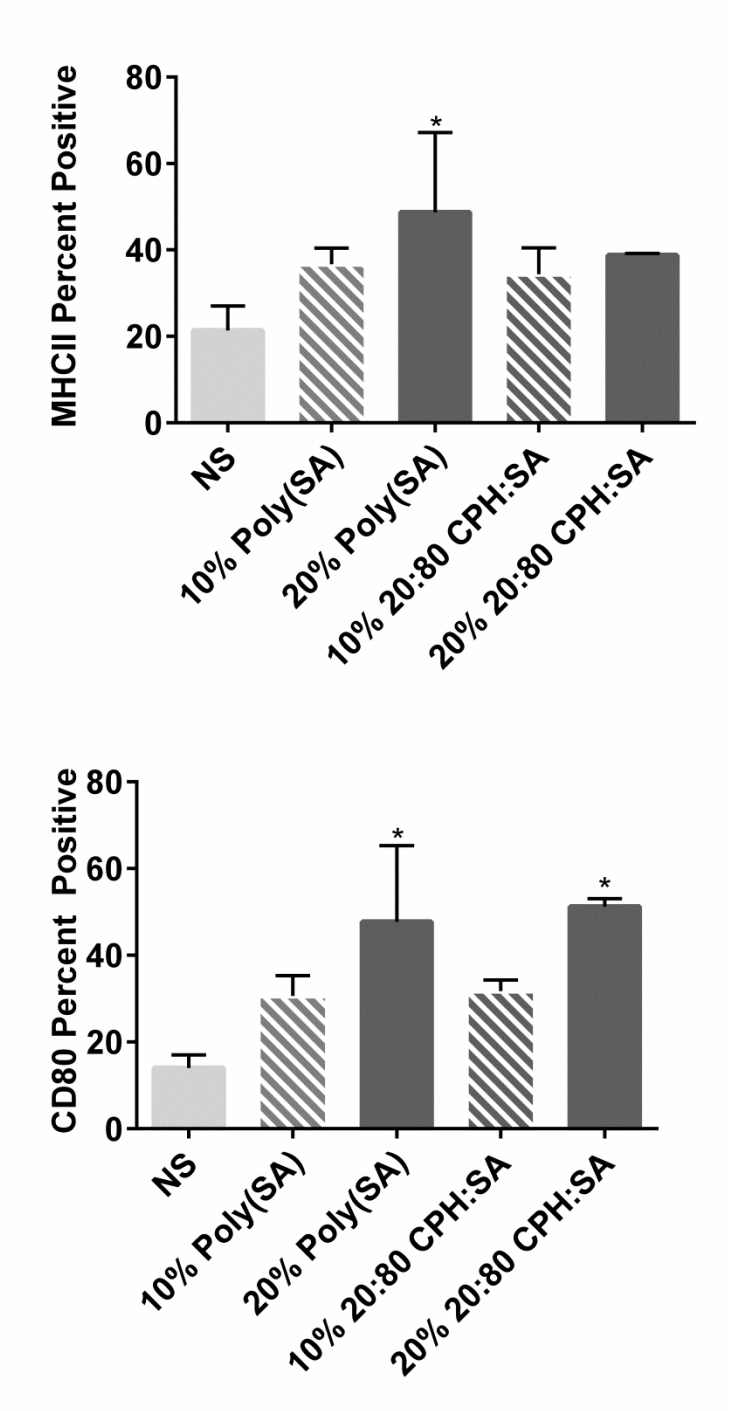

Figure 5.4 Activation profiles of bone marrow-derived dendritic cells after stimulation with PROMPT formulations (n = 3 ± SD *p≤0.05)

5.3.2.2 Cytokine secretion

In addition to expression of surface markers for antigen presentation and activation, secretion of cytokines is essential to enhance $CD4^+$ T cell activation and subsequent promotion of B-cell antibody production (18). Production of IL-2 and TNF-α was significantly higher for formulations synthesized with 10% instead of 20% feed ratio of the nanoparticles (**Figure 5.5**). Both TNF-α and IL-2 are important in early DC maturation and induction of T cell differentiation, and can ultimately influence the bias and magnitude of immune response (19, 20). Microgels synthesized with the lower nanoparticle feed ratio have higher P(MAA-g-EG) content per mass, therefore it is possible that the increase cytokine production induced by 10% microgels as compared to 20% formulations is associated with the higher concentration of polymer, which could increase local acidification and trigger cytokine production in response to the local stress.

All PROMPT formulations except for 20%Poly(SA) stimulated significant IL-6 production, which is important in both innate and adaptive immune response by contributing to systemic inflammation and promoting humoral immune response (21, 22). Additionally, all PROMPT formulations stimulated IL-1β significantly compared to nonstimulated DCs, which also has implications for early stages of immune response to infections towards B-cell maturation and proliferation (19, 20).

Secretion of IL-10, IL-12p70 and IFNγ were not as high as the other cytokine concentration in supernatant (<10pg/mL), but there were still differences between the particle treatments worth discussing (**Figure 5.6**). IL-10, which is associated with inhibition of pro-inflammatory cytokine production and can suppress immune response, demonstrated a response dependent on the relative amount of polymer in the 10% nanoparticle formulations, regardless of their chemistry. IL-12p70 demonstrated a

chemistry-dependent activation, favoring the mort hydrophobic 20:80 CPH:SA based formulations than those with Poly(SA), which has been observed in previous work (9). Lastly, all PROMPT formulations stimulated significant IFN-γ production compared to nonstimulated DCs. IFN-γ has been demonstrated to be important for mucosal defense to both viral and bacterial infections (23, 24).

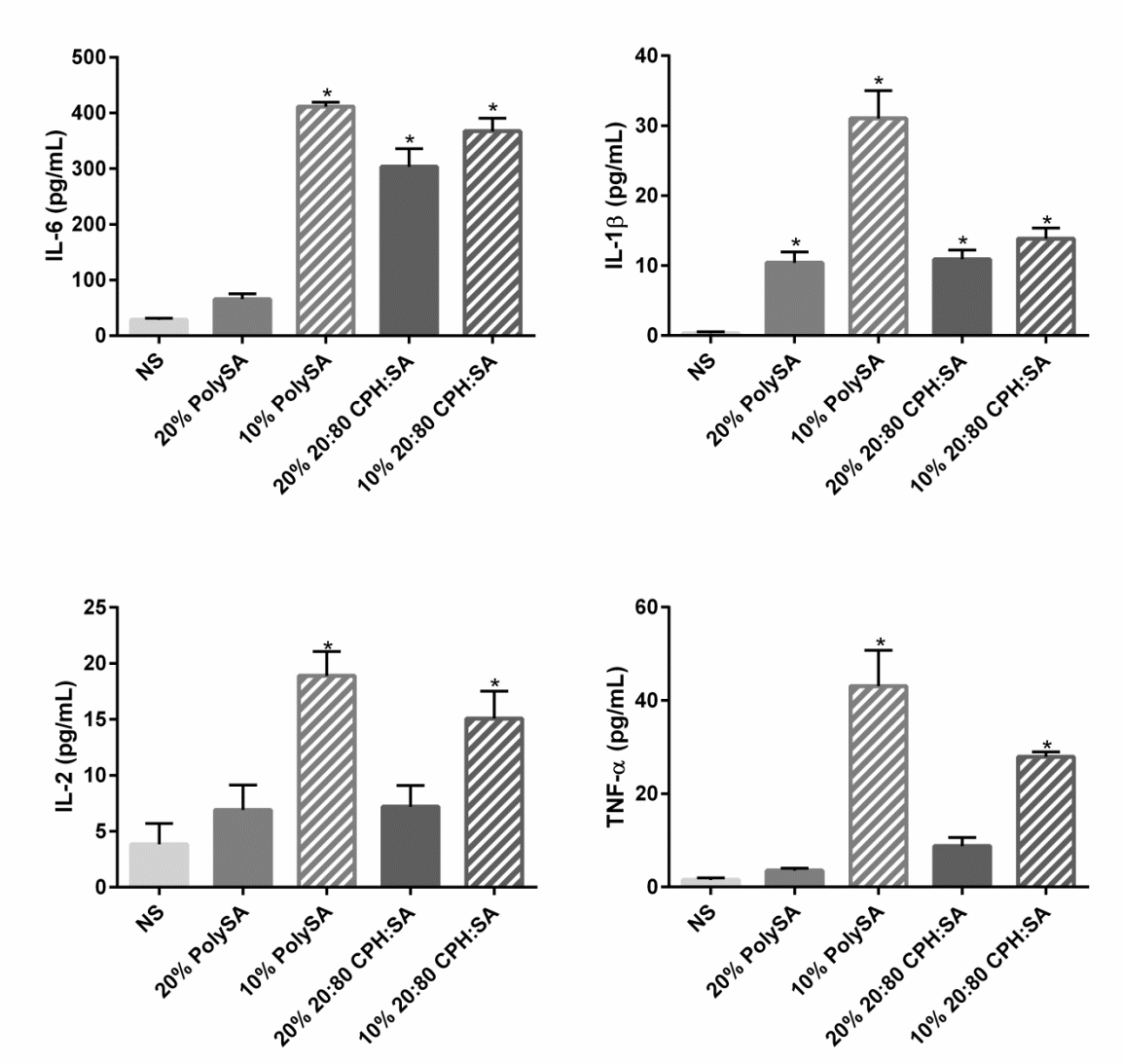

Figure 5.5 Cytokine secretion profiles from bone marrow-derived dendritic cells after stimulation with PROMPT formulations (n = 3 ± S.E.M *p≤0.05)

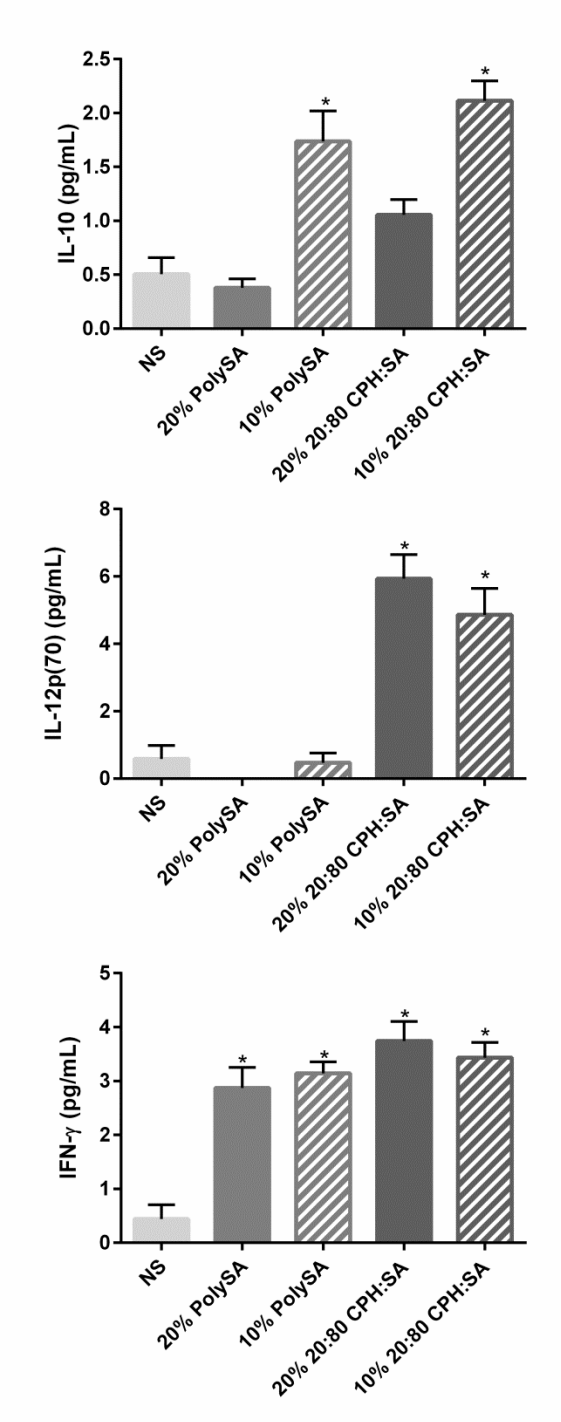

Figure 5.6 Cytokine secretion profiles from bone marrow-derived dendritic cells after stimulation with PROMPT formulations (n = 3 ± S.E.M *p≤0.05)

5.4 CONCLUSIONS

PROMPT composite microgels formulations exhibited minimal-to-no effect on cellular proliferation and membrane integrity at concentrations as high as 1 mg/mL when evaluated with two model cell lines: Caco-2 intestinal epithelial cells and RAW 264.7 macrophages. The cytocompatibility results are encouraging that PROMPT microgels are suitable carriers for oral vaccine applications, and defined tolerable concentrations for subsequent *in vitro* stimulation experiments with BMDCs. PROMPT formulations synthesized with 20% feed ratio of nanoparticles resulted in higher nanoparticle internalization as well as higher cell surface marker expression than those synthesized with 10% feed ratio of nanoparticles. PROMPT formulations were also able to stimulate cytokine production. These studies show that PROMPT composite microgel formulations are biocompatible and have the ability to release nanoparticles for internalization and activation of dendritic cells, which is known to be beneficial for the induction of efficacious immune responses.

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Chapter 6: *In vivo* Evaluation of Microencapsulated Polyanhydride Nanoparticles as an Oral Vaccine Strategy

6.1 INTRODUCTION

Oral vaccination poses numerous advantages over the more commonly used parenteral route of administration. While parenteral vaccination can induce efficient systemic protection, injection is painful, invasive and cannot confer protective mucosal immunity (1). Most diseases for which vaccines are not yet available or elicit incomplete protection are associated with infectious agents that are restricted to mucosal membranes (2, 3). Therefore, it is desirable to generate local mucosal immune response that can impede infection and disease development.

In addition to offering a less intrusive vaccine with higher patient compliance, oral administration mimics the immune response elicited by natural infection to induce both mucosal and systemic immune response. However, antigens administered via the mucosal route are generally poorly immunogenic. Additionally, antigen degradation and poor uptake into lymphoid tissue of the gastrointestinal (GI) tract have hindered development of oral vaccine formulations.

The use of micro- and nano-particulate delivery systems has been explored to address the challenges associated with mucosal immunization (4, 5). Biodegradable polymeric particles have demonstrated promise, serving as antigen delivery vehicles and adjuvants by protecting the antigenic payload through transit, enhancing antigen presentation, and inducing protective immune response compared to soluble protein. Specifically, polyanhydride nanoparticle-based vaccines have shown the successful encapsulation and release of a diversity of antigens, activation of B and T cells, and

ability to induce both antibody and cell-mediated immune responses when administered intranasally (6-10).

Polyanhydride nanovaccines have demonstrated their potential to elicit protective immunity after intranasal administration. However, oral delivery poses additional challenges that could impair translation of the technology. Oral formulations are exposed to the gastric environment before reaching the intestine, where dilution in intestinal fluid can reduce the local antigen concentration and, therefore, increase the possibility of inducing tolerance. Polymeric coating has been investigated as a strategy to achieve oral delivery of nanoparticulate vaccines. Specifically, pH responsive Eudragit® and derivatives have been used to achieve nanoparticle protection and depot release for chitosan (11, 12) and PLGA nanoparticles (13), respectively. The work reported herein used a copolymer of poly(methacrylic acid) (PMAA) with poly(ethylene glycol) (PEG) tethers, henceforth designated as P(MAA-g-EG), to facilitate oral delivery of polyanhydride nanoparticles. P(MAA-g-EG) possesses similar pH responsive behavior to Eudragit® formulations due to the presence of MAA, which enables the targeted intestinal release of the formulations. Inclusion of PEG tethers could provide additional advantages such as promoting mucoadhesion in the GI tract to facilitate nanoparticle release proximal to mucosal tissue for persistence, as well as improve likelihood of sampling by M cells in the small intestine. Additionally, PEG possesses molecular components that have been correlated with "pathogen-like behavior", including backbone oxygen moieties and aliphatic carbon (9).

The present research describes the *in vivo* evaluation of the composite nanoparticle-in-microgel formulation, designated as Polyanhydride-Releasing Oral MicroParticle Technology (PROMPT). A single immunization with formulations

containing the model antigen ovalbumin (ova) was administered. Ova-specific IgG and IgA production were measured as indicators of systemic and mucosal immune response, respectively. Additionally, production of inflammatory cytokines and a histopathological analysis of critical organs for filtration and purification, specifically the liver and kidney, were assessed to provide insight on potentially deleterious effects of PROMPT vaccine formulations.

6.2 Experimental Methods

6.2.1 Materials

All reagents were used as received, unless specified. Chemicals for composite microgel synthesis include methacrylic acid (MAA), 1-hydroxycyclohexyl phenyl ketone (Irgacure® 184), Span® 80, and albumin from chicken egg white were obtained from Sigma-Aldrich (St.Louis, MO), and polyethylene glycol monomethyl ether methacrylate (PEGMMA, ME 1000) was purchased from Polysciences Inc. (Warrington, PA). Goat serum, trypsin inhibitor type II, phenylmethylsulfonyl fluoride (PMSF), pefabloc SC, ethylenediaminetetraacetic acid (EDTA), bovine serum albumin (BSA) p-nitrophenyl phosphate substrate, diethanolamine substrate buffer and gelatin from cold water fish skin were obtained from Sigma Aldrich (St. Louis, MO). All other solvents and buffers were also obtained from Thermo Fisher Scientific (Waltham, MA). Polyanhydride polymers were synthesized by collaborators at Iowa State University.

6.2.2 Animals

All animal experiments were conducted as approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Texas at Austin. Female Balb/c mice, ages 6-8 weeks old, were obtained from The Jackson Laboratory (Bar Harbor,

ME). Mice were housed in specific pathogen-free conditions, where all bedding, caging and feed were sterilized prior to use. All mice underwent a 48-72 hour adjustment period after arrival before the enrollment in any experimental protocol.

6.2.3 Synthesis

6.2.3.1 Polyanhydride Nanoparticle Synthesis

Polyanhydride particles were synthesized by anti-solvent nanoencapsulation as described previously (14, 15). Prior to encapsulation, endotoxin was removed from ova using Pierce™ High Capacity Endotoxin Removal Spin Columns and quantified by Pierce™ LAL Chromogenic Endotoxin Quantitation Kit (Thermo Fisher Scientific, Waltham, MA). Protein was considered to be "endotoxin-free" below 0.01 ng/mL and encapsulated into nanoparticles for immunization. Briefly, either 20:80 CPH:SA or Poly(SA) (20mg/mL) were dissolved in methylene chloride with ova (5 wt%) and Span80 ® (1% v/v). The polymer solution was sonicated at 50 Hz for 30 seconds using a probe sonicator (Fisher Scientific Model 50 Sonic Dismembrator), then rapidly poured into a pentane bath at a solvent to non-solvent ratio of 1:250. Particles were collected by vacuum filtration (Whatman 50, Fisher Scientific), and characterized using scanning electron microscopy (SEM, Zeiss Supra40 Scanning Electron Microscope, Oberkochen, Germany).

6.2.3.2 PROMPT Composite Microgel Synthesis

Antisolvent precipitation was used to produce particle dispersions of polyanhydride nanoparticles stabilized by P(MAA-g-EG). P(MAA-g-EG) dissolved in deionized water (pH 6) at 20 mg/mL. Polyanhydride nanoparticles were added at 20 wt% relative to P(MAA-g-EG), and sonicated at 50 Hz for 30 seconds to achieve a dispersed

solution. The anti-solvent 0.1N HCl was used at a volumetric ratio of 1:100. Upon addition of solvent into the antisolvent phase, particles rapidly form and begin to flocculate.

Flocs were collected by centrifugation (4500 rcf x 5 min), then freeze-dried. The final particles were obtained by crushing the particle pellet with a mortar and pestle to achieve a fine powder. PROMPT formulations are denoted in this work as either PROMPT 20:80 CPH:SA or PROMPT Poly(SA) based on the nanoparticle cargo.

6.2.4 Mouse treatment

6.2.4.1 Oral administration of particle formulations

Female Balb/c mice, ages 6-8 weeks old, were immunized by oral gavage after a two hour fasting period. The oral gavage procedure was performed using a curved, stainless steel, 1.5", 22-guage needle with a 2.4 mm ball tip (Squirrel Store, Alabaster, AL), with a maximum dosing volume of 10 mL/kg, or 200 μL per mouse. Prior to administration, the gavage needle was dipped into a 1 g/mL sucrose solution to minimize rodent stress during the procedure (16). After gavage, the mice were observed for 5-10 minutes for any symptoms of respiratory distress. Treatment groups consisted of 6 mice dosed with ova encapsulated into 5 mg PROMPT formulations, 1mg nanoparticles, or as a soluble dose suspended in 10X PBS. Control mice received PBS alone.

6.2.4.2 Saphenous Vein Blood Collection

Blood samples to evaluate the cytokine secretion and serum IgG were collected prior to immunization, at 24 and 72 hours post-immunization, and then weekly thereafter for the duration of the study in order to evaluate cytokine secretion and serum IgG. Blood was collected from the saphenous vein by gently restraining the animals and

immobilizing the hind leg, which was shaved to expose the saphenous vein. A sterile 23-gauge needle was used to puncture the saphenous vein and the blood was collected using heparin coated capillary tubes. Collected blood was allowed to clot in low adhesion microcentrifuge tubes on ice for one hour. Serum was then collected by centrifuging samples at 10,000 x g for 10 minutes. The collected serum removed and stored at -80^{o}C until analysis.

6.2.4.3 Fecal Pellet Collection

Feces from each mouse were collected at weeks 4 and 8. Seven fecal pellets were collected per mouse and dried overnight. Pellets were emulsified in 600 µl of ice-cold PBS containing 0.2 mg/mL trypsin inhibitor, 1% (w/v) bovine serum albumin, 25 mM EDTA and 0.035mg/mL Pefabloc in PBS and 50% glycerol at 4^{o}C overnight. Debris was removed by centrifugation at 15,000 g for 10 minutes and supernatant collected and stored at -20^{o}C.

6.2.4.4 Intestinal Lavage

Intestinal fluids were collected by a method adapted from Lycke, et al (17). The small intestine from the stomach to the cecum was removed and clamped on one end. The intestines were carefully injected with 3mL of a protease inhibitor solution consisting of 0.1 mg/mL trypsin inhibitor, 50mM EDTA and 1mM PMSF in PBS, before clamping the other end of the intestine and incubating for 10 minutes at room temperature. The intestinal content was transferred to a test tube, vigorously vortexed, and then centrifuged for 10 min at 2000 rcf. The supernatant was transferred to microcentrifuge tubes and sodium azide was added to a total concentration of 0.001%. The mixture was incubated

for 15 minutes before addition of 50 µL goat serum per mL of solution, and then stored at -80^{o}C until analysis.

6.2.5 ELISA for Ova-specific IgG Antibody Titer

High-binding 96-well plates (Costar 3590) were coated with 5µg/mL ova overnight at 4^{o}C. Plates were washed with phosphate buffered saline containing 0.5% Tween 20 at a pH of 7.4 (PBST). Plates were blocked with 2% gelatin for two hours at room temperature and then washed again three times with PBST. Serial dilutions of serum samples in PBST with 1% heat inactivated normal goat serum were incubated overnight at 4^{o}C. Plates were washed four times prior to addition of alkaline phosphatase-conjugated AffiniPure goat anti-mouse IgG (H&L), (Jackson ImmunoResearch Laboratories, West Grove, PA) diluted 1:1000 in PBST with 1% heat inactivated normal goat serum for two hours at room temperature. Plates were washed four times with PBST and 100 µL p-nitrophenyl phosphate substrate (1 mg/mL) in diethanolamine substrate buffer was added to each well. Changes in optical density were measured at 405 nm using a Biotek Cytation 3 Cell Imaging Multi-Mode Reader (Winoosky, VT, USA).

6.2.6 Mucosal Antibody (IgA) Determination

High-binding 96-well plates (Costar 3590) were coated with 5 µg/mL ova overnight at 4^{o}C. Plates were washed with PBST, blocked with 2% gelatin for two hours at room temperature, and then washed again three times with PBST. Serial dilutions of either fecal pellet or intestinal lavage samples diluted in PBST were incubated overnight at 4^{o}C. Plates were washed four times prior to a two hour incubation with biotin rat anti-mouse IgA diluted 1:2000 (Clone C10-1, BD Biosciences, San Jose, CA). Plates were washed and streptavidin-HRP diluted 1:1000 (BD Biosciences) incubated for two hours.

Plates were washed and color development achieved by addition of TMB substrate (BD Biosciences). Changes in optical density were measured at 405 nm using a Biotek Cytation 3 Cell Imaging Multi-Mode Reader (Winoosky, VT, USA).

6.2.7 Assessment of Cytokine Levels

Blood serum samples obtained 24 and 72 hours post-immunization were compared to baseline using a Bio-Plex Pro Mouse Cytokine Panel A 6-Plex assay (IL-1β, IL-6, IL-10, IL-17A IFN-γ, and TNF-α), per manufacturer's instructions, and data acquired using a BioPlex200® Multiplex System (Bio-Rad, Hercules, CA).

6.2.8 Histopathology

Kidneys and liver were harvested and fixed in formalin. The tissues were sent to the University of Texas Health Science Center San Antonio (UTHSCSA) for paraffin embedding, sectioning and H&E staining. A blind histopathology analysis of the tissues was performed to assess both inflammatory markers and indications of organ failure, including tissue necrosis and distribution of inflammatory cells.

6.2.9 Statistical Analysis

Statistical analysis was used to analyze the cell surface marker expression and cytokine secretion data. Two-way ANOVA and Dunnett's test were used to determine statistical significance among treatments and p-values <0.05 were considered significant.

6.3 Results and Discussion

6.3.1 Ova-Specific Serum Antibody (IgG) Titers

One of the primary benefits of vaccines administered via a mucosal route is the potential to elicit systemic immune protection in addition to the local mucosal response. **Figure 6.1** shows the serum titers after oral immunization of mice by a single dose of ova

loaded into either nanoparticles alone (20:80 CPH:SA or Poly(SA)), or nanoparticles loaded at 20% feed ratio into composite PROMPT microgels (PROMPT 20:80 CPH:SA or PROMPT Poly(SA)). Soluble ova and PBS were included as controls. Anti-ova IgG response peaked at six weeks for PROMPT Poly(SA) composite microgels, in which serum IgG response was significantly higher than all other formulations. All other formulations, including soluble ova, failed to elicit ova-specific IgG titers above the baseline, as measured in mice treated with PBS only.

Previous studies have demonstrated Poly(SA) chemistries are more rapidly internalized than more hydrophobic chemistries (18), which could be particularly beneficial in the narrow intestinal residence time. Additionally, it has been suggested that while hydrophobic chemistries can increase the association of nanoparticles with the mucosal barrier, this association could ultimately impede the necessary mucus penetration required for subsequent cellular uptake (19). The fact that PROMPT Poly(SA) induced a systemic immune response, while Poly(SA) nanoparticles did not, indicates that depot delivery achieved by microencapsulation can improve the response.

It should be noted that while the theoretical loading of all formulations was matched at 50 μg/mL and encapsulated into either 1 mg of nanoparticles or 5mg of microgels, the actual dosage of encapsulated ovalbumin depended on the encapsulation efficiency of the microgels and was, consequently, lower (see Chapter 4). Therefore, mice that received PROMPT Poly(SA) formulations actually received a dose of only approximately 13 μg of ova, instead of the 50 μg or 42 μg administered via soluble or nanoparticle-encapsulated doses, respectively. The difference in antigen ultimately delivered in PROMPT Poly(SA) as compared to Poly(SA) nanoparticles or soluble ova underscore the importance of a suitable oral delivery vehicle for effective immunization

using polyanhydride nanoparticles, as well as the potential dose-sparing capability of the composite materials.

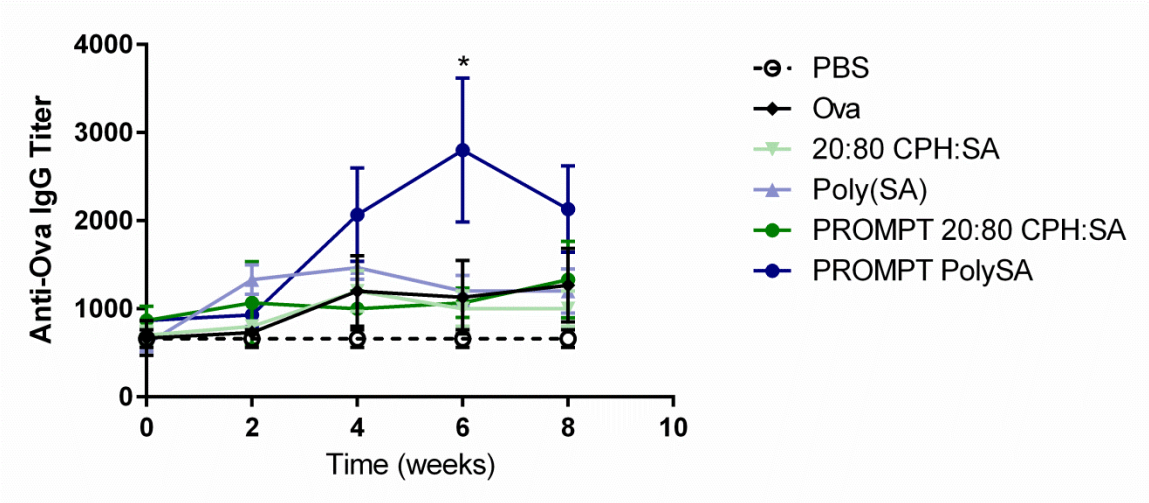

Figure 6.1 Ova-specific IgG in serum after single immunization of BALB/c mice (n=6 ±SEM; *p≤0.05. Immunization was performed at day 0 by oral gavage of formulations containing 50 μg ova. Dashed lines indicate baseline levels detected by ova-specific ELISA in mice treated with PBS.

6.3.2 Ova-Specific Intestinal Antibody (IgA) Titers

The potential of PROMPT formulations to induce local mucosal immune response was evaluated in fecal pellet extractions obtained at weeks four and eight, as well as from intestinal fluid harvested at week eight (**Figure 6.2**). As observed for serum IgG analysis, only PROMPT Poly(SA) formulations induced statistically significant ova-specific IgA titers. PROMPT 20:80 CPH:SA also elicited detectable IgA levels at weeks four and either, while Poly(SA) nanoparticles alone elicited detectable IgA at week four which

dissipated by week eight. These results suggest that simple entrapment of antigen in nanoparticles was not sufficient for localized immune response.

It is likely that the local dose of either soluble ova or nanoparticles alone in the intestine were two low to induce an immune response, which can lead to induction of tolerance rather than protection in the gastrointestinal mucosa (20, 21). PROMPT microgels, conversely, appear to facilitate mucosal antibody production. It is likely that microgel delivery enables a bolus dose of nanoparticles upon transit to the small intestine, which achieves a dose in the appropriate range for protective response. Additionally, the presence of PEG tethers in the P(MAA-g-EG) coating could provide advantages to facilitate nanoparticle interaction with GI mucosal surfaces, increasing both residence time and local proximity (22, 23).

However, encapsulation of the nanoparticles in P(MAA-g-EG) alone is not enough to elicit the desired response. While the PROMPT 20:80 CPH:SA formulation provided small and sustained IgA production, it did not achieve the significant immune response observed in PROMPT Poly(SA). Therefore, the chemistry of the nanoparticles themselves plays a key role in the ultimate effectiveness of the formulation. The Poly(SA) chemistry, compared to the 20:80 CPH:SA, seems to be advantageous in generating a localized immune response. Studies on copolymers of poly(SA) and PEG have demonstrate ability to rapidly transport in mucus, which could be impaired by the presence of the hydrophobic CPH moieties (24).

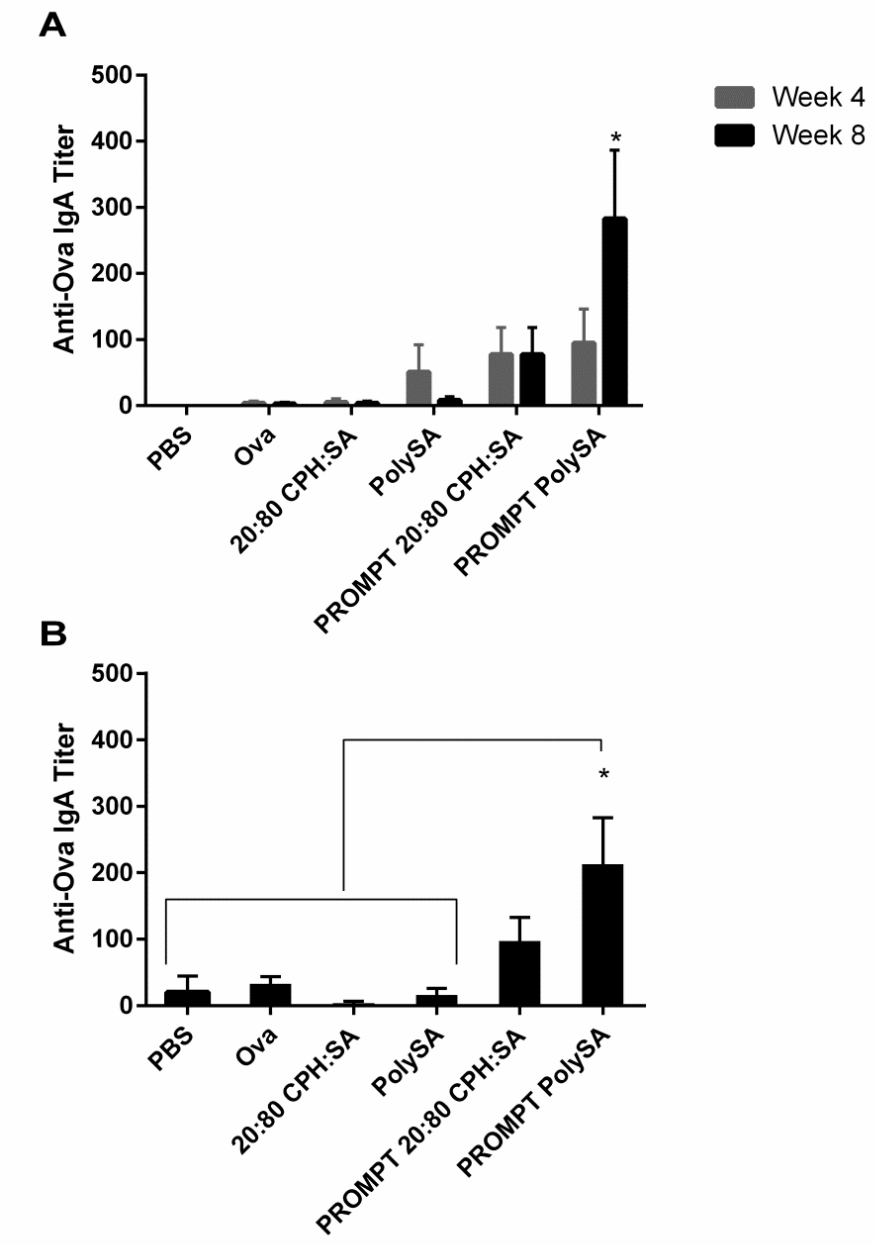

Figure 6.2 Ova-specific IgA in (A) fecal pellet extract and (B) intestinal fluid after single immunization of BALB/c mice (n=6 ± SEM;*$p<0.05$). Immunization was performed at day 0 by oral gavage of formulations containing 50 μg ova. Dashed lines indicate baseline levels detected by ova-specific ELISA in mice treated with PBS.

6.3.3 Cytokine Release

Development of vaccines that enhance immunogenicity while minimizing reactogenicity is an important consideration for development of adjuvants and delivery systems. Cytokines comprise a family of proteins that are critical in cellular signaling and regulation of host immune response to infection, inflammation and trauma (25, 26). Evaluating serum cytokines can provide valuable information about local and systemic inflammatory environment induced by vaccine adjuvant systems. Additionally, cytokine microenvironments play a large role in shaping the antigen-specific adaptive immune response, directing Th1 or Th2 bias (27). Cytokine production occurs within hours of stimulus and concentration quickly dissipates due to the short-circulating *in vivo* half-live (26).

In order to evaluate the inflammatory response to administration of PROMPT formulations, blood serum concentration of pro-inflammatory cytokines (IL-1β, IL-6, TNF-α), anti-inflammatory cytokine (IL-10) and additional cytokines (IL-6, IL-17) were measured at 24 hours (**Figure 6.3**) and 72 hours (**Figure 6.4**) post-immunization. These time points were selected to capture information on systemic cytokine levels within the window of response time to the stimulus – immunization – and in which levels should return to baseline. Cytokine primary source, target and biological role are summarized in **Table 6.1**.

Average cytokine concentration was slightly increased at 24 hours, as compared to 72 hours for IL-1β, IL-6, IL-10, and TNF-α, across all formulations and controls. This minor but universal increase in cytokines can be attributed to the stress of the oral gavage procedure itself. Additionally, cytokine concentration was not significantly different for any animals receiving formulations or control compared to baseline (t=0) pooled

measurements. Serum levels of IL-17A and IFN-γ were mostly below the detectable range. These results indicate that the PROMPT microgels do not possess significant immunomodulatory functions, nor do they cause immunotoxicity and are therefore safe oral delivery vehicles.

Table 6.1. List of evaluated cytokines, their principal production source, primary activity, and broad grouping. Adapted from Zhang, et al. (28) Abbreviations: IL – interleukin, TNF – tumor necrosis factor, IFN –interferon, Th –helper T cells, NK– natural killer, MHC – major histology complex, DC – dendritic cell, APC – antigen presenting cell

Cytokine	**Source**	**Target**	**Biological Role**
IL-1β	Macrophages, monocytes, DCs	Th, NK, and B cells	Pro-inflammatory; Th cell stimulation, NK cell activation, B cell maturation and proliferation
IL-6	Activated Th2 Cells, APCs,	Plasma cells, B cells	Pro-inflammatory and anti-inflammatory; Differentiation and antibody secretion
IL-17A	Th17 cells	Monocytes, neutrophils	Pro-inflammatory; induce cytokine production by epithelia, endothelia, fibroblasts
IL-10	Th2 cells, CD8+ T and B cells, macrophages	Macrophages, APC	Anti-inflammatory; inhibits cytokine production, promotes B cell proliferation and antibody production, suppresses cellular immunity
IFNγ	Activated Th1, NK	Macrophages and other cells	Inhibitor of viral replication, cell proliferation and IL-5 induced isotype switching
TNFα	Macrophages	Tumor cells, macrophages	Cytotoxicity, induction of cytokine secretion; inflammation and pain

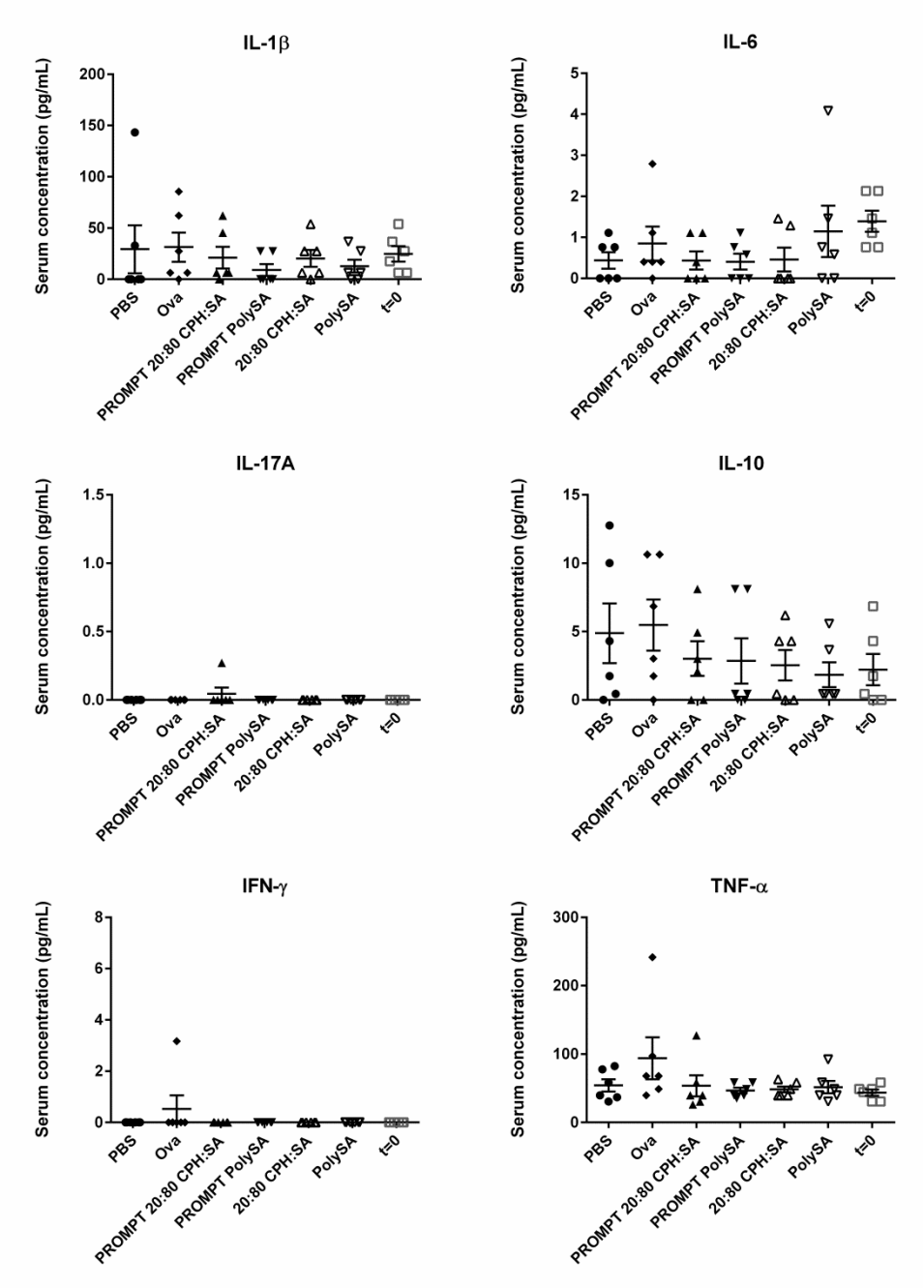

Figure 6.3 Cytokine expression levels at time 0 and 24 hours after oral administration of vaccine formulations and controls. T=0 blood samples were pooled. (n=6)

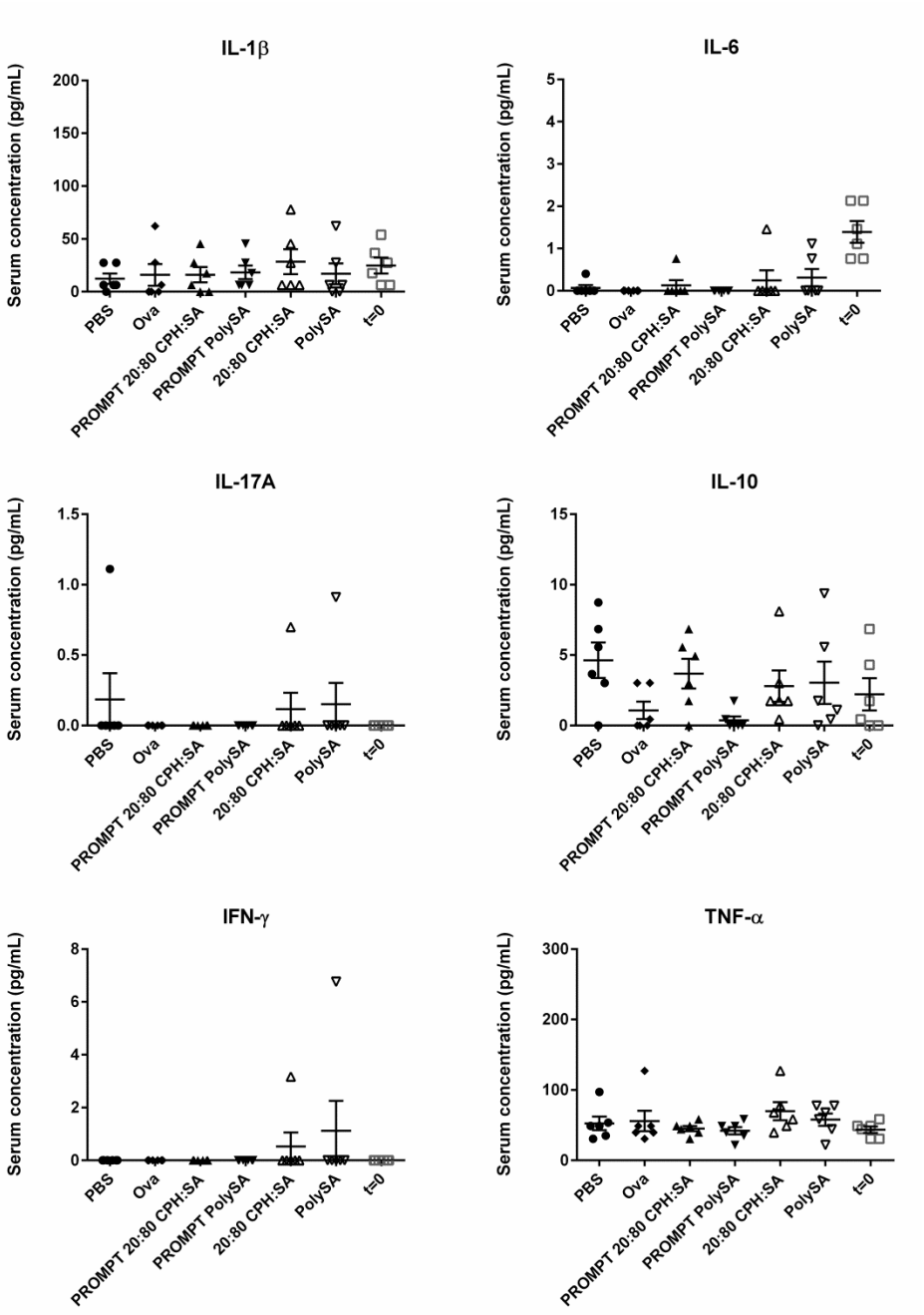

Figure 6.4 Cytokine expression levels at time 0 and 72 hours after oral administration of vaccine formulations and controls. T=0 blood samples were pooled. (n=6)

6.3.4 Histopathology

Polyanhydride nanoparticles have been demonstrated to persist *in vivo* following subcutaneous or intranasal administration, and depending on the route of administration, can distribute away from the administration site (29, 30). While increased biodistribution could be advantageous for more robust immune protection, accumulation of particles in the liver or kidney could prove detrimental to normal physiological processes. Liver and kidney tissues were examined for evidence of necrosis, cellular alterations, or inflammation. Across all treatment groups, there was no evidence of acute inflammation. Rare tubular casts and cytoplasmic eosinophilia were present in all kidney samples, though any morphological changes were not associated with parenchymal changes to tubular epithelial cells and glomeruli. Similarly, there was evidence of rare and subtle changes in the liver tissues characterized by the presence of random small collections of lymphoid cells in the parenchyma and portal areas of the liver. However, these changes were attributed to normal aging as opposed to pathological findings (31). Overall, the histological evaluation of mice administered the various particle formulations revealed normal histological features similar to those observed in saline controls.

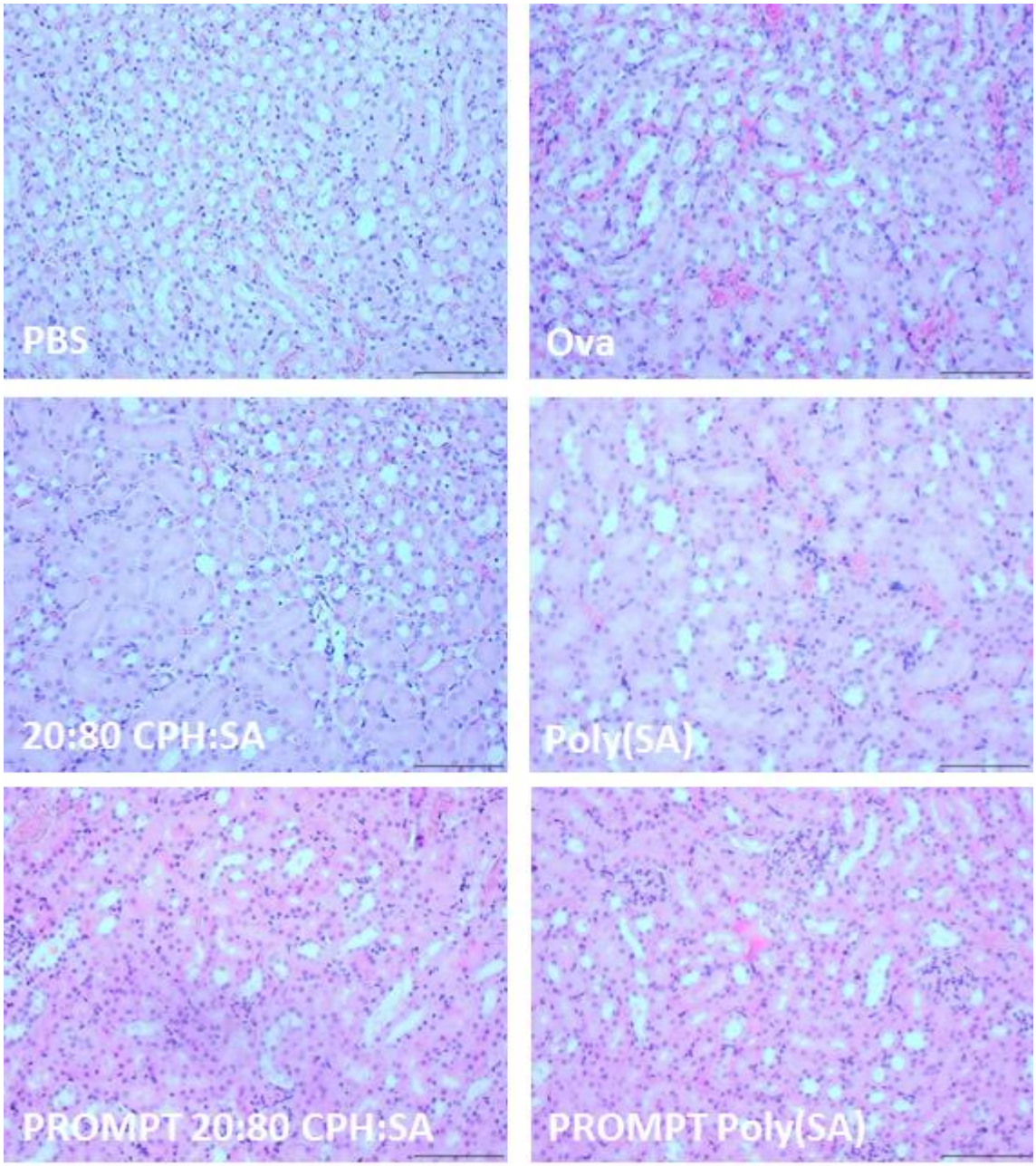

Figure 6.5 Administration of PROMPT formulations did not induce any significant histological alterations in kidneys. All samples were evaluated for evidence of necrosis, cellular alterations, inflammation, and location and distribution of histological changes. Scale bar 10μm.

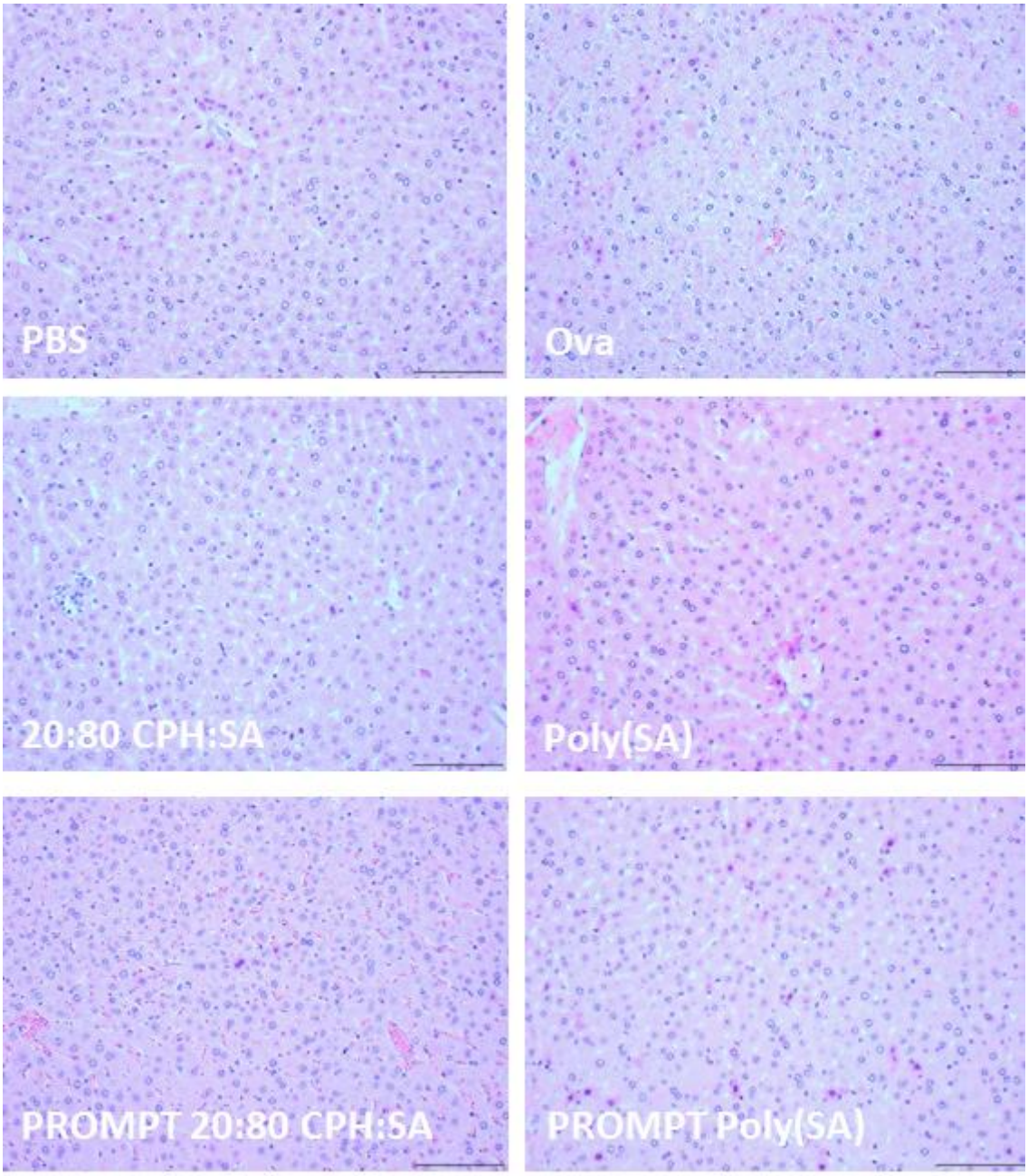

Figure 6.6 Administration of PROMPT formulations did not induce any significant histological alterations in the liver. All samples were evaluated for evidence of necrosis, cellular alterations, inflammation, and location and distribution of histological changes. Scale bar 10μm.

6.4 CONCLUSIONS

The potential of PROMPT composite microgels to serve as oral vaccine carriers was evaluated after a single immunization with the model antigen ova. PROMPT Poly(SA) particles were the only formulation to induce systemic ova-specific IgG production. More promising, both PROMPT formulations induced measurable mucosal ova-specific IgA in both fecal matter and intestinal washes, with PROMPT Poly(SA) eliciting significantly higher IgA production compared to soluble ova, nanoparticles alone, or PROMPT 20:80 CPH:SA. The trend of IgA production observed after administration of PROMPT formulations support the benefits of microgel encapsulation of nanoparticles for effective local mucosal protection. Furthermore, formulations containing Poly(SA) demonstrate more promise than 20:80 CPH:SA formulations for IgA production.

Cytokine levels measured after formulation administration showed no significant changes in expression to indicate inflammatory response, indicating the potential for these formulations to induce protective immune response without systemic reactogenicity. Additionally, histopathological evaluation of kidney and liver demonstrated no evidence of inflammation. Overall, these studies indicate that PROMPT microgel formulations warrant further study towards development of an adaptable oral vaccine platform with subunit antigen.

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Chapter 7: Conclusions and Recommendations for Future Research

7.1 Conclusions

Oral vaccination represents the most attractive mode of administration due to the fact that oral delivery is noninvasive and safe, improving both patient compliance and clinical practicality. Oral delivery has also been demonstrated to enhance the mucosal immune response for improved local protection against pathogens that invade via the gut (e.g. enteropathogens). However, there are relatively few vaccines available via the oral route, and no oral vaccine formulations based on subunit vaccines. The challenges to development of oral formulations are attributed to i) the obstacles for a vaccine, particularly a protein or peptide, in the harsh environment of the gastrointestinal (GI) tract, including acidic pH and digestive enzymes; and ii) crossing the tightly regulated epithelial layer to enter the gut-associated lymphoid tissue where the mucosal immune responses may be induced.

Development of polymeric nanoparticles for subunit vaccine delivery is a promising strategy. Nanoparticles afford protection of the antigen from degradation or clearance, can increase cellular uptake by inclusion of pathogen-mimicking characteristics (e.g. size, shape), and can be targeted to antigen presenting cells. However, formulating technology that enables oral vaccination in the gut remains a substantial challenge. Nanoparticle technologies still have limitations such as poor aqueous solubility, burst release, and insufficient protection of the encapsulated antigen. One strategy to improve the efficacy of nanoparticle-based vaccines is adding a polymeric coating, which can improve nanoparticle stability in contact with physiological fluids in the gastrointestinal tract and minimize premature release of the antigen (1-5).

The goal of this work was to develop, synthesize and characterize a composite approach to achieve oral delivery of nanoparticle-based vaccines. Specifically, polyanhydride nanoparticles based on poly(sebacic acid) (SA) and 1,6-bis(*p*-carboxyphenoxy)hexane (CPH) have been developed by Professor Balaji Narasimhan and his research team at Iowa State University for use as vaccine platforms via subcutaneous or intranasal administration (6-10). These nanoparticles have demonstrated the ability to stabilize encapsulated antigens, sustain protein release, and stimulate humoral and cellular response. The Peppas lab has developed a class of pH-responsive hydrogels to protect sensitive therapeutics for oral delivery (11, 12). However, these networks are limited to delivery of low molecular weight macromolecules due to limitations in the mesh size.

Inspired by the use of crosslinked hydrogel networks for pH-sensitive protein delivery, polyanionic microgels were synthesized using copolymers of methacrylic acid (MAA) and poly(ethylene glycol) monomethyl ether monomethacrylate (PEGMMA), denoted as P(MAA-g-EG). Microencapsulation of the nanoparticles was done via pH-triggered self-assembly. In contrast to crosslinked networks, the self-assembly process enabled facile inclusion of nanoparticles without subsequent purification steps and overcame the limitations of release associated with crosslinked networks. The composite particles are called Polyanhydride-Releasing Oral MicroParticle Technology, or PROMPT.

Several formulations were synthesized by varying the feed ratios of the polyanhydride nanoparticles and exploring two different polyanhydride compositions: Poly(SA) and 20:80 CPH:SA. The composition and molecular weight of P(MAA-g-EG) were verified by ^{13}C NMR and GPC, respectively, prior to PROMPT synthesis. SEM

micrographs demonstrated the self-assembly process produced a polydisperse population of microgels with nanoparticles adsorbed to the surface, while confocal microscopy confirmed nanoparticle inclusion throughout the microgel. FT-IR further confirmed the inclusion of complexed P(MAA-g-EG) and nanoparticles into the PROMPT microparticles. The feed ratio of nanoparticles had a significant effect on the inclusion of the more hydrophobic 20:80 CPH:SA nanoparticles, but not Poly(SA). Both fluorescence and brightfield microscopy verified pH-dependent dissociation occurred rapidly upon transition from acidic to neutral pH environments.

The suitability of PROMPT formulations for oral administration was assessed in release studies using the model antigen, ovalbumin. Poly(SA) nanoparticles had higher encapsulation efficiency than 20:80 CPH:SA, as well as slightly higher burst release, though both compositions demonstrated an initial burst of 60-70% and then sustained antigen delivery thereafter. All of the PROMPT formulations demonstrated reduced burst release compared to the nanoparticles alone, resulting in less than 50% cumulative mass fraction released. These results demonstrated that protection by P(MAA-g-EG) microencapsulation has the potential to slow the solvent diffusion to nanoparticles, thereby mitigating the degree of burst release and enabling a more sustained delivery of antigen. Formulations synthesized with a 20% feed ratio of nanoparticles exhibited lower fractional burst release compared to those synthesized with 10% feed ratio, attributed to the higher P(MAA-g-EG) content per mass facilitating more rapid dissociation of the microgels.

In dynamic experiments mimicking gastrointestinal conditions, PROMPT formulations released negligible ovalbumin in gastric conditions. Upon transition to intestinal conditions, PROMPT formulations exhibited sustained release kinetics after the

initial burst of protein. Microgels synthesized with 20% feed ratio of nanoparticles released more protein than the 10% microgels. Additionally, 20:80 CPH:SA containing formulations released more ova than Poly(SA) microgels at the same feed ratio. Therefore, the increased incorporation efficiency for 20:80 CPH:SA into microgels offset both the lower encapsulation efficiency of ova into the nanoparticles and the slower release kinetics of the more hydrophobic 20:80 CPH:SA chemistry.

Upon confirming that PROMPT formulations could release the nanoparticles and antigen in pH-dependent manner, their biocompatibility was studied *in vitro* using two representative cell lines: Caco-2 intestinal epithelial cells and RAW 264.7 macrophages. There was minimal effect on cellular proliferation or membrane integrity at concentrations up to 1mg/mL during the incubation with Caco-2 cells. Concentration-dependent perturbation of cellular proliferation was observed when incubated with RAW 264.7 macrophages for 24 hours, though these effects were attributed to the static nature of the cell culture conditions affecting nutrient exchange. However, there were no deleterious effects on cellular proliferation at lower concentrations, and no impact on membrane integrity for the extended incubation period.

The capability of PROMPT formulations to stimulate antigen presenting cells was evaluated with bone marrow-derived dendritic cells. PROMPT formulations synthesized with 20% feed ratio of nanoparticles result in higher nanoparticle internalization as well as higher cell surface marker expression than those synthesized with 10% feed ratio of nanoparticles. Immunostimulatory capability was further confirmed by cytokine secretion, although no clear trends emerged regarding the nanoparticle composition or feed ratio into PROMPT microparticles.

An *in vivo* assessment of PROMPT formulations was performed to determine the ability to elicit systemic and mucosal immune response after oral administration. The 20% PROMPT formulations were selected due to the higher total antigen loading and lower burst release. Only the PROMPT Poly(SA) formulation induced statistically significant titers for both serum IgG and mucosal IgA, while both PROMPT formulations induced measurable IgA titers compared to the controls of soluble ova or nanoparticles alone. These results are particularly promising given that while the theoretical loading of all formulations was matched at 50 μg, the actual dosage of ovalbumin encapsulated depended on the encapsulation efficiency of the microgels and was, consequently, much lower. Therefore, the capability of PROMPT formulations to induce antibody production at a lower dose of encapsulated antigen than the nanoparticles underscore the importance of a suitable oral delivery vehicle for effective immunization using polyanhydride nanoparticles, as well as the potential dose-sparing capability of the composite materials. Furthermore, both analysis of cytokine profile and histopathological analysis of liver and kidneys indicate PROMPT formulations did not induce any damage or chronic inflammation.

In summary, a novel composite system was developed based on pH-responsive microencapsulation of polyanhydride nanoparticles. Synthesis of PROMPT formulations by self-assembly enabled facile synthesis of composite microparticles that did not require additional purification steps, readily incorporated two different polyanhydride compositions, and demonstrated rapid pH-dependent dissociation for depot release of the nanoparticle payload. Higher nanoparticle content in PROMPT microparticles was favorable for reduced burst release of the encapsulated antigen, higher total antigen loading, as well as increased cell surface marker expression. Additionally,

microencapsulation of polyanhydride nanoparticle was necessary to induce both serum IgG and mucosal IgA responses. These composite formulations have potential as a vehicle for oral vaccination with subunit antigens.

7.2 Recommendations for Future Research

Future work on these systems could be directed towards more controlled self-assembly. For example, the synthesis of block copolymers of MAA and PEG could allow for micellization and, therefore, more ordered morphology of the resulting microparticles. The ionizable MAA blocks should still form a hydrophobic core in acidic pH for inclusion of nanoparticles, with hydrophilic PEG blocks in the corona to minimize nanoparticle adhesion to the surface. Self-assembly of the amphiphilic block copolymers would enable more complete microencapsulation of the nanoparticles to further minimize the burst release associated with nanoparticles. There is currently ongoing work in the lab towards development of block co-polymers for self-assembly.

Additionally, the nature of the self-assembly process allows for facile co-encapsulation of additional molecules, which has been demonstrated in pilot experiments with retinoic acid (data not shown). This could allow for inclusion and exploration of costimulatory molecules or mucosal adjuvants in future work.

Alternative polyanhydride nanoparticle chemistries could be explored, since nanoparticle chemistry had an effect on both the microparticle encapsulation efficiency and immunomodulatory capabilities. For example, despite the 20:80 CPH:SA based PROMPT formulations demonstrating higher nanoparticle incorporation and, therefore, higher total antigen dose, the Poly(SA)-based PROMPT formulation elicited higher protective antibody titers *in vivo*. It is possible that the hydrophobicity of the 20:80

CPH:SA nanoparticles impaired mucus penetration and cellular internalization. Therefore, it could be desirable to explore other more amphiphilic polyanhydride chemistries. For example, copolymers including 1,8-bis(*p*-carboxyphenoxy)-3,6-dioxaoctane (CPTEG) (e.g. 20:80 CPHTEG:CPH and 50:50 CPTEG:CPH) have been demonstrated to stimulate strong cellular and humoral responses (13-15).

The *in vivo* evaluation of these systems could be enhanced by inclusion of a higher dose of ovalbumin or using alternative immunization schedules. Ideally, these formulations would enable single dose immunization. However, including co-administration of soluble antigen with the particles or administration of a "booster" dose would likely stimulate higher antibody titers in order to better compare the protective response elicited by the various formulation compositions. Evaluation of immune response could also be expanded to evaluate activation of T cells, which are critical in induction of high-affinity antibodies and immune memory.

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Appendix A. Development of hydrolytically degradable hydrogel carriers for targeted intestinal release

A.1 INTRODUCTION

Development of biodegradable crosslinked hydrogels are of particular interest for drug delivery applications, particularly in consideration of larger payloads whose diffusion into and out of the network could be prohibited by maximum achievable mesh size. There are a number of strategies to incorporate biodegradable cleavage points. Ultimately, the choice of the appropriate labile chemistry within the crosslinking agent allows for the network to respond in a predictable manner to the environmental stimuli of interest, providing advantages for regulated release and/or targeted delivery of encapsulated payloads. For example, photolabile crosslinks enable externally triggered degradation (1, 2)), while inclusion of azoaromatic crosslinking agents or certain peptide sequences allow for degradation in the presence of specific enzymes in either site or disease specific manner (3-6).

There are number of pH-responsive degradable cross linkers described in the literature for drug delivery applications. For example, acid-labile acetal crosslinkers allow for selective delivery of therapeutic content intracellularly in disease states such as tumors and inflammatory tissues (7). There are also base-labile crosslinkers based on ester, amide or carbonate linkages, though degradation time at physiological pH is often on the order of days or weeks (8) (9).

The chemical crosslinker, N,O-dimethacryloyl hydroxylamine (DMHA), is a base-labile crosslinking agent of interest for degradation in physiological conditions. DMHA undergoes hydrolysis above pH 5 and is stable below that pH. It has been explored for the development of biodegradable drug carriers, demonstrating capability to

improve delivery of both encapsulated low molecular weight and macromolecular drugs (10-14).

This work explores the inclusion of DMHA in pH-responsive anionic poly(methacrylic acid-grafted-polyethylene glycol), or P(MAA-g-EG), hydrogel particles for a composite oral delivery system of nanoparticle vaccines.

A.2 Experimental Methods

A.2.1 Materials

All reagents were used as received. Methacrylic acid (MAA) and 1-hydroxycyclohexyl phenyl ketone (Irgacure® 184) were obtained from Sigma-Aldrich (St.Louis, MO). Polyethylene glycol monomethyl ether methacrylate (PEGMMA, ME 1000) was purchased from Polysciences Inc. (Warrington, PA). All other solvents and buffers were also obtained from Thermo Fisher Scientific (Waltham, MA).

A.2.2 Synthesis of N,O- Dimethacryloylhydroxylamine

DMHA was graciously prepared by the laboratory of Professor Emmanuel Akala at Howard University, as published previously and demonstrated in Figure A.1 (11-13). Briefly, DMHA is formed from methacryloylation of hydroxylamine. In a typical synthesis, hydroxylamine hydrochloride (2.5 g, 0.036 mol) was dissolved in pyridine (26.1 mL, 0.3298 mol). Methacryloyl chloride (8.3 mL, 0.085 mol) was added dropwise with a syringe while the reaction was maintained by an ice bath. After addition of the methacryloyl chloride, the reaction was allowed to proceed for two hours at room temperature. After the reaction period, chloroform (28.4 mL) was added and the resulting solution was washed twice with distilled water, dried with anhydrous magnesium sulphate and evaporated under vacuum to obtain a solid crystalline material.

Purification of the crystals was carried out by the addition of cold hexane and ethylacetate (5:1) to the solid and placed in the freezer for 1 hour. White crystals were recovered by vacuum filtration.

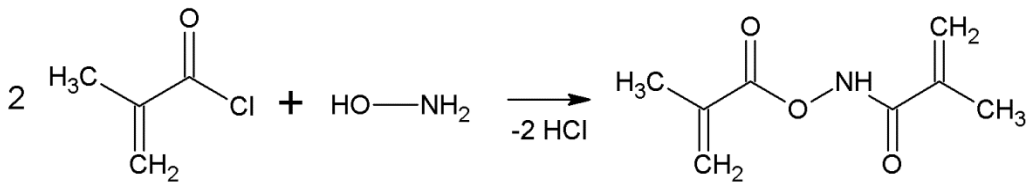

Figure A.1 Preparation of hydolyzable crosslinking agent DMHA by the reaction of hydroxylamine hydrochloride with methacryoyl chloride in pyridine.

A.2.3 Characterization of DMHA crosslinking agent

Fourier-transform infrared (FT-IR) spectrophotometric analysis was used to verify structure of DMHA crosslinking agent by averaging 64 scans on a Nicolet iS10 FT-IR Spectrometer (Thermo Fisher Scientitifiec, Waltham, MA, USA) outfitted with Smart iTX accessory for measurement in ATR mode. Proton nuclear magnetic resonance (^{1}H-NMR) using a VARIAN DirectDrive 400 MHZ spectrophotometer. DMHA samples were dissolved at 15 mg/mL in deuterated dimethyl sulfoxide.

A.2.4 Polyanhydride Nanoparticle Synthesis

Polyanhydride particles were synthesized by anti-solvent nanoencapsulation as described previously (15, 16). For these studies 20:80 CPH:SA (20 mg/mL) was dissolved in methylene chloride with Span® 80 (1% v/v) and quantum dots (λ_{em}= 625 nm) (1% v/v). The polymer solution was sonicated at 50 Hz for 30 seconds using a probe sonicator (Fisher Scientific Model 50 Sonic Dismembrator) and then rapidly poured into a pentane bath at a solvent to non-solvent ratio of 1:250. Resulting particles were collected by filtration (Whatman 50, Fisher Scientific).

A.2.5 Synthesis of hydrolytically degradable hydrogel carriers for targeted intestinal delivery

Hydrolyzable networks were synthesized by photoinitiated, free-radical polymerization of monomer solutions containing MAA, PEGMMA, and DMHA. MAA and PEGMMA were mixed in a 1:1 molar ratio in 1:1 (w/w) deionized water and ethanol solution to yield a 1:1 (w/w) total monomer to solvent ratio. The photoinitiator Irgacure® 184 was added at 1 wt% with respect to total monomer weight. DMHA was added in 10% (by volume) solution of DMSO at crosslinking densities between 0.5-2 mol% relative to total monomer content. The mixture was homogenized by sonication, loaded into the sealed and oxygen-deficient environment of an MBraun® glovebox (MBraun Inc., Garching, Germany), and purged with nitrogen for 5 minutes. Finally, the solution was pipetted between glass slides, separated by a 0.7 mm Teflon® spacer, and placed into a UV flood source at 70% intensity for 30 minutes.

Following polymerization, the film was removed from the slides and either punched into discs (6mm diameter) or left as a bulk film for purification and drying. Hydrogels were purified from unreacted reagents by washes in ethanol (5 washes with solution changes every 30 minutes), and then gradually transitioned to aqueous solution (0.01M HCl, pH 2.0) for the next 5 washes at 30 minutes intervals. Finally, dried bulk films were crushed into microparticles using a mortar and pestle and sieved into distinct size ranges, ranging from ~30-90 μm, and stored in a desiccator at room temperature. As an experimental control, P(MAA-g-EG) hydrogels were synthesized with the non-degradable crosslinker tetraethyleneglycol dimethacrylate (TEGDMA) at 0.75 mol% and prepared in the same manner.

Synthesis was slightly modified for inclusion of polyanhydride nanoparticles into the hydrogel matrix. Briefly, nanoparticles were incorporated into the pre-polymer

solution at 5-20% by weight with respect to total precursor concentration. The solution was homogenized by sonication for 5-10 minutes, followed by polymerization steps described previously and ultimate crushing of microparticles containing nanoparticles.

A.2.6 Relative turbidity degradation studies

Turbidity measurements were used to evaluate the time-dependent degradation of microparticles. Microgels (90-150 μm) were suspended in either 1X phosphate buffered saline (PBS) or 0.1N hydrochloric acid (HCl) at a concentration of 5 mg/mL. One hundred microliters of each sample solution was added to a 96-well plate in triplicate and absorbance was measured at 250 nm at two minute intervals over the course of 90 minutes using a Cytation 3 Cell Imaging Multi-Mode Reader (BioTek, Winooski, VT). Statistical analysis was performed to compare formulations using ANOVA to compare multiple groups and post-hoc Dunnett's test to confirm statistical significance, with p-values < 0.05 considered significant.

A.2.7 Microparticle morphology

Scanning electron microscopy (SEM) was used to examine the surface morphology, particle shape, and size of microparticle formulations in the dried state. Microparticles were examined at various time points after incubation in PBS (pH 7.4) to evaluate degradation over time. After samples were exposed to PBS for the designated amount of time, 1N hydrochloric acid was added dropwise until the solution reached a pH of 2 and remaining particles were collapsed. Particles were collected by centrifugation and then re-suspended in ethanol. SEM stubs were prepared using a drop-casting technique, dried, and then coated with 8nm of platinum/palladium using a

Cressington 208HR sputter coater (Watford, England, UK). Coated samples were imaged with a Zeiss Supra 40VP Scanning Electron Microscope (Oberkochen, Germany).

A.2.8 pH-dependent hydrolytic degradation studies

Gravimetric degradation experiments were carried out to observe the rate of hydrolytic degradation as a function of DMHA crosslinking density. Hydrogel discs (6mm) were dried in a vacuum oven were weighed (M_o) and placed into 37^{o}C PBS (pH 7.4). At each time point, samples were removed, freeze dried, and then weighed to determine fraction of initial mass remaining (M_t/M_o). Triplicate samples were used for each time point.

A.2.9 Verification of Nanoparticle Incorporation

Fluorescence microscopy was used to verify successful incorporation of nanoparticles (encapsulating quantum dots) into the hydrogel network. Nanoparticle distribution in the microgel population was determined by ImageStream®X Mark II Imaging Flow Cytometer (EMD Millipore, Darmstadt, Germany).

A.3. Results and Discussion

A.3.1 DMHA Characterization

Both the FT-IR and ^{1}H NMR spectra of the crosslinking agent DMHA were consistent with the expected structure and those previously reported (11, 17). The FT-IR spectrum shows the presence of C=C stretch at 1626 cm^{-1}, as well as both ester and amide C=0 stretches at 1766 cm^{-1} and 1663 cm^{-1}, respectively. The proton NMR spectrum confirms the presence of a divinylic structure, and is consistent with previously published structures ^{1}H NMR (400 MHz, C_2D_6OS) δ 1.86 [s, 3H, CH2=C(CH3)CO-NH-

O-CO-C(CH3)=CH2] 1.93 [s, 3H, CH2=C(CH3)CO-NH-COC(CH3)=CH2], 5.48 [s, 1H, CH2=C(CH3)CO-NH-CO-C(CH3)=CH2] 5.72 [s, 1H,CH2=C(CH3)CO-NH-CO-C(CH3)=CH2] 5.85 [s, 1H, CH2=C(CH3)CO-NH-COC(CH3)=CH2] 6.16 [s, 1H, CH2=C(CH3)CO-NH-CO-C(CH3)=CH2] 9.20 [s, 1H, CH2=C(CH3)CO-NH-CO-C(CH3)=CH2]. Characterization was performed for every batch of DMHA received, as well as for polymerizations after long periods of DMHA storage to ensure the crosslinker remained intact. Representative spectra are depicted in Figures A.2 and A.3

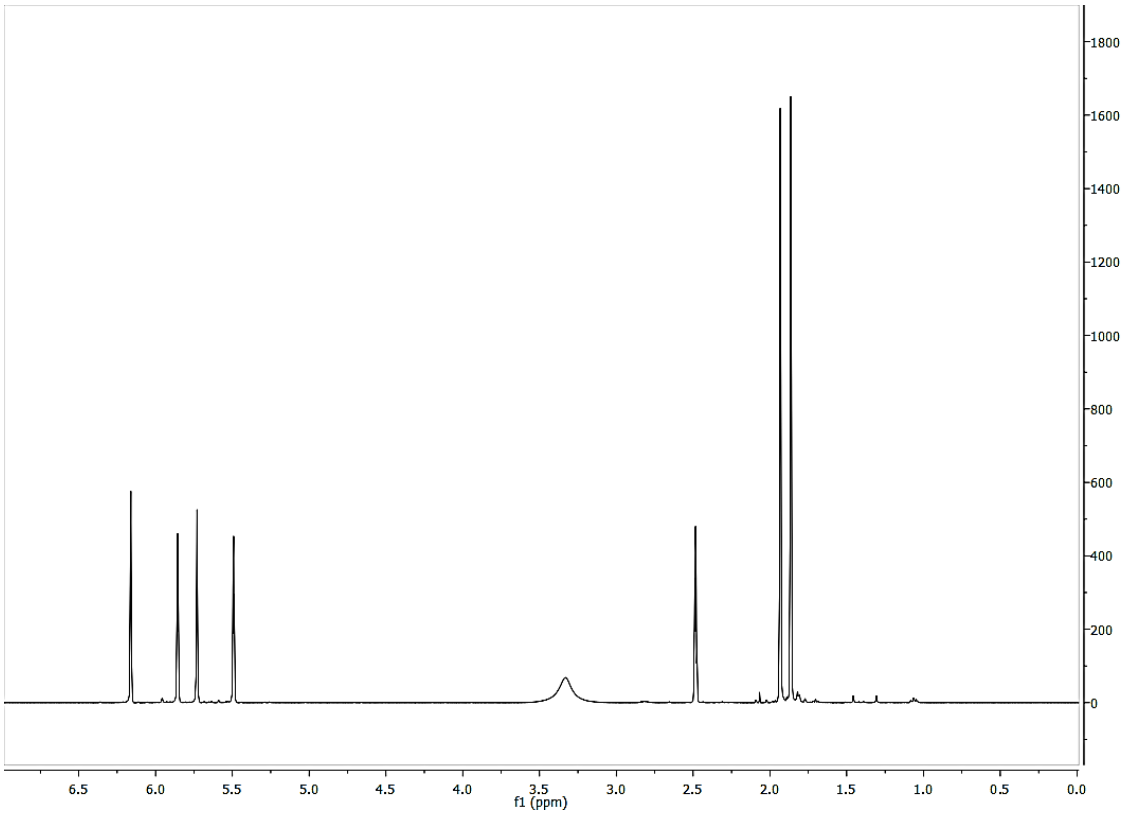

Figure A.2 Proton NMR spectrum of DMHA crosslinker (400 MHz, C_2D_6OS)

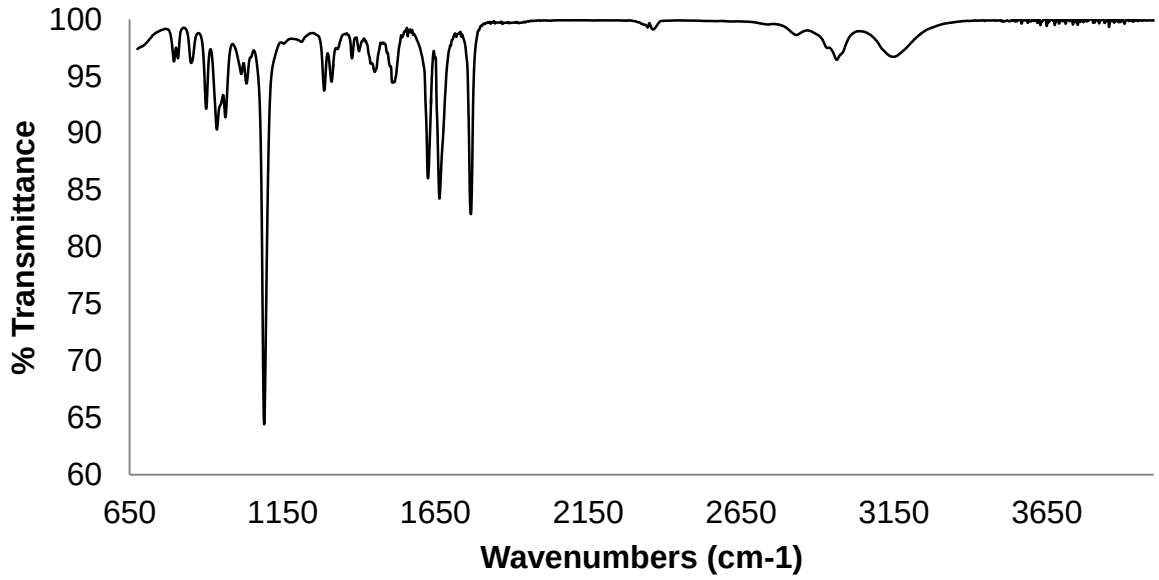

Figure A.3 Representative FTIR spectra of DMHA crosslinker.

A.3.2 Turbidimetric Measurements

Turbidity measurements are often used to evaluate temperature-dependent phase transitions of thermoresponsive polymers such as poly(*N*-isopropylacrylamide*)* (18, 19). More recently, turbidity has been used to measure evaluate degradation of particles as a function of time (6, 20). As reported by Klinger and Landfester (20), decreased turbidity over time is observed as the network loosens, swelling of the gel increases and the contrast between the refractive indices of the solvent and the polymer decreases. Mathematically, turbidity is described by the following equation (21):

$$\tau = \frac{\varphi Q_{ext} 3}{2d} \qquad \text{(Equation A.1)}$$

Where φ is the volume fraction of the particles, Q_{ext} is the Mie extinction efficiency, and d is the particle diameter. Q_{ext} is a function of both particle size and the ratio of the refractive indices of the particles and the solvent, $n_p/n_{0,}$ and decreases as either of those parameters decrease.

It was hypothesized that as crosslinking points were cleaved, resulting in smaller particles and eventually linear polymer, that turbidity would decrease over time correlating with the extent of degradation. Experimentally, turbidity was determined by measuring the absorbance of the degrading microgels at 250 nm, the peak absorbance for the incident light. Turbidity τ(t) was calculated as the intensity ratio of transmitted light of sample (I_t) to transmitted light of pure solvent (I_o), as follows:

$$\tau(t) = -\ln\left(\frac{I_t}{I_0}\right) = -\ln\left(\frac{10^{A,blank)}}{10^{A,sample}}\right) \quad \text{(Equation A.2)}$$

Relative turbidity enables better comparison between samples that vary in particle size and was calculated as:

$$\tau_{rel} = \frac{\tau(t)}{\tau(t=0)} * 100 \quad \text{(Equation A.3)}$$

and then plotted as a function of time.

The turbidity of P(MAA-g-EG) 1%DMHA, 2%DMHA and 0.75%TEGDMA hydrogel microparticles (90-150 μm) at 5 mg/mL concentration were evaluated (Figure A.4). As expected, relative turbidity decreased for samples incubated in PBS with values plateauing after 20-40 minutes. However, differences between swelling and degradation were difficult to discern using this technique, as evidenced by the nearly identical trends

observed for 2%DMHA and non-degradable 0.75%TEGDMA microgels. The 1%DMHA microgels had statistically significant reduction in turbidity as compared to the other two formulations starting at 12 minutes, which could be attributed either to increased swelling capability associated with decreased crosslinking density or as degradation of the crosslinking points, or some combination of the two phenomena.

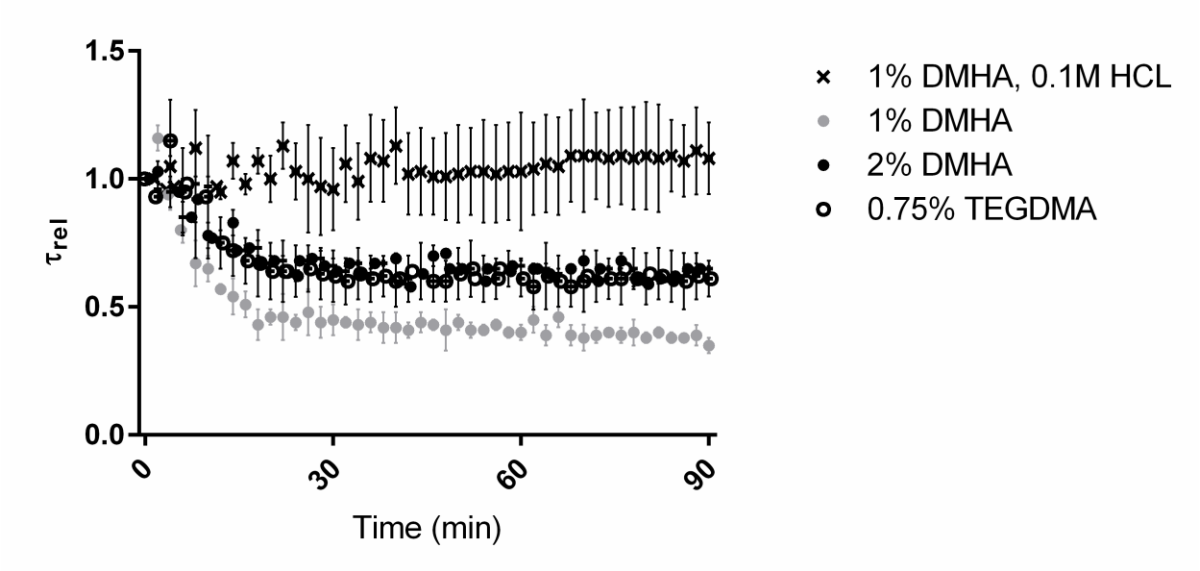

Figure A.4 Relative turbidity of P(MAA-g-EG) microgel formulations prepared at 5 mg/mL concentration in 1X phosphate buffered saline (37°C, pH 7.4 n=3 +/- S.E.M.) unless specified as 0.1M HCl (37°C, pH 1.2, n=3 +/- S.E.M). Absorbance readings were measured at 250 nm for 90 minutes.

A.3.3 SEM

Microparticle morphology of P(MAA-g-EG) microgels containing 2mol% DMHA were examined using SEM at various time points throughout the degradation process (Figure 6). Microgels crushed to 90-150 μm by mortar and pestle have irregular morphology. Figures 6 B) and C) show microgels after 5 and 24 hours of exposure to

degrading conditions (pH 7.4), respectively. However, it should be noted that since water must be used instead of PBS to prepare samples for SEM, swelling and deprotonation of MAA acidified the solution and slowed the pH dependent hydrolysis, indicated by minimal changes to particle morphology within 5 hours (Figure 6B). However, within 24 hours, particles were substantially smaller than original particles (Figure 6C).

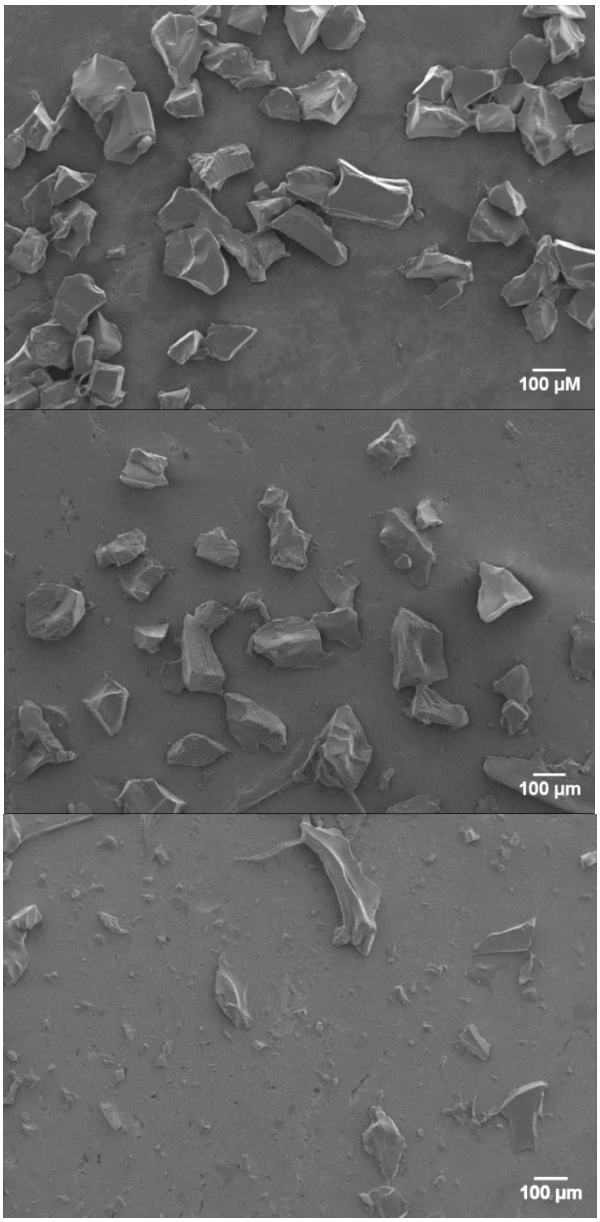

Figure A.5 Representative scanning electron microscopy (SEM) images of P(MAA-g-EG) microgels containing 2 mol% DMHA at 150x magnification. A) Gels crushed to 90-150 µm by mortar and pestle have irregular morphology. B) and C) illustrate microgels after 5 and 24 hours of exposure to degrading conditions (pH 7.4), respectively. Particle morphology does not change substantially by 5 hours, but particles are substantially smaller fragments by 24 hours.

A.3.4 Gravimetric Analysis

Gravimetric analysis was used to determine how the hydrolytic degradation of the hydrogel network could be modulated by crosslinking density, with the goal of releasing the encapsulated payload within the relatively narrow residence time of 3-6 hours within the small intestine (22). It was expected that reducing the crosslinking density would result in faster degradation of the hydrogel networks, due to increased swelling capacity and fewer scission points (13).

The expected trend was observed for DMHA crosslinking densities between 1 and 4% (Figure A.6). The 4% gels burst into pieces within the first hour of submersion into degrading conditions, but maintained weight for the duration of the study thereafter. At lower crosslinking densities, the gels remained intact during initial swelling and slowly degraded over time, with the 1% gel degrading completely within 6 hours. While 6 hours exceeds the intestinal residence time, microparticles have a much larger surface area to volume ratio than the hydrogel discs and therefore are expected to degrade at faster timescales.

Interestingly, further reducing the crosslinking below 1% had the unexpected result of increasing the degradation time. Previous studies on degradable ionic hydrogel networks have demonstrated the complex behavior of degradable networks, noting deviation from theoretical predictions of macroscopic properties based on microscopic crosslinking density during late stages of degradation (23). These deviations have been attributed to chemical and structural changes occurring within the network, such as buildup of negatively charged carboxyl groups within the network that can expand and then stiffen the networks. It is also possible that the longer polymer chains synthesized as

a result of the decreased crosslinking density physically hindered hydration of the network, and accordingly decreased the rate of crosslinker hydrolysis.

In follow up studies to investigate the surprising result, a new batch of DMHA crosslinker was used to synthesize the hydrogels. Though similar trends were observed, the degradation time was inconsistent with previous experiments, increasing from 6 to 8 hours for complete degradation of the 1% DMHA hydrogels and even longer for the 0.75% DMHA hydrogels (Figure A.7). Though the chemical evaluation of the crosslinkers matched, the incorporation into the hydrogel matrix appeared to be different enough to affect the degradation time.

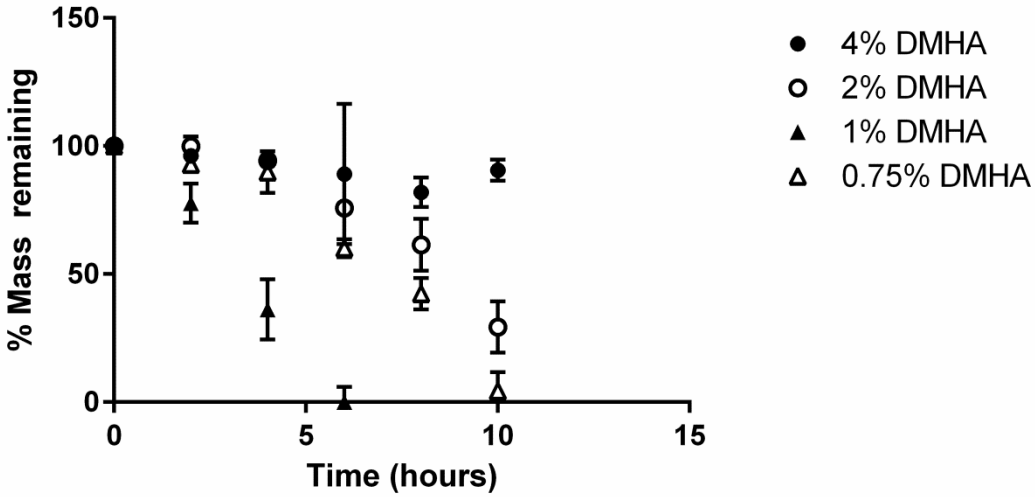

Figure A.6. Degradation of hydrogel discs was measured as fraction mass remaining as a function of time in hydrolytic conditions (PBS, pH 7.4).

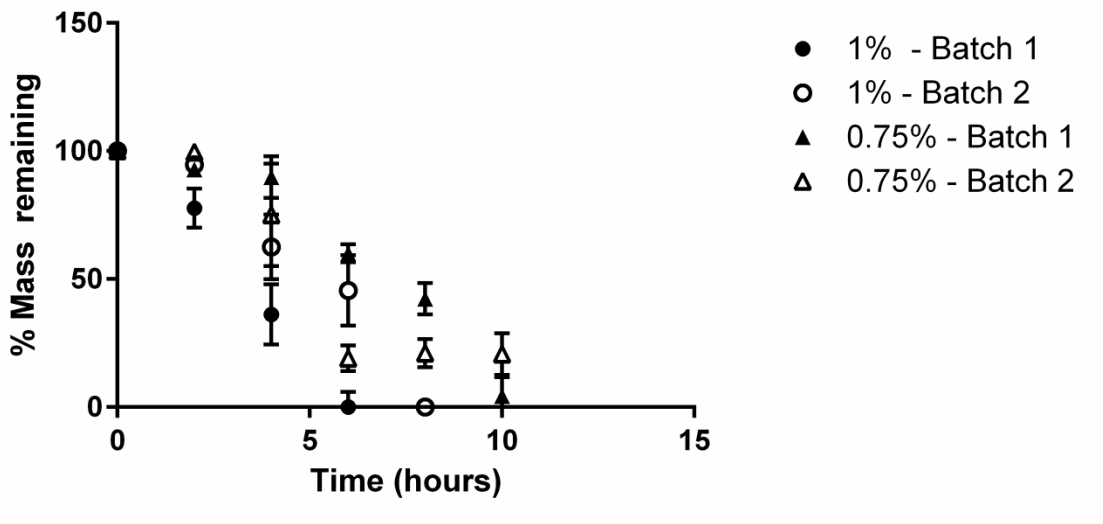

Figure A.7. Degradation of hydrogel discs measured as fraction mass remaining as a function of time for hydrogels composed with two different batches of DMHA.

A.3.5 NANOPARTICLE INCORPORATION

Both fluorescence microscopy and imaging flow cytometry were used to evaluate nanoparticle incorporation into the microgels. Fluorescence microscopy confirmed that nanoparticles were successfully encapsulated into the crushed microgels (Figure A.8). However, nanoparticles aggregated during the synthesis resulting in very unequal distribution of the nanoparticles within the microgel population. Imaging flow cytometry further confirmed nanoparticle aggregation affected nanoparticle distribution both within a single particle and among the particle populations. Figure A.9 demonstrates a representative population of P(MAA-g-EG) microgels synthesized with 20:80 CPH:SA nanoparticles at 20 wt% with respect to the monomer content. On average, 64% of the

microgels contained “fluorescent events” indicative of nanoparticle incorporation, with fluorescence intensity varying substantially between individual microgels.

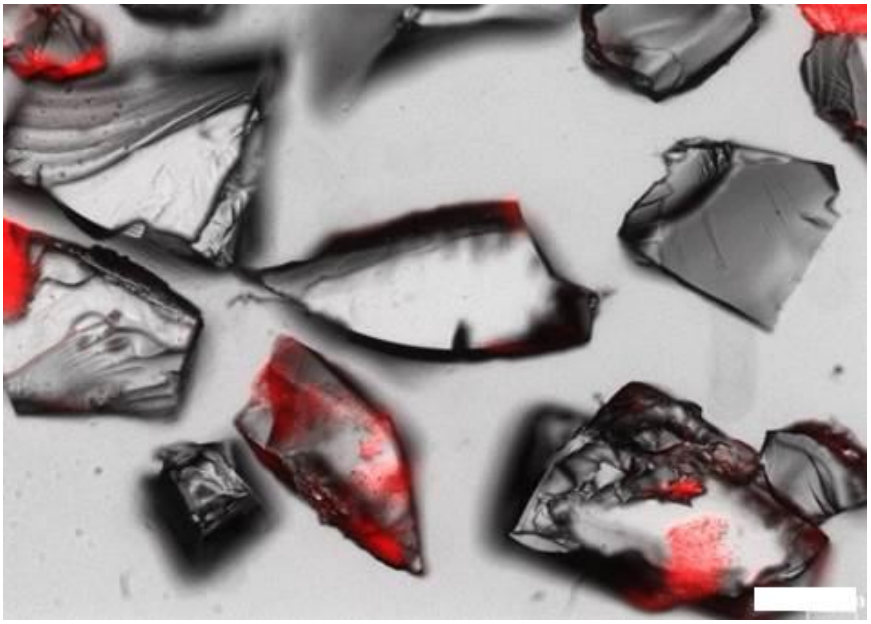

Figure A.8 Fluorescent light microscopy verified nanoparticle incorporation into microgels. Scale bar: 100 μm

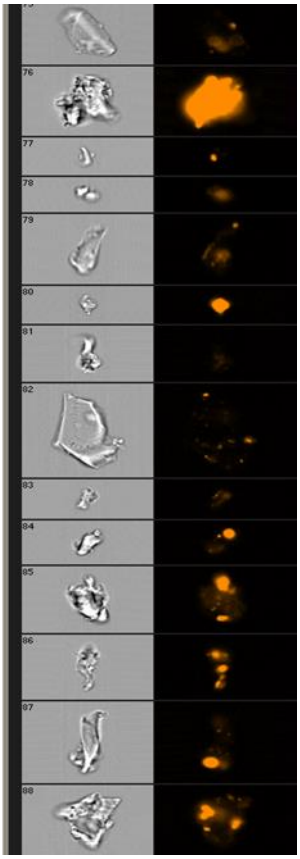

Figure A.9 Representative data from imaging flow cytometry of P(MAA-g-EG) microgels encapsulating 20:80 CPH:SA nanoparticles demonstrate that 64% of microgels encapsulate nanoparticles.

A.4. CONCLUSIONS

Biodegradable hydrogel films and microparticles were synthesized using the base-labile crosslinker, DMHA. The pH-dependent degradation of microgels was assessed by scanning electron microscopy and turbidity, though swelling and degradation behavior were indistinguishable in the latter. Gravimetric analysis confirmed degradation kinetics were tunable by modifying the crosslinking density. Hydrogels crosslinked with 1% DMHA degraded within the least amount of time, which is desirable for targeted delivery within the ~4 hour intestinal residence time. However, the degradation kinetics were inconsistent between batches of the hydrolysable crosslinker. Furthermore, inclusion of the nanoparticles into microgels was variable due to aggregation during the polymerization process, resulting in an average of 64% microgels containing nanoparticles. In order to achieve targeted intestinal delivery of the nanoparticle vaccines, it is desirable to achieve complete degradation within the intestinal residence time to ensure release of the nanoparticles. Furthermore, microgel encapsulation of the nanoparticles needs to be more efficient and consistent for appropriate dosage calculations. Therefore, it was determined that inclusion of DMHA as a crosslinker was not a suitable strategy for oral delivery system of nanoparticle vaccines.

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Vita

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