

**Development and Characterization of Novel
Self-Encapsulating Poly(lactic-co-glycolic acid)
Microspheres for Vaccine Delivery**

by

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In loving memory of my grandfather,

George Agius

You are missed.

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List of Common Abbreviations

APC	Antigen-presenting cell
BAL	Bronchial alveolar lavage
CaPh	Calcium phosphate adjuvant gel
CTB	Cholera toxin B
DC	Dendritic cell
dd	Double-distilled
ELISA	Enzyme-linked immunosorbent assay
FBS	Fetal bovine serum
GM-CSF	Granulocyte-macrophage colony-stimulating factor
ICP-OES	Inductively coupled plasma-optical emission spectrometry
LPS	Lipopolysaccharide
MHC	Major histocompatibility complex
MOPS	3-(N-morpholino)propanesulfonic acid
MPLA	Monophosphoryl lipid A
MPs	Microspheres
OVA	Ovalbumin
PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PLGA	Poly(lactic- <i>co</i> -glycolic acid)
PRR	Pathogen recognition receptor
PVA	Polyvinyl alcohol
SE	Self-encapsulating
SE-HPLC	Size exclusion-high performance liquid chromatography

TAP	Transporter-associated protein complex
T _g	Glass transition temperature
Th	T helper cell
TLR	Toll-like receptor

Abstract

Microspheres composed of poly(lactic-*co*-glycolic acid) (PLGA) have been a major area of interest for vaccine delivery due to their ability for (1) controlled release of antigen, which may enable a decrease in the number of doses required for protective immunity, and (2) enhancing the immune response against poorly immunogenic antigens. One disadvantage to this delivery system is that antigens encapsulated within PLGA microspheres by traditional techniques are susceptible to instability due to harsh processing conditions, including shear stress and exposure to organic solvent. Our lab has developed a novel method, termed “self-encapsulation,” for the remote loading of large molecules into pre-made PLGA microspheres. These microspheres contain a protein-trapping agent and interconnecting pore network. Simple mixing of the microspheres in an aqueous solution of the antigen enables the protein to diffuse into the polymer pores and bind to the trapping agent. Subsequent heating of the system above the glass transition temperature (T_g) of the polymer causes the pores to close, sealing the protein inside the microspheres. This project further expands upon the self-encapsulation approach by exploring its application to vaccine delivery. A formulation of self-encapsulating microspheres was developed for internalization by antigen-presenting cells (APCs), key cells for antigen processing and adaptive immunity. Using ovalbumin as a model antigen, formulation characteristics, including self-encapsulation ability and

protein release kinetics, were defined. Stability of encapsulated protein was investigated and maintenance of antigenicity during release was confirmed. *In vitro* studies showed successful internalization of the microspheres by murine APCs. To examine the type and magnitude of the immune response induced by the microspheres, C57BL/6 mice were immunized with the formulation by intranasal and subcutaneous administration. Intranasal delivery of the microspheres resulted in a Th2-skewed response in serum and local mucosa. In comparison, subcutaneous delivery of the formulation resulted in a significant ovalbumin-specific cellular immune response and serum IgG subclass titers associated with a mixed Th1/Th2 response. Additionally, the microspheres showed promising ability for single-time injection, which could improve convenience and patient compliance. Due to the results of our studies, this formulation of self-encapsulating microspheres was concluded to have potential for further vaccine development.

Chapter 1

Introduction

1.1 Motivation

Vaccines are one of the most successful public health interventions, with an estimated 2-3 million deaths alone averted each year due to immunization against diphtheria, pertussis, tetanus, and measles [1]. Despite this, there are still many challenges to overcome in regards to vaccine coverage and protection from infectious diseases. Factors including the vaccine delivery method (e.g. administration by needle), product thermostability during storage and transport, and multiple doses for full immunization all affect the completeness of vaccine coverage [2]. In addition, there is also a need for new and improved vaccines. There are some infectious diseases for which vaccines are nonexistent and others for which the vaccines could be made more effective in their protective immune response ([2], [3]).

Following these challenges in vaccine coverage and efficacy has come the development of novel approaches to vaccine delivery, with the goal of improving vaccine performance [3]. Antigen delivery systems (e.g. microspheres, nanoparticles, and liposomes) and adjuvants (e.g. aluminum and calcium compounds) have been widely explored as approaches to improve the immune response generated by the antigen. This is especially true for subunit- and recombinant protein-based vaccines, which are generally poorly immunogenic on their own, namely due to difficulty activating antigen-presenting

cells and their short *in vivo* half-life ([4], [5]). Adjuvants and delivery systems can improve antibody- and cell-mediated immunity, decrease the amount of antigen required in a dose, and decrease the number of doses necessary for immunization, among other benefits [3].

Polymeric microspheres and nanoparticles have been widely researched for their use as vaccine delivery systems. One of the most commonly used polymers for this application is poly(lactic-*co*-glycolic acid) (PLGA), a biodegradable and biocompatible polymer ([4], [6]). PLGA particles are advantageous in their ability to control antigen delivery in both time and location [7]. The kinetics of PLGA degradation in the body can be tuned by the ratio of lactic to glycolic acid in the polymer [6]. By controlling the rate of degradation, one can also control the rate of release of the antigen cargo and exposure of the antigen to the immune system. For example, the release can be made continuous to mimic an infection or pulsatile to mimic booster administration [7]. The particles can also control where the antigen is delivered, such as directly to antigen-presenting cells (APCs), protecting the antigen from degradation until it is delivered to the cell ([7], [6]).

Despite the benefits of PLGA particles, there are a number of challenges facing the development of this delivery system. One of the key challenges is antigen instability, which can occur during microsphere production, lyophilization and storage of the microspheres, and during antigen release [8]. Traditional microsphere production methods, during which the antigen is encapsulated by the polymer, expose the antigen to detrimental conditions. These include forces from emulsification procedures and exposure to aqueous/organic solvent interfaces ([8],[9]). Protein instability takes such

forms as aggregation and protein unfolding (i.e. loss of native structure) ([10], [11]). These factors create quite a task for delivering protein in its antigenic state.

In an effort to improve protein stability in PLGA particles, the Schwendeman group has developed an encapsulation paradigm, termed “self-healing microencapsulation” or “self-encapsulation,” that bypasses many of the challenges of traditional PLGA encapsulation techniques ([8], [9]). This method relies on simple aqueous mixing of the antigen with pre-formed PLGA particles to load the antigen into the microspheres, thereby avoiding many sources of destabilization. The antigen does not encounter the detrimental shear stresses for emulsification, organic solvent, or phase interfaces characteristic of traditional encapsulation [9].

In this work, we investigate the application of self-healing microencapsulation to the delivery of vaccine antigens. Due to its gentle processing conditions, self-encapsulation is a promising approach for maintaining the stability and immunogenicity of encapsulated antigens ([8],[9]). For this project, a self-encapsulating PLGA microsphere formulation was developed for internalization by dendritic cells, key antigen-presenting cells. We specifically explore the type of *in vivo* immune response generated in mice after subcutaneous and intranasal administration of the microsphere formulation.

1.2 An Overview of the Immune Response

In order to appreciate the role that antigen delivery systems, such as PLGA microspheres, play in vaccine delivery, it is important to understand how the immune system operates. The immune system can be divided into two arms: the innate and the adaptive immune response, known respectively as “natural” and “acquired” immunity

[12]. These two systems work together to recognize and eliminate a threat and protect from re-infection ([13],[6]).

1.2.1 Innate and Adaptive Immunity

Though the innate and adaptive responses behave as an integrated system, it is the innate immune response that acts as a first line of defense against invading microorganisms. Its purpose is to recognize the presence of a pathogen and then eliminate the pathogen through mechanisms that include chemical mediators and effector cells, which fight against the pathogen in a non-specific way [5]. The innate immune system reacts quickly, usually within a few hours, and is responsible for differentiating self from infectious non-self and relaying this information to the adaptive immune system [5].

Pathogen detection is achieved through non-specific receptors on innate immune cells, which recognize pathogen-associated structures. These structures, known as pathogen-associated molecular patterns (PAMPs), are recognized by pattern recognition receptors (PRRs) on effector cells [13]. PRRs include the toll-like receptors (TLRs) and the mannose receptor. Eleven TLRs have been identified thus far and differ based upon their cellular localization and the type of pathogen they recognize. For example, TLR2 and TLR4 are found on the cell surface and detect bacterial lipoproteins and Gram-negative lipopolysaccharide (LPS), respectively, whereas TLR9 is found within the cell and recognizes bacterial and viral DNA ([5], [14], [12]). The innate immune system can thus discriminate against the different types of pathogens to ensure that the appropriate adaptive immune response is generated.

The adaptive immune response provides specialized defense and long-lasting immunity against a specific pathogen. Though the adaptive immune response is slower

(on the order of days to weeks), the immunological memory it provides allows for a stronger and quicker response upon future encounters with the pathogen [13]. Antigen-presenting cells, namely dendritic cells (DCs), act as a bridge between the innate and the adaptive immune systems, stimulating the cells of the adaptive response that are responsible for the production of cellular and humoral immunological memory. The adaptive response itself is facilitated by B and T lymphocytes [5]. The process of antigen presentation and activation of T and B cells are discussed in the following sections.

1.2.2 Dendritic Cells and Antigen Presentation

Dendritic cells are considered to be the most effective antigen-presenting cells and are critical for the induction of the adaptive immune response ([15],[16], [17]). DCs are responsible for monitoring peripheral tissues for foreign antigen. Upon detection of foreign antigen, DCs internalize the antigen and travel to lymph nodes to present the antigen to T cells for induction of the adaptive response. Initially, dendritic cells are in an immature form when scanning the peripheral tissues and are highly endocytic in order to more efficiently internalize antigen [15]. Upon antigen uptake, the cells undergo a maturation process whereby their phenotype and function change.

As DCs mature after antigen uptake, there is a decrease in their ability to internalize antigen and an increase in their ability to process and present antigen ([15], [5]). Antigen processing and presentation are crucial in the lymphoid tissue, where the DCs migrate following antigen capture. Mature DCs express major histocompatibility complex (MHC) molecules, MHC-peptide complexes, and costimulatory molecules, which are necessary for antigen presentation and DC interaction with T cells [15]. Since DCs control the quality of the antigen-directed immune response, certain features affect

the maturation of dendritic cells to direct the type of immune response generated [15]. For example, TLR ligands give the dendritic cell information as to the nature (e.g. bacterial or viral) of the infecting pathogen ([5], [13]). This information transfer, in turn, allows the DC to activate the appropriate T cells and generate a more effective immune response.

Following uptake, antigens undergo different processing and presentation depending on the nature of the antigen. For example, exogenously-produced antigens (i.e. soluble and particulate antigens) are degraded by endosomal proteases and loaded onto MHC class II molecules to be presented on the cell surface as a MHC class II:peptide complex ([5], [16]). This complex then proceeds to activate CD4⁺ T helper cells. On the other hand, intracellular antigen (i.e. cytosolic) is processed by cytosolic proteasomes and then transported to the endoplasmic reticulum via the transporter-associated protein (TAP) complex to be loaded onto MHC class I molecules ([5], [16]). It is subsequently presented on the DC surface as a MHC class I:peptide complex that can then activate CD8⁺ T cells. Exogenous antigens are not limited to MHC class II complexes: they can also be loaded onto MHC class I molecules and presented to CD8⁺ T cells by a process called cross-presentation. This occurs when exogenous antigens escape endosomes and are degraded by cytosolic proteasomes, or when the antigen is degraded in endosomes and exchanged with peptide loaded onto MHC class I molecules ([5], [16], [18]). Dendritic cells are well known for their ability to carry out either method of cross-presentation [5].

1.2.3 T and B Lymphocytes

Mature DCs are considered to be the most potent activators of naïve T cells. A single DC is capable of activating 100-1000 T cells [5]. Activation and clonal expansion of T cells by DCs requires two signals: the first signal comes from the stimulation of the T cell receptor and its co-receptor by the MHC:peptide surface complex, while the second signal comes from ligation of CD28 on T cells by B7 molecules (CD80 + CD86) on DCs ([5], [16]).

Once activated, naïve CD4⁺ and CD8⁺ T cells lead to different types of immune responses. The T cells are so named due to the expression of different costimulatory molecules on their surfaces: CD4⁺ T cells express CD4, whereas CD8⁺ cells express CD8. It is CD8⁺ T cells that recognize antigen bound to MHC class I molecules. Since this antigen is generally of intracellular origin, CD8⁺ T cells are cytotoxic in function so as to eliminate cells infected with the intracellular pathogen ([5], [19]). Conversely, CD4⁺ T cells recognize MHC class II-associated peptide from extracellular pathogens.

The CD4⁺ T cell response can be divided into two effector cells types: type 1 (Th1) and type 2 (Th2) T helper cells ([5], [19]). These cells secrete different cytokines that help direct the immune response. Th1 cells are associated with IFN- γ , IL-12 and TNF- α , whereas cytokines for Th2 cells include IL-4, IL-5, IL-10 and IL-13 ([5], [20]). The Th1 response is a pro-inflammatory response and enhances cellular immunity [20]. On the other hand, the Th2 response involves the stimulation of B cells and the subsequent production of antibodies for targeting extracellular pathogens [20].

B cells are responsible for the production of antibodies, i.e. humoral immunity. Pathogens are recognized by receptors on the surface of B cells in a very pathogen-

specific manner. It is important to note that B cells recognize pathogenic protein in its native form [5]. When binding between the pathogen and a B cell receptor occurs, the B cell internalizes and processes the pathogen to present its epitopes in complex with MHC class II molecules on the cell surface [21]. A helper CD4⁺ T cell with the ability to recognize the specific peptide being presented by the B cell can activate the B cell to produce antibodies against that pathogen ([5], [21]). The function of antibodies is to bind and neutralize toxins and eliminate whole pathogens (e.g. bacteria). Five different classes of antibodies exist: IgA, IgD, IgE, IgG, and IgM. IgG is most abundant in internal body fluids, such as the blood and lymph, while IgA is principally found at mucosal surfaces [21].

The advantage of T and B lymphocyte activation is the formation of immunological memory. One result of the expansion of pathogen-specific T and B cells is the production of long-lived memory cells. Upon re-infection, these cells react much faster and more efficiently than naïve immune cells in eliminating the pathogen. This immunological memory is the goal of vaccination.

1.3 New Generation Vaccines

Vaccines function by delivering an antigen to the immune system in such a way so as to stimulate an antigen-specific, adaptive immune response for long-term protection against that antigen [22]. Many traditional vaccines consist of live attenuated pathogens, whole inactivated organisms, or inactivated bacterial toxins. These vaccines come with certain drawbacks, including safety concerns surrounding reactogenicity and, for some, a limitation to humoral immunity ([6], [23], [24]).

Recent advances have led to new vaccine approaches that are based on recombinant proteins or peptides. While they are advantageous in their reduced toxicity, these new vaccines are also poorly immunogenic on their own ([23], [4], [5]). To improve their immunogenicity, new vaccines will require the aid of safe and effective adjuvants and antigen delivery systems to induce a more potent immune response ([23], [3]).

1.4 Adjuvants and Antigen Delivery Systems

Adjuvants are defined as agents that act in combination with antigens to enhance and direct the immune response against that antigen ([3], [23]). Adjuvants include mineral salts (e.g. aluminum and calcium compounds), immunostimulatory agents (e.g. TLR ligands), and particulate delivery systems (e.g. polymer particles) [23]. Adjuvants can function to (a) create an antigen depot for slow release of the antigen to the immune system, (b) deliver the antigen to APCs and improve antigen internalization, and (c) direct and enhance the immune response generated by the antigen [6]. The following sections provide a discussion of some of the main classes of adjuvants.

1.4.1 Mineral Salt Adjuvants

Aluminum salts, namely aluminum hydroxide and aluminum phosphate, are the most common of the mineral salt adjuvants. They have been used in many vaccines licensed for clinical use, including diphtheria toxoid, tetanus toxoid and acellular pertussis (DTaP) and hepatitis B ([25], [26]). Aluminum salts possess an adjuvant effect in their ability to (a) act as an antigen depot, (b) present a dense population of antigen to recognition receptors, and (c) be of sufficient size for phagocytosis by APCs [27].

Limitations of aluminum salts include the potential formation of granulomas at the site of injection and the production of IgE-mediated allergic reactions ([22], [28]). In addition, the adjuvant is associated with humoral or Th2-type immune responses and, thus, is not as effective against intracellular pathogens, which require a cell-based immune response ([3], [29]).

Calcium phosphate has been studied as an alternative to aluminum adjuvants and has been used in childhood diphtheria-tetanus-pertussis vaccines ([22], [28]). Its properties are similar to those of the aluminum salts, though it does offer certain advantages. Calcium phosphate is a normal constituent of the body, therefore it is biocompatible and biodegradable [30]. In addition, calcium phosphate does not elicit the IgE response associated with aluminum adjuvants [26].

1.4.2 Immunopotentiators

This class of adjuvants activates immune cells through direct interaction with recognition receptors, such as the toll-like receptors, and has the ability to guide the immune response [3]. Immunopotentiators are typically pathogen-derived, such as monophosphoryl lipid A (MPLA) and CpG oligonucleotides. MPLA, a TLR4 agonist, is a safer derivative of gram-negative lipopolysaccharide (LPS) and has been approved for use in Europe and the US ([31], [32], [33]). CpG (cytosine-phosphate-guanine) is a common motif in bacterial and viral DNA and is recognized by TLR9 ([31], [32], [34]). MPLA and CpG are associated with enhancing Th1 and cell-based immune responses ([35], [36]). Immunopotentiators may be combined along with other adjuvants for a greater immunogenic effect.

1.4.3 Particulate Delivery Systems

Polymeric microparticles and nanoparticles have been studied as antigen delivery systems for many years. Particulate delivery systems offer many advantages, including (a) protecting antigen that may be susceptible to degradation upon injection, (b) controlled release of antigen to prolong its exposure to the immune system, (c) incorporation of immunostimulatory agents for added adjuvant effect, and (d) surface conjugation of antigen to present it in a similar manner as the pathogen ([19], [6], [32]). Perhaps the most widely studied polymer is PLGA, which is the focus of the remaining sections.

1.5 Overview of PLGA

PLGA has been studied for the purpose of drug delivery since the 1960s [37]. It is an aliphatic polyester composed of different ratios of L-lactic, D-lactic, and glycolic acid [17]. In an aqueous environment, PLGA is hydrolyzed into its acid monomers, which are then metabolized by the citric acid cycle [38]. Due to the biodegradable and biocompatible nature of the polymer, these particles present an excellent safety profile. Indeed, PLGA is used in numerous products that are approved for clinical use by both the US Food and Drug Administration (FDA) and the European Medical Agency (EMA) ([39], [40]). PLGA presents a promising strategy for the purpose of vaccine delivery.

1.5.1 Preparation of PLGA Particles

There are three methods commonly used for encapsulating protein and peptide antigens into PLGA particles: the w/o/w double emulsion process, phase separation (coacervation), and spray drying (Figure 1) ([41],[42]). For the w/o/w technique, an

aqueous solution of antigen is added to PLGA dissolved in an organic solvent. The two solutions are vigorously mixed to form the first w/o emulsion. This primary emulsion is then added to an aqueous solution of emulsifier and mixed again to form the w/o/w emulsion. The secondary emulsion is then stirred in an aqueous bath (with or without surfactant) for multiple hours to allow for solvent evaporation, during which time the particles harden. After solvent evaporation, the particles can be collected by filtration or centrifugation. The w/o/w method is best used to encapsulate water-soluble drugs such as proteins and peptides [42].

Phase separation and spray drying also involve the formation of a w/o emulsion. For the phase separation method, an organic non-solvent is added to the polymer solution to induce phase separation and the formation of coacervate droplets, which encapsulate the protein or peptide [42]. Spray drying forms PLGA particles by atomizing the w/o emulsion into a stream of hot air; this induces rapid solvent evaporation ([41],[42]). Both methods can be successfully used to encapsulate peptide and protein antigens [42].

1.5.2 Controlled Antigen Release

Protein is released from PLGA microspheres in three phases: an initial burst, diffusion controlled release, and then erosion controlled release [43]. Burst release is associated with the diffusion of protein at the outmost layers of the polymer ([43], [44]). The protein release then enters a lag phase as the polymer undergoes hydrolysis [45]. Degradation of the polymer occurs by bulk erosion. During bulk erosion, water enters into the polymer matrix faster than hydrolysis of the polymer chains [46]. Consequently, erosion occurs throughout the entire polymer matrix until a critical molecular weight is reached. At this point, the degradation products are small enough to be soluble in water.

This causes the formation of a porous network within the polymer structure, allowing for the release of encapsulated antigen through diffusion ([47], [48], [49]). During erosion-controlled release, the polymer fully hydrolyzes, eventually leading to the end of protein release [45].

The rate of polymer erosion – and, consequently, the protein release profile – is influenced by the molecular weight, crystallinity, and hydrophobicity of the polymer ([50], [51]). PLGA with a lower molecular weight and higher hydrophilicity will degrade more quickly. Glycolic acid, which has no methyl group on the α -carbon, is more hydrophilic than lactic acid. Hence, a higher ratio of glycolic acid will cause faster degradation with the absence of polymer crystallinity. Similarly, PLGA containing amorphous D,L-lactic acid degrades faster than polymer containing only crystalline L-lactic acid owing to the inhibition of molecular movements and water entry into the polymer crystallites ([17], [45]).

1.6 PLGA Particles as Antigen Delivery Systems

It has been well established that PLGA particulates with adsorbed or encapsulated antigen provide an enhanced immune response relative to the soluble antigen alone. Advantages to using PLGA particulates include targeting of particulates for uptake by APCs, incorporating immunostimulators into the delivery system, and controlled release of antigen ([41], [3], [52]).

1.6.1 Uptake of PLGA Particles by APCs

As activators of the adaptive immune system, APCs are important targets for vaccines. PLGA particulates can be modified in their design to enhance their

internalization by APCs. A key factor in facilitating internalization is particle size. Particle sizes typically fall into the following three ranges: (1) 10-100 μm for antigen depots, (2) 0.5-10 μm for delivery to APCs, and (3) 50-500 nm for delivery to APCs and circulation in the blood ([53], [54]). Many studies have shown that PLGA particulates of a size less than 10 μm are preferentially internalized by APCs ([55], [17], [56], [52], [57], [6], [58], [59]). APCs internalize particles by endocytosis, with particles smaller than 0.5 μm being taken up by receptor-mediated endocytosis and particles larger than 0.5 μm being internalized by both receptor-mediated and receptor-independent endocytosis (including phagocytosis and macropinocytosis) [60].

To further facilitate uptake, the particle surface can be decorated with ligands that bind to receptors found on the surface of APCs. Dendritic cells, which are the most potent of APCs, express different surface receptors, such as the mannose receptor, DC-SIGN, and DEC-205. Particles affixed with ligands of these receptors (e.g. a ligand with a terminal mannose) or antibodies against these receptors (e.g. an anti-DEC-205 antibody) can enhance the specificity of the particles for APCs and improve their internalization through receptor-mediated endocytosis ([53], [17]).

1.6.2 Incorporation of Immunostimulators

Co-delivery of immunostimulators with an antigen in PLGA particulates enables a guided and more robust antigen-directed immune response to develop. Oral vaccination of mice with particles containing ovalbumin and MPLA resulted in both a stronger IgG and IgA response compared to soluble ovalbumin and ovalbumin encapsulated without MPLA [61]. Subcutaneous vaccination of mice with PLGA nanoparticles containing the melanoma antigen tyrosinase-related protein 2 (TRP2) and the TLR4 ligand 7-acyl lipid

A induced a CD8⁺ T cell response against the antigen. This T cell response was stronger compared to that produced by soluble antigen alone [62]. Mice vaccinated with tetanus toxoid co-encapsulated with CpG ODN in PLGA nanoparticles were found to produce both a Th1 and Th2 type response, with a bias towards Th1 [36].

Due to the controlled-release nature of PLGA particles, delivery of TLR ligands by this delivery system limits the amount of TLR ligand released systemically. This is beneficial due to the non-specific immune activity and toxicity that may otherwise develop. In addition, PLGA particulates allow for the simultaneous delivery of multiple TLR ligands, which opens up more possibilities for directing the immune response.

1.7 Limitations of PLGA Particulate Vaccines

Though a promising vaccine delivery system, PLGA particulates do possess certain limitations. Two limitations that will be discussed here are cost and antigen instability.

1.7.1 Cost

Advancement of a PLGA antigen delivery system into the clinic could be prevented if production costs for the delivery system are too high. Acceptable cost is dependent upon the vaccine at hand – for example, the acceptable cost for a cancer vaccine may be much higher than that for a routine childhood immunization. Currently, cost is a significant issue when developing PLGA delivery systems, in particular because most microencapsulation methods require aseptic manufacturing conditions ([63], [64]). This adds significant cost, particularly for the aseptic processing of organic solvents [9].

1.7.2 Antigen Instability

Release of stable antigen from PLGA particles has proven to be a challenge to the field. Ideally, released antigen will have an intact primary and three-dimensional structure where linear and conformational epitopes are to be found. However, instability can occur at a number of stages, including during encapsulation of the antigen, lyophilization, storage of the protein-loaded microspheres, and antigen release [8]. During traditional encapsulation methods, forces from emulsification, exposure to water-oil interfaces, and elevated temperatures can denature proteins ([8],[9]). Further, without the addition of stabilizing agents, conditions during lyophilization and storage can also cause the antigen to lose its native structure [11]. Instability most commonly takes the form of aggregation (covalent and non-covalent), protein unfolding, and hydrolysis of the peptide backbone ([10], [11]). During release, water content in the polymer and the microclimate pH in the polymer pores affect the antigen stability. Increasing water content makes the protein more flexible and, hence, prone to destabilization [11]. As acid byproducts are produced during polymer hydrolysis, the microclimate pH is lowered and may lead to protein denaturation [11]. All of these factors make it difficult to deliver protein with its antigenicity unaffected.

1.8 Overcoming the Limitations: Active Self-Microencapsulation

One way to overcome both high cost and antigen instability is to employ simpler methods for encapsulating antigen into the PLGA carrier. One such method is the active self-microencapsulation paradigm described by the Schwendeman laboratory. The potential advantages of this method include reduced manufacturing costs and improved stability of encapsulated proteins ([11],[9], [8])

Active self-microencapsulation relies on the ability of the polymer to self-heal at higher temperatures in aqueous media. PLGA particles are first made by the w/o/w method without the addition of protein (i.e. “blank” particles). Trehalose is included in the formulation as a porosigen to help create a porous network within the particles. The osmotic pressure created by the trehalose causes an influx of water through the polymer phase and into any accessible pores. In response to this stress, the polymer swells and new, interconnected pores form to relieve the pressure. Incorporated into the formulation is a protein-trapping agent, such as an aluminum or calcium salt adjuvant, which is accessible through the pore network. Once the blank particles have been prepared, they can be lyophilized and stored for later use (provided necessary lyoprotective measures are taken). Additionally, at this stage, the blank particles can undergo sterilization (Figure 2).

To load antigen, the particles are simply incubated in an aqueous solution of the protein. The mixture is initially incubated at a temperature much lower than the glass transition temperature (T_g) of the polymer. This step allows the protein to enter through the open pores of the particles and bind to the protein-trapping agent for improved loading [8]. Next, the mixture is incubated at a temperature higher than the T_g . When a polymer is at a temperature above its T_g , the polymer chains become mobile and rearrange ([11],[9], [8]). It is at this step that self-healing of the polymer occurs and the pores close (i.e. heal). The result is protein loaded within the particles (Figure 3).

Due to the nature of active self-microencapsulation, peptide and proteins encapsulated by this method encounter fewer sources of destabilization. The protein does not encounter the detrimental micronization forces or the water-oil interfaces of encapsulation. The protein also avoids destabilization due to lyophilization conditions

since the blank particles are lyophilized before the addition of antigen. This encapsulation paradigm also simplifies sterilization of the product. Sterilization has been a challenge due to the denaturing effect it can have on antigen encapsulated in particles. However, by this novel method, the blank particles can be sterilized before the introduction of the antigen ([11], [9], [65]). In addition, it is expected that the protein-trapping agent improves protein stability by immobilizing the antigen, making it less prone to unfolding and aggregation. Thus, active self- microencapsulation is a promising approach for antigen encapsulation, particularly for maintaining the stability and immunogenicity of proteins.

1.9 Hypothesis and Thesis Objectives

PLGA particles are a promising delivery system for the development of new generation vaccines, particularly for poorly immunogenic protein and peptide antigens. The self-encapsulation method has demonstrated potential of overcoming certain limitations of traditional PLGA particles, namely antigen instability. It is hypothesized in this thesis that, by employing active self-encapsulation of antigen in calcium phosphate/PLGA microspheres of a size range suitable for targeting antigen-presenting cells, the resulting vaccine will have improved antigen stability and immunogenicity relative to both traditional PLGA microspheres and free calcium adjuvant. The purpose of this thesis is to test this hypothesis. The following chapters discuss the investigation of a self-encapsulating PLGA microsphere formulation for delivery of antigen to APCs and the type of immune response generated after subcutaneous and intranasal administration. It is noted that while three separate investigations are described here (formulation

characterization, *in vitro* cell studies, and *in vivo* immunization studies), they were combined in a two-chapter format for publication purposes.

1.10 Figures

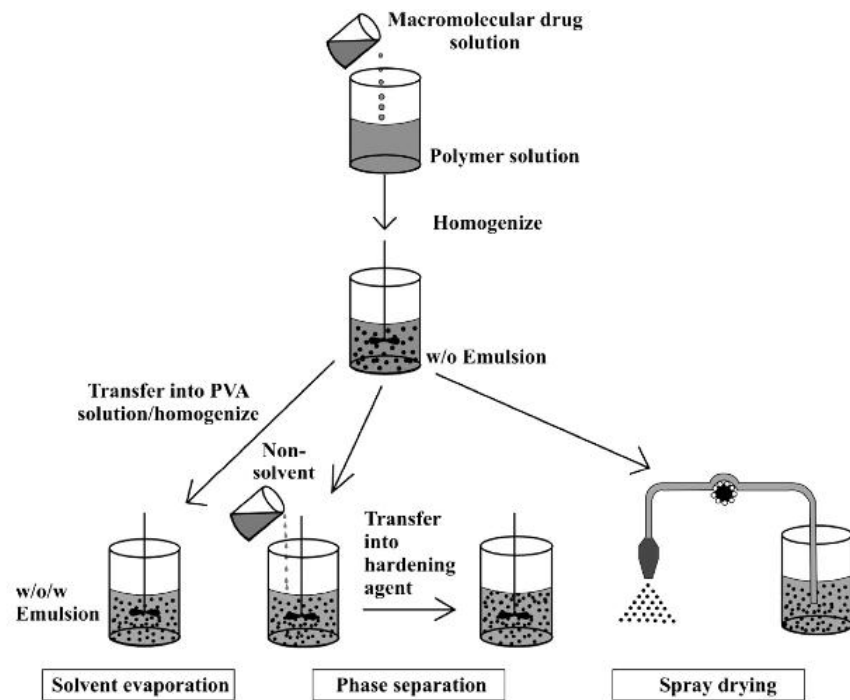


Figure 1-1. Diagram of common microencapsulation techniques: solvent evaporation, phase separation, and spray drying. Adapted from [41].

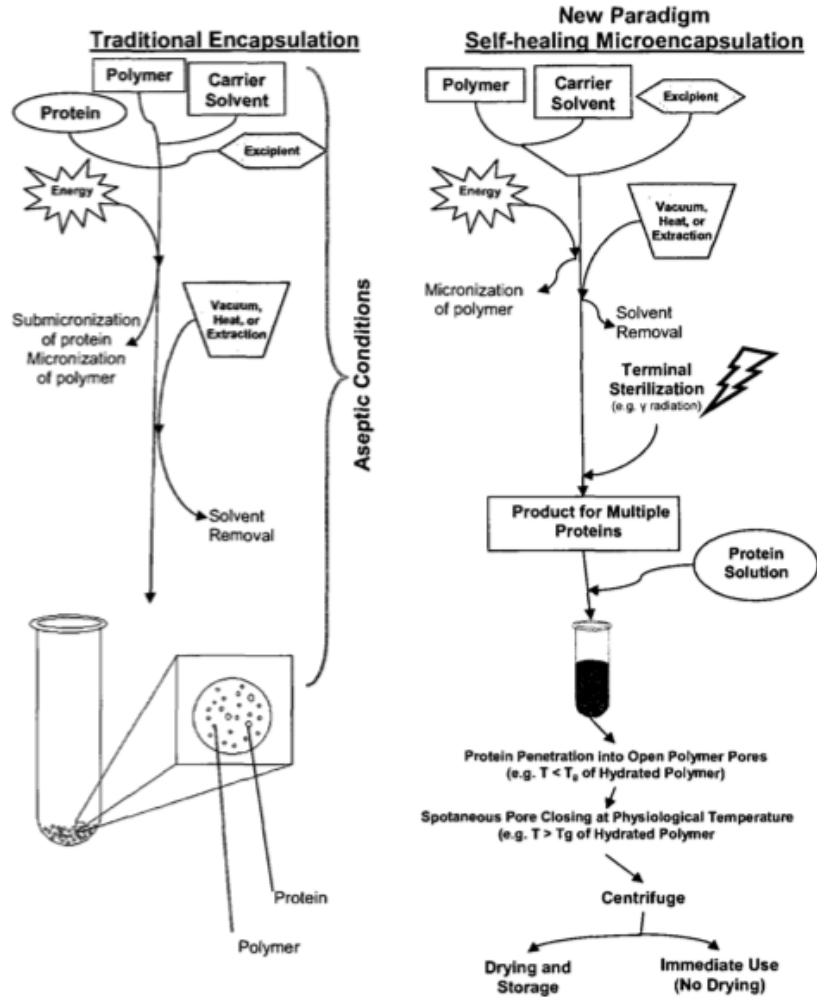


Figure 1-2. Comparison of the self-encapsulation process to traditional encapsulation. Unlike traditional methods, self-encapsulation offers the ability to sterilize the microspheres prior to antigen loading. Adapted from [11].

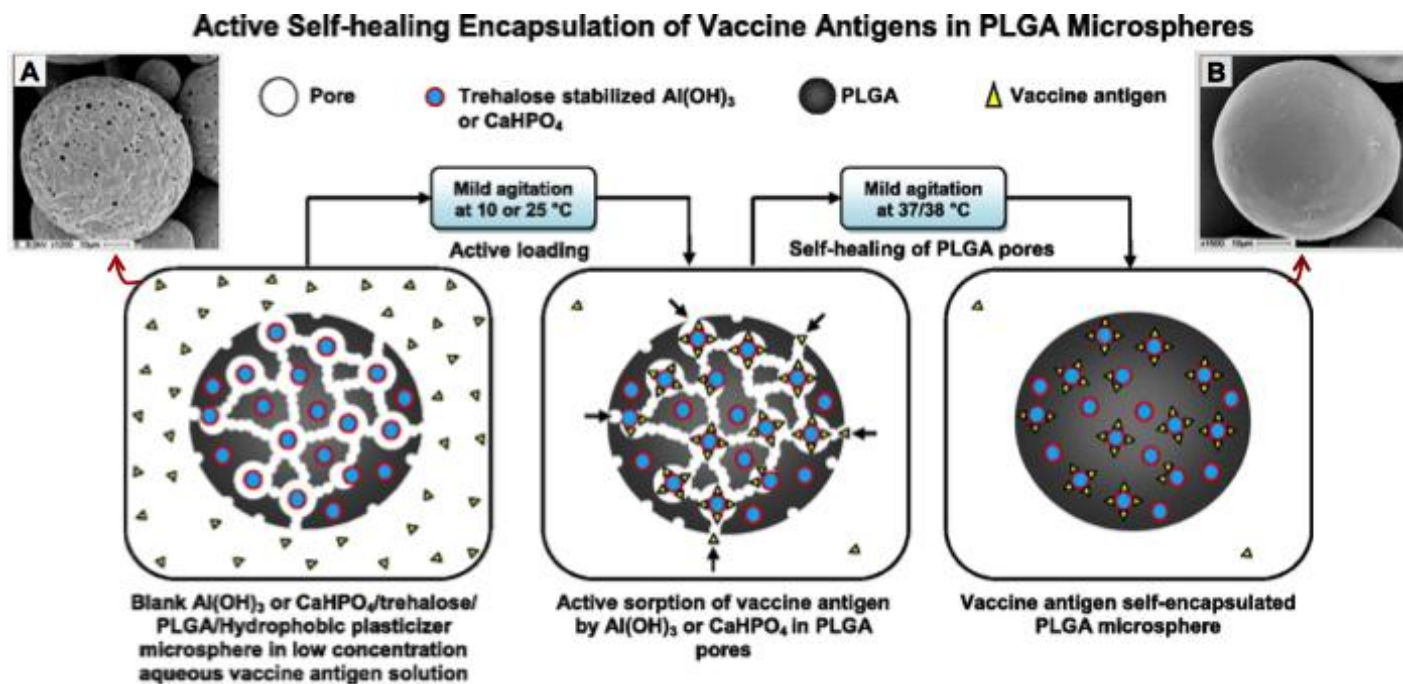


Figure 1-3. Diagram of the stages of self-encapsulation. Porous microspheres (inset A), with an internal protein-trapping agent (aluminum or calcium phosphate adjuvant), are mixed in an aqueous solution of antigen under mild agitation. This mixture is initially incubated at lower temperatures for active loading to take place. During this step, the antigen diffuses into the polymer pores and sorbs to the protein-trapping agent. The mixture is then heated at a temperature above the glass-transition temperature of the polymer for the pores to heal (close), thereby encapsulating the antigen within the microspheres (inset B). Adapted from ([8], [9]).

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Chapter 2

Development and Characterization of Self-Encapsulating PLGA Microspheres for Vaccine Delivery

2.1 Abstract

Conventional preparation methods for microspheres of poly(lactic-*co*-glycolic acid) (PLGA) generally subject the antigen to destabilizing conditions, including organic solvent and shear stresses. Recently, we developed a technique, termed “self-encapsulation,” for the loading of large molecules into pre-made, porous PLGA microspheres. By applying this loading method, the antigen can better maintain its bioactivity and immunoreactivity since it bypasses many of the denaturing conditions of conventional techniques. In this study, we further expand upon this approach by developing a formulation of self-encapsulating (SE) microspheres for vaccine delivery. The formulation was characterized, and an intranasal immunization study was performed in mice to investigate the immune response generated by the microspheres. CaHPO₄ adjuvant gel was incorporated into the microspheres as a protein-trapping agent for improved encapsulation efficiency. The formulation demonstrated continuous release of OVA model antigen over a 49-day period. Released OVA maintained its antigenicity over the first 21 days of release, confirming the stability of the aqueous SE method. Mice were immunized intranasally with prime and booster doses of OVA (10 µg), loaded into

microspheres or co-administered with cholera toxin B (CTB) mucosal adjuvant. Microspheres generated a Th2-type response in both serum and local mucosa, with IgG antibody responses approaching those generated by CTB. This formulation of self-encapsulating microspheres shows promise for further study as a vaccine delivery system.

2.2 Introduction

Vaccination is one of the most successful public health interventions, as evidenced by the eradication of smallpox and polio, which is approaching eradication. Despite these successes, there is still much room for improving vaccine coverage and efficacy. One major challenge to vaccine coverage is that many vaccines require multiple immunizations for protective immunity ([1], [2]). Non-compliance with vaccination programs can reach 70% in some developing countries, meaning that a large part of the population is under-immunized ([1], [2]). In addition, new generation vaccines will have quite a challenge inducing potent immunity considering poorly immunogenic antigens and complex vaccine targets [3].

Understanding these difficulties has led to the development of novel approaches to vaccine delivery, with the goal of improving vaccine performance [4]. Antigen delivery systems (e.g. microspheres, nanoparticles, and liposomes) and adjuvants (e.g. aluminum and calcium compounds) have been widely explored as approaches to improve the immune response generated by the antigen. This is especially true for subunit- and recombinant protein antigens, which are generally poorly immunogenic on their own due to difficulty activating antigen-presenting cells and their short *in vivo* half-life ([5], [6]). Adjuvants and delivery systems may improve antibody- and cell-mediated immunity,

decrease the amount of antigen required in a dose, and decrease the number of doses necessary for immunization, among other benefits [4].

Polymeric microspheres and nanoparticles have been widely researched for their use as vaccine delivery systems. One of the most commonly used polymers for this application is poly(lactic-*co*-glycolic acid) (PLGA) ([5], [1]). PLGA particles have been of interest due to their biodegradable and biocompatible nature and their proven safety record in humans [7]. Depending on polymer molecular weight and the ratio of lactic to glycolic acid components, the release of antigen from PLGA particles can occur over a few days or be sustained for over a year [8]. In addition, studies performed with PLGA microspheres have demonstrated their capacity to induce both antibody- and cell-mediated immune responses in mice [9].

Despite the benefits of PLGA particles, there are a number of challenges facing the development of this delivery system. One of the key challenges is antigen instability, which can occur during microsphere production, lyophilization and storage of the microspheres, and during antigen release ([10], [7]). Traditional microsphere production methods expose the antigen to detrimental conditions, which include forces from emulsification procedures and exposure to aqueous/organic solvent interfaces ([10],[11]). Protein instability takes such forms as aggregation and structural unfolding and can result in a significant loss of protein antigenicity ([12], [13]).

In an effort to improve protein stability in PLGA particles, our group has developed an encapsulation paradigm, termed “self-encapsulation,” that bypasses many of the challenges of traditional PLGA encapsulation techniques ([10], [11]). This method relies on the ability of polymer surface pores to heal (close) at temperatures above the

glass transition temperature (T_g) of the polymer [14]. The self-encapsulating microspheres contain an interconnecting pore network and an inner protein-trapping agent, such as an aluminum- or calcium-based adjuvant gel, that is accessible through the pores. Through simple mixing of the microspheres in an aqueous solution of antigen, the antigen diffuses into the pores and binds to the trapping agent. Subsequent heating of the system above the T_g results in pore healing and closure of loaded protein within the microsphere. Due to its gentle processing conditions, self-encapsulation is a promising approach for maintaining the antigenicity of encapsulated antigen.

In this work, we investigate the application of self-healing microencapsulation to the delivery of vaccine antigens. Herein, we describe the development and characterization of a formulation of self-encapsulating microspheres for antigen delivery. Formulation characteristics were studied, including the (1) incorporation of protein-trapping agent (CaHPO_4 adjuvant gel), (2) encapsulation of ovalbumin model antigen, (3) release kinetics, and (4) stability and antigenicity of released ovalbumin. The formulation was then tested in an intranasal immunization study in mice to explore the type and quality of the immune response generated.

2.3 Materials and Methods

2.3.1 Materials

PLGA 50:50 (i. v. = 0.60 dL/g, $M_w = 53.4$ kDa, ester terminated) was purchased from Lactel (Durect Corporation, USA). Poly(vinyl alcohol) (PVA) (88% hydrolyzed, $M_w = 25$ kDa) was purchased from Polysciences Inc. (USA). Alexa Fluor 647-OVA was purchased from Molecular Probes (Thermo Fisher Scientific Inc., USA). Cholera toxin B

subunit, ovalbumin grade V, and rhodamine 6G were purchased from Sigma (USA). Endofit OVA for immunization studies was purchased from Invivogen (USA). Biotinylated goat anti-mouse IgG, IgG₁, IgG_{2C}, and IgA were purchased from Southern Biotech (USA). RPMI 1640 medium, heat-inactivated FBS, L-glutamine, penicillin/streptomycin, 2-mercaptoethanol, and ACK lysis buffer were purchased from Invitrogen (Thermo Fisher Scientific Inc., USA). PMA/ionomycin (Cell Stimulation Cocktail) was purchased from eBioscience (USA). All other reagents and solvents purchased from commercial suppliers and were of analytical grade or higher.

2.3.2 Preparation of CaHPO₄ Adjuvant Gel

CaHPO₄ adjuvant gel was prepared as described in [15]. Equal parts Na₂HPO₄ and CaCl₂ were rapidly mixed together, and the pH immediately adjusted to 6.8-7.0 with NaOH. The resulting precipitate was washed 5 times with sterile 0.9% NaCl to remove excess phosphate and then resuspended in 0.9% NaCl to the desired concentration.

2.3.3 Preparation of Self-Encapsulating PLGA Microspheres

Porous, self-encapsulating PLGA microspheres were prepared by the w/o/w double emulsion-solvent evaporation technique. Calcium phosphate adjuvant gel (CaHPO₄) was included as a protein-trapping agent and trehalose as a porosigen. The inner water phase contained calcium phosphate gel (28 mg/mL) and trehalose (28 mg/mL) in 25 mM succinate buffer (pH 4.0). One hundred microliters of inner water phase was added to 1 mL of 50 mg/mL PLGA in methylene chloride. The mixture was sonicated at 50% amplitude for 1 minute using a Sonics Vibra-Cell VC130 Ultrasonic Processor (Sonics & Materials Inc., USA) to form the first emulsion. Four milliliters of

5% (w/v) PVA solution (used as an emulsion stabilizer) was added to the primary w/o emulsion, and the mixture was vortexed (Genie 2, Scientific Industries Inc., USA) for 30 seconds to produce the w/o/w double emulsion. The w/o/w emulsion was poured into 100 mL of chilled 0.5% (w/v) PVA under rapid stirring and hardened at room temperature for 6 hours. Microspheres were passed through a 10- μ m mesh sieve, and the filtrate was collected and centrifuged (7000 rpm for 5 minutes). The microspheres in the pellet were washed repeatedly with double-distilled (dd) H₂O and then lyophilized (FreeZone 2.5, Labconco, USA).

2.3.4 Determination of CaHPO₄ Loading by ICP-OES

Loading of CaHPO₄ in PLGA microspheres was determined by inductively coupled plasma-optical emission spectrometry (ICP-OES). Briefly, approximately 10 mg of microspheres were added to 1 mL acetone, vortexed, and then centrifuged at 7000 rpm for 5 minutes. The supernatant polymer solution was removed and the pellet was washed twice more with 1 mL acetone. The resulting residue was dried in a fume hood to allow for evaporation of the acetone. One milliliter of 30% HCl was added to each sample to dissolve the residue. The samples were then diluted with ddH₂O to bring the theoretical concentration of Ca ion in the samples to between 1 and 15 ppm. The concentration of Ca ion was then analyzed by ICP-OES (Perkin-Elmer Optima 2000 DV with Winlab software). Each sample was measured three times and analysis was done in triplicate.

2.3.5 Distribution of CaHPO₄ Gel within Microspheres

To view the distribution of CaHPO₄ gel within the particles after microsphere preparation, the gel was first made fluorescent by pre-loading it with a fluorescent

ovalbumin conjugate. CaHPO₄ gel was incubated with an Alexa Fluor 647-OVA conjugate at a ratio to meet the capacity of the gel for the OVA. The mixture was incubated at 37°C for 2 hours under mild agitation on a table-top shaker. Following loading of the protein onto the gel, the gel was then used in the inner water phase for microsphere preparation. Fluorophore-loaded PLGA microparticles were produced by encapsulating Rhodamine 6G in the oil phase. Lyophilized microspheres were resuspended in ddH₂O and placed on a glass slide and covered with a coverslip. The sample was viewed using a Nikon A-1 spectral confocal microscope with NIS Elements software (Nikon Instruments).

2.3.6 Scanning Electron Microscopy

The surface morphology of microspheres was examined using a Hitachi S3200N scanning electron microscope (SEM) (Hitachi, Japan). Briefly, lyophilized microspheres were fixed on a brass stub using double-sided carbon adhesive tape. The sample was made electrically conductive by coating with a thin layer of gold for 120 seconds at 40 W under vacuum. Images were taken at an excitation voltage of 8.0 kV. EDAX® software was used to obtain the final image.

2.3.7 Microsphere Size and Zeta Potential Analysis

The volume median diameter of the microspheres was measured using a Mastersizer 2000 (Malvern Instruments Ltd, UK). Ten milligrams of lyophilized microspheres were suspended in 5 mL 0.5% PVA solution and briefly vortexed. Six measurements were performed per sample at a stir speed of 2000 rpm and sampling time of 15 seconds. Zeta potential was measured with a Zetasizer Nano ZSP (Malvern

Instruments Ltd, UK). Lyophilized microspheres were suspended in ddH₂O (0.1% mass) and placed in a disposable folded capillary zeta cell (Malvern Instruments Ltd, UK). Each sample was measured six times.

2.3.8 Active Self-Encapsulation of Ovalbumin by PLGA Microspheres

Active encapsulation of ovalbumin (OVA) by PLGA microspheres was carried out in two phases: an initial incubation of the porous microspheres in protein solution at a temperature below the polymer T_g for protein sorption onto the CaHPO₄ gel, and then incubation at a temperature above the T_g for the polymer pores to heal. Briefly, approximately 20 mg of lyophilized microspheres were incubated in an OVA solution (0.5 mg/mL OVA in 0.4 mL 10 mM MOPS buffer, pH 7.4) at 4°C for 24 hours and then 25°C for 24 hours ($T < T_g$), followed by incubation at 42°C for 48 hours ($T > T_g$). Incubation was performed under constant agitation using a rigged rotator (Glas-Col, USA).

2.3.9 Determination of Protein Loading and Encapsulation Efficiency

After self-encapsulation of the protein, the microsphere samples were centrifuged at 7000 rpm for 5 minutes. The supernatant was passed through a low protein-binding Durapore (PVDF) membrane-based syringe filter unit (Millipore Corporation, USA) and the filtrate collected. The microspheres were then washed once with ddH₂O and the rinse filtered and collected. A modified Bradford assay was used to determine OVA concentration in the filtrates. Coomassie Plus® reagent (Thermo Fisher Scientific, USA) was added to the appropriate volume of standard OVA solution or filtrate sample in a 96-well plate (Nunc, Thermo Scientific, USA). After 10 minutes, the absorbance was read at

595 nm using a Dynex II MRX microplate reader (Dynex Technology Inc., USA). The mass of OVA encapsulated by the microspheres was calculated by subtracting the mass of OVA in the filtrates from the mass of OVA in the initial loading solution. Percent w/w loading and encapsulation efficiency (EE) were quantified with the following formulas:

$$\% \text{ w/w loading: } \frac{\text{mass of OVA encapsulated by the microspheres}}{\text{mass of microspheres in loading sample} + \text{mass of OVA encapsulated}} \times 100$$

$$\% \text{ EE: } \frac{\text{mass of OVA encapsulated by the microspheres}}{\text{mass of OVA in initial loading solution}} \times 100$$

2.3.10 Capacity of Unencapsulated CaHPO₄ Gel for Ovalbumin

To determine the capacity of unencapsulated CaHPO₄ gel for OVA, 1 mg of gel was incubated with 200 µg OVA (0.5 mg/mL in 10 mM MOPS buffer, pH 7.4) at 25°C for 24 hours under mild agitation. Following incubation, the mixture was centrifuged at 7000 rpm for 5 minutes. The mass of OVA remaining in the supernatant was determined using a modified Bradford assay. Based upon the mass of OVA in the original loading solution and the mass of OVA in the supernatant, the amount of OVA loaded onto the gel was calculated. The capacity of the gel for OVA was determined by taking the ratio of OVA loaded and the mass of gel present in the loading mixture.

2.3.11 Distribution of Encapsulated Ovalbumin within Microspheres

To observe the distribution of self-encapsulated OVA within microspheres, fluorescently-labeled OVA was loaded into microspheres containing a rhodamine dye in

the polymer phase and observed by confocal microscopy. Briefly, rhodamine 6G was added to the oil phase during microsphere production to dye the polymer. The microspheres were then used to load Alexa Fluor 647-OVA using the above self-encapsulation process. Directly following encapsulation, the loaded microspheres were centrifuged at 7000 rpm for 5 minutes and the supernatant removed. Next, the microspheres were rinsed once with ddH₂O. Following centrifugation and removal of the supernatant, the microspheres were resuspended in ddH₂O and placed on a glass slide and covered with a coverslip. The sample was viewed using a Nikon A-1 spectral confocal microscope with NIS Elements software (Nikon Instruments).

2.3.12 Size Exclusion-High Performance Liquid Chromatography of Ovalbumin

Size exclusion-high performance liquid chromatography (SE-HPLC) was performed using a TSKgel G3000SWxl column (Tosoh Bioscience, USA) on a Waters HPLC system (Waters, USA). The mobile phase consisted of PBS, pH 7.4, at a flow rate of 0.7 mL/min and injection volume of 50 μ L. Protein detection by UV was done at 210 and 280 nm.

2.3.13 Evaluation of Ovalbumin Stability During Self-Encapsulation

Ovalbumin stability in the conditions for self-encapsulation was investigated by incubating OVA solutions at the different temperatures required for the encapsulation process. OVA solutions (0.5 mg/mL) in 10 mM MOPS buffer, pH 7.4, were incubated under mild agitation on a rotator. One sample each was incubated at 4°C for 24 hours, 25°C for 24 hours, 42°C for 48 hours, and 4°C for 24 hours + 25°C for 24 hours + 42°C for 48 hours. A separate sample was incubated at 80°C for 24 hours to induce

denaturation. The samples were then analyzed by SE-HPLC to examine OVA aggregation.

2.3.14 *In Vitro* Release of Ovalbumin from Self-Encapsulating Microspheres

Approximately 20 mg of OVA-encapsulated microspheres were incubated in 0.5 mL PBS, pH 7.4, at 37 °C under constant agitation (240 rpm/min). At different incubation times (1, 3, 5, and 7 days, and then every 7 days until day 49), the mixture was centrifuged at 7000 rpm for 5 minutes and the supernatant was collected through a low protein-binding Durapore (PVDF) membrane-based syringe filter unit (Millipore Corporation, USA). Fresh release media (0.5 mL) was then added to the sample and the microspheres were resuspended to continue the release study. The OVA content in the supernatants was analyzed by SE-HPLC.

2.3.15 ELISA for Quantifying Release of Antigenic Ovalbumin

In vitro release samples collected and analyzed by SE-HPLC, as described above, were further analyzed by enzyme-linked immunosorbent assay (ELISA) to quantify the amount of antigenic OVA in each sample. Samples were collected at time points of 1, 3, 5, 7, 14, and 21 days. Antigenic OVA was detected using a commercial chicken egg ovalbumin ELISA kit (Alpha Diagnostic International, USA), which was used according to the manufacturer's instructions. Samples were diluted with the sample diluent provided with the kit to fall within the working range of the assay and then assayed in duplicate. The dilution was based upon the calculated concentration of OVA in the samples determined by SE-HPLC. The ELISA plate was read at 405 nm using a Synergy Neo plate reader with Gen5 software (Biotek Instruments Inc, USA).

2.3.16 Extraction of Unreleased Protein

At the end of release, microspheres were dissolved in acetone (3 washes at 1 mL acetone per wash) to remove the polymer. The CaHPO₄ gel and OVA pellet was allowed to dry in a fume hood before re-suspension in 1 mL 10% w/v sodium citrate to elute soluble OVA from the gel. The samples were analyzed by SE-HPLC to determine remaining soluble OVA. The residue was then dissolved in 0.1 mL of a denaturing (6 M urea, 1 mM EDTA) plus reducing agent (10 mM dithiothreitol) to dissolve any noncovalent and disulfide-bonded aggregates, respectively. The aggregated OVA was quantified using a modified Bradford assay as previously described.

2.3.17 Immunization Study

Female C57BL/6 mice, 6-7 weeks old, were purchased from Harlan Laboratories, Inc. and handled according to the University of Michigan Institutional Animal Care guidelines. Mice (10 mice per group) were immunized intranasally (i.n.) with 15 µL (~7.5 µL per nare) of sterile PBS, cholera toxin B (CTB) co-administered with OVA (10 µg CTB + 10 µg OVA), or microspheres loaded with OVA (10 µg OVA). A booster dose was given three weeks later on day 21 after primary immunization. On days 20 and 41, blood samples were collected by submandibular bleed for analysis of serum antibody titers. Blood was collected into Microvette 500 Z-Gel serum collection tubes (Sarstedt, Germany) and centrifuged at 10,000 x g for 5 minutes to separate the serum. Serum was stored at -80°C until analysis.

2.3.18 Bronchial Alveolar Lavage

Mice were euthanized on day 42 and bronchial alveolar lavage (BAL) was performed for analysis of mucosal antibody titers. Briefly, the trachea were cannulated and lavage was carried out with approximately 0.75 mL of PBS. The bronchial alveolar lavage fluid (BALF) was centrifuged and the supernatant collected and stored at -80°C until analysis with ELISA.

2.3.19 Measurement of Antibody Titers

Serum and BALF samples were sent to the Immunology Core at the University of Michigan Cancer Center for ELISA analysis. The assays were performed using a standard ELISA protocol. Briefly, OVA (10 µg/mL in carbonate/bicarbonate buffer) was used to coat 384-well plates overnight at 4°C. Plates were washed and residual binding sites blocked with 0.2% casein in Tris-buffered saline (TBS) for at least one hour. The same buffer was used for sample dilutions. Ig detection antibodies were minimally diluted to the manufacturer's suggested working range. IgG, IgG₁, and IgG_{2c} response was measured for both serum and BALF samples, while IgA response was also included for BALF.

2.3.20 Cytokine Response

On day 42, mouse spleens were collected under sterile conditions. Splenocytes were isolated by gentle disruption of the spleen through a mesh filter, and red blood cells were lysed with ACK lysis buffer and washed. Cells were plated at 5×10^5 cells/well in a 96-well tissue culture plate (Corning Inc., USA) with RPMI 1640 medium supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, 100 U/mL penicillin, 100 µg/mL

streptomycin, and 55 μ M 2-mercaptoethanol. Cells were tested in duplicate and treated with medium (negative control), 2 μ L/mL PMA/ionomycin (positive control), 25 μ g/mL whole OVA, or 2 μ g/mL OVA MHC class I or class II peptide. After 96 hours of incubation (37°C, 5% CO₂), the well supernatants were collected and stored at -80°C until analysis. Samples were submitted to the Immunology Core at the University of Michigan Cancer Center for ELISA analysis of cytokine response.

2.3.21 Statistical Analysis

Statistical analyses were carried out using GraphPad Prism 6.0g software. Two-way ANOVA with Bonferroni's post-test was used to compare multiple groups. Values are reported as mean \pm SEM (standard error of the mean).

2.4 Results and Discussion

2.4.1 Preparation and Characterization of Self-Encapsulating Microspheres

Self-encapsulating PLGA microspheres were prepared by a standard double emulsion-solvent evaporation technique. Trehalose was added to the inner water phase to act as a porosigen to create the interconnected pore network in the microspheres. This pore network is necessary for the protein in the loading solution to diffuse through the pores into the microspheres during self-encapsulation [11]. As shown in Figure 1(a), scanning electron microscopy was used to verify that the formulation produced particles that displayed a spherical morphology and were porous in nature.

2.4.1.1 Incorporation of CaHPO₄ Adjuvant Gel

Calcium phosphate (CaHPO₄) adjuvant gel was present in the inner water phase to be incorporated into the microspheres. The purpose of the gel was to act as a protein-trapping agent within the microsphere pores. In previous work done by our lab, use of a protein-trapping agent, such as Al(OH)₃ or CaHPO₄ adjuvant gels, improved the encapsulation efficiency of the self-encapsulating microspheres compared to microspheres without an inner trapping agent ([11], [10]). To distinguish the two strategies, use of a protein-trapping agent is termed “active” self-encapsulation.

Calcium phosphate adjuvant was selected as the protein-trapping agent in this study. Calcium phosphate is an alternative to the aluminum-based adjuvants. It is well-tolerated and readily resorbed, being a natural constituent of the body [15]. CaHPO₄ has been used in France for many years as an adjuvant with diphtheria-tetanus-pertussis (DTP) vaccines [16]. Unlike aluminum adjuvants, calcium phosphate does not lead to enhanced IgE production in animals and humans [15]. The adjuvant is thought to act as an antigen depot and improve uptake of the antigen by antigen-presenting cells (APCs) ([1], [17], [15]).

The CaHPO₄ gel was prepared by rapid mixing (<10 seconds) of equimolar solutions of disodium hydrogen phosphate and calcium chloride, as described by Gupta *et al.* [15]. Gel prepared by rapid mixing was found to have much better adsorption of diphtheria toxoid compared to gel made by slower mixing (10 minutes) [15]. The adjuvant was then washed thoroughly with 0.9% sodium chloride to remove excess phosphate ions that would interfere with antigen adsorption.

Successful incorporation of the adjuvant in the microspheres was confirmed by ICP-OES (Table 1). In addition to measurement of the % w/w loading of the adjuvant ($2.2 \pm 0.1\%$), the distribution of the gel within the microspheres was viewed by confocal microscopy (Figure 2a). The CaHPO_4 gel loaded with Alexa Fluor 488-ovalbumin was used in the inner water phase to be incorporated into microspheres. The gel was observed to be loaded within both smaller and larger microspheres and to be homogeneously distributed.

2.4.1.2 Microsphere Size and Zeta Potential

The formulation parameters were designed to produce microspheres of a size range smaller than 10 μm . In particular, sonication was used to form the primary emulsion since greater agitation is known to generate smaller particles [18]. A smaller microsphere size was desired for improved immune response as research has shown that particles smaller than 10 μm are preferentially phagocytosed by APCs compared to larger particles, with smaller particles eliciting a stronger immune response ([19], [20], [21], [22], [23]). The median microsphere diameter was found to be within the desired size range ($7.05 \pm 0.31 \mu\text{m}$), with a zeta potential of $-21.9 \pm 2.1 \text{ mV}$ (Table 1).

Based upon their size, microspheres may carry out a number of functions. For example, microspheres can (1) act as a depot for continued release of antigen, prolonging exposure of the immune system to the antigen; and (2) facilitate uptake of the antigen by APCs, improving delivery of the antigen to the lymphoid organs [24]. Consequently, microsphere size is an important consideration for antigen delivery.

2.4.2 Self-Encapsulation of Ovalbumin Model Antigen

Ovalbumin was loaded into microspheres by the active self-encapsulation process. Lyophilized microspheres were initially incubated in a solution of ovalbumin at lower temperatures, with mild mixing, to allow the protein to diffuse through the pores and sorb onto the CaHPO₄ gel. Following this stage, the mixture was then incubated at a temperature above the T_g of the polymer in order for the microsphere pores to heal, thereby trapping the antigen within the particle. SEM was used to verify pore closure after self-encapsulation (Figure 1b).

Results of the self-encapsulation are shown in Table 2. The mass of ovalbumin loaded into the microspheres was assessed by measuring the mass loss from the original loading solution. The measured % w/w loading of ovalbumin was found to be 0.60 ± 0.05%. By comparing the ovalbumin capacity of the microspheres to that of the unencapsulated CaHPO₄ gel (Table 2), it suggests that incorporation of the gel within the microspheres did not adversely affect the ability of the gel to sorb the protein. CaHPO₄ may adsorb antigen by electrostatic interaction, hydrophobic interaction, and ligand exchange [17]. The latter occurs by exchange of hydroxyl groups in the gel with phosphate groups in the protein. Commercial ovalbumin, such as the type used in this study, contains 0-2 mol PO₄ covalently bound per mol of protein [25].

Self-encapsulation of ovalbumin was also monitored by confocal microscopy. Microspheres were made fluorescent by addition of rhodamine 6G into the polymer phase during microsphere preparation, and the self-encapsulation process was carried out with Alexa Fluor 448-ovalbumin. Incorporation of the protein into the microspheres was

confirmed by confocal microscopy (Figure 2b). Orthogonal images verified that protein was internal to the microspheres and not on the particle surface.

2.4.3 Release of Encapsulated Ovalbumin

Sustained release of ovalbumin from the microspheres was observed over a 49-day period, with a moderate burst release of 28% (Figure 3). Ovalbumin in the *in vitro* release media was quantified by SE-HPLC, and the % cumulative release over time was calculated based on the mass of ovalbumin loaded into the microspheres. By the end of the study at day 49, 70% of the encapsulated ovalbumin was released.

At the conclusion of the release study, the remaining unreleased ovalbumin was extracted from the polymer and desorbed from the CaHPO₄ gel. The fraction of soluble and insoluble (covalent and non-covalent aggregates) remaining was determined. No unreleased soluble ovalbumin was detected, however a small fraction of insoluble aggregates (3%) was discovered after extraction (data not shown). Following mass balance, a total of 73% of the encapsulated ovalbumin was accounted for after the study.

The stability of ovalbumin at the temperatures of self-encapsulation was studied by incubating the protein in loading solution buffer at the different process temperatures for the specified periods (Figure 4). Samples of ovalbumin were treated separately at (1) 4°C for 24 hours, (2) 25°C for 24 hours, (3) 42°C for 48 hours, and (4) 4°C (24 hours) + 25°C (24 hours) + 42°C (48 hours) (the full self-encapsulation process). A separate sample of protein was also incubated at 80°C for 24 hours to purposefully induce denaturation and aggregation [26]. As can be seen from the overlaid HP-SEC chromatograms of the samples in Figure 4, aggregation of the protein was not observed

during the self-encapsulation process, as compared to the protein sample denatured at 80°C.

2.4.4 Antigenic Ovalbumin Released from Microspheres

The stability of the ovalbumin released from the microspheres was analyzed by measuring the antigenicity of the protein by ELISA. Previous work from our lab has shown improved stability of protein loaded by self-encapsulation compared to traditional encapsulation methods. Self-encapsulated tetanus toxoid maintained >96% of its antigenic activity over 28 days of release compared to only 27% for tetanus toxoid loaded by a traditional w/o/w method [10].

The antigenicity of released ovalbumin was measured over 21 days. The protein was analyzed by HP-SEC to measure total protein released and by ELISA to determine the fraction of released protein that was antigenic. From the plots shown in Figure 5, there was excellent agreement between measured total protein release and antigenic protein. This suggests that self-encapsulation of the ovalbumin did not negatively affect the stability of the model antigen.

2.4.5 Serum and Mucosal Antibody Response in Mice Following Intranasal Immunization with Microspheres

Female C57BL/6 mice were immunized via intranasal administration of microspheres loaded with OVA (10 µg OVA per dose). A booster dose was given three weeks later after primary immunization (Figure 6). The immune response generated by immunization with microspheres was compared to that for naïve mice (PBS control group) and mice immunized with cholera toxin B (CTB) (10 µg CTB + 10 µg ovalbumin).

Cholera toxin is a gold-standard adjuvant for stimulation of mucosal immune response in pre-clinical studies, but its use is limited due to its toxicity in humans [27]. The B subunit of cholera toxin maintains intranasal adjuvanticity but lacks the toxicity of the whole toxin [28].

Intranasal immunization is an attractive route for vaccine administration since the nasal cavity is easily accessible and has a high density of dendritic cells [29]. Intranasal vaccination has shown the ability to produce an antibody response in the serum and in both local and distal mucosal secretions [30]. Since many infections occur at mucosal surfaces, induction of a mucosal immune response is important for protection against the infectious agent [31].

Following prime and booster intranasal immunization of mice, the serum anti-ovalbumin antibody response was measured (Figure 7). The total IgG response was analyzed (Figure 7a), as well as titers for IgG₁ and IgG_{2C} subclasses (Figures 7b and 7c, respectively). Subclass titers provide information about the polarization of the Th response, with IgG₁ associated with a Th2-type response and IgG_{2C} associated with a Th1 response ([32], [33]). For both the CTB- and microsphere-immunized mice, the post-prime antibody response was not significantly different than the baseline response produced by the PBS control group. However, a significant total IgG and IgG₁ response was observed for both groups following booster administration. The response following immunization with microspheres approached that for the mice immunized with CTB, a strong mucosal adjuvant. Neither group displayed a significant IgG_{2C} response after prime or boost vaccination, suggesting that the response for both groups was Th2-biased.

This is confirmed in the literature since CTB is known to trigger a more Th2-type response ([34], [35]).

Apart from systemic immunity, local mucosal response was also analyzed by determining anti-ovalbumin antibody titers in bronchial alveolar lavage fluid collected after booster administration (Figure 8). The mucosal antibody response reflected the serum response in that a Th2-bias was observed due to predominant anti-ovalbumin IgG₁ titers and no detectable levels of IgG_{2C}. In addition, the IgG and IgG₁ antibody titers for the microsphere-immunized group once again approached the response generated by the CTB-immunized mice. No detectable levels of anti-ovalbumin IgA were found in the lavage fluid (data not shown), indicating a stronger systemic versus mucosal response to intranasal immunization.

2.4.6 Cytokine Response after *Ex Vivo* Stimulation of Splenocytes

The immune response was further assessed by studying the cytokine profiles of splenocytes restimulated *ex vivo*. Splenocytes were collected from immunized mice three weeks after booster administration and restimulated with whole ovalbumin and MHC class I- and II-restricted ovalbumin peptides. Splenocytes from CTB-immunized mice produced significant levels of IL-2, IL-6, IL-10, and IFN- γ compared to the PBS control group (Figure 9). While splenocytes from microsphere-immunized mice secreted detectable levels of the four cytokines, only IL-10 was produced at a statistically significant level compared to the control group. IL-2 and IFN- γ are secreted by Th1 cells, while IL-6 and IL-10 are indicative of a Th2-skewed response ([36], [37]). Hence, the cytokine results confirm the Th2-biased response suggested by the serum and mucosal antibody data for the microsphere-immunized mice.

2.5 Conclusions

Active self-encapsulation of protein within PLGA microspheres is a promising alternative approach to traditional encapsulation techniques. Conventional encapsulation methods subject the protein to detrimental conditions that can cause instability and loss of antigenicity. Previous work by our group has shown improved stability of vaccine antigen loaded into microspheres by self-encapsulation [10].

This study built upon our previous work to develop a formulation of self-encapsulating microspheres for vaccine delivery, which showed unprecedented stability of tetanus toxoid in PLGA [10]. The formulation used in this study demonstrated the ability to maintain the antigenicity of released model antigen and generated encouraging results from a proof-of-concept intranasal immunization study. This research motivates further investigation of this formulation for delivery of clinically relevant antigens.

2.6 Figures

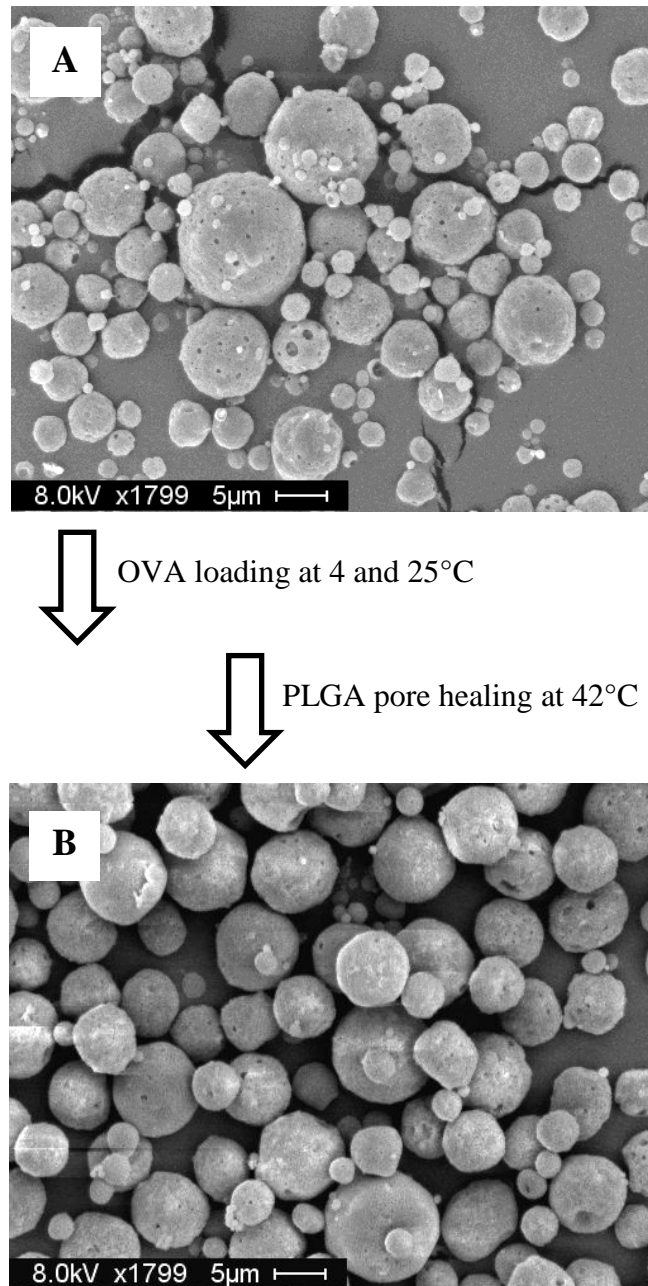


Figure 2-1. SEM images of the self-encapsulation of OVA by PLGA microspheres loaded with CaHPO_4 adjuvant gel as the protein-trapping agent. Images were taken prior to self-encapsulation of OVA (A) and after encapsulation of OVA and pore healing (B). Scale bars represent 5 μm .

Table 2-1. Composition, size, and zeta potential of the self-encapsulating PLGA microsphere formulation. Data represent mean \pm SEM, $n = 3$.

PLGA concentration (mg in 1 mL)	Volume of inner water phase (mL)	Trehalose loading (wt. %)^a	Measured CaHPO₄ loading (wt. %)^b	Volume median diameter (μm)	Zeta potential (mV)
50	0.1	5	2.2 \pm 0.1	7.05 \pm 0.31	-21.9 \pm 2.1

^aTheoretical loading

^bTheoretical loading = 5 wt. %

Table 2-2. Active self-encapsulation of ovalbumin (OVA) by PLGA microspheres. Self-encapsulation was performed sequentially at 4, 25, and 42°C to load ovalbumin into the microspheres and then heal the polymer pores. Data represent mean \pm SEM, $n = 3$.

Loading Solution			OVA Loading				
Mass of microspheres in sample (mg)	OVA concentration (mg/mL)	Volume (mL)	Theoretical % (w/w) loading ^a	Measured % (w/w) loading	% Encapsulation efficiency ^b	Capacity of CaHPO ₄ within microspheres ^{c,d}	Capacity of unencapsulated CaHPO ₄ gel ^{c,e}
20	0.5	0.4	1.0	0.60 \pm 0.05	55.6 \pm 6.0	0.27 \pm 0.03	0.209 \pm 0.004

^a (mg of total OVA in loading solution)/(mg of microspheres in sample + mg of total OVA in solution)

^b Efficiency relative to original mass of ovalbumin in loading solution

^c mg OVA loaded/mg CaHPO₄ gel

^d Based upon the determined mass of CaHPO₄ gel loaded within the microspheres

^e Determined for OVA loaded onto unencapsulated CaHPO₄ gel

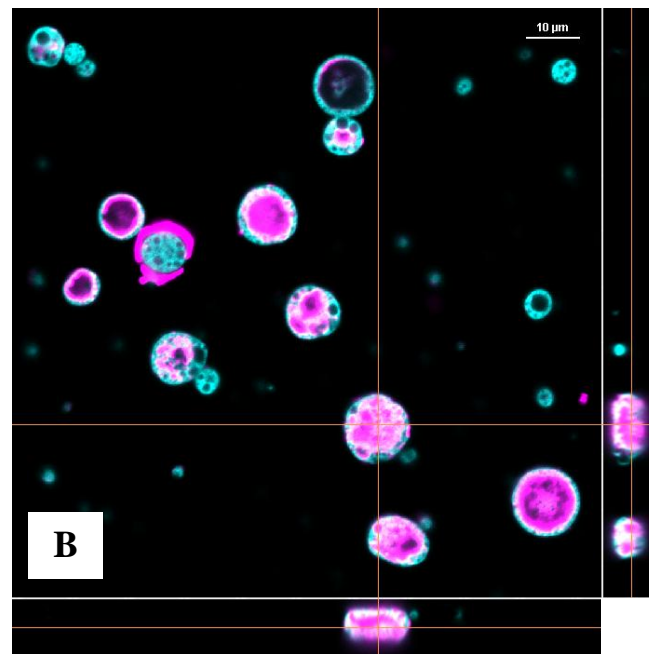
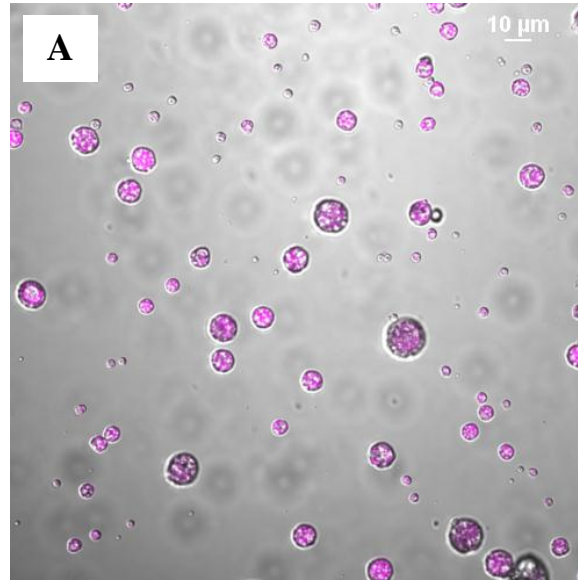


Figure 2-2. (A) Confocal microscopy (fluorescent and brightfield image overlay) of the distribution of calcium phosphate gel (violet) in microspheres. The gel was pre-loaded with Alexa Fluor 647-ovalbumin prior to incorporation in the inner water phase for microsphere production. (B) Confocal microscopy image of the distribution of Alexa Fluor 647-ovalbumin (violet) loaded within rhodamine-labeled PLGA microspheres (cyan) by self-encapsulation. Orthogonal images are shown in the right and bottom panel. Scale bar represents 10 μm.

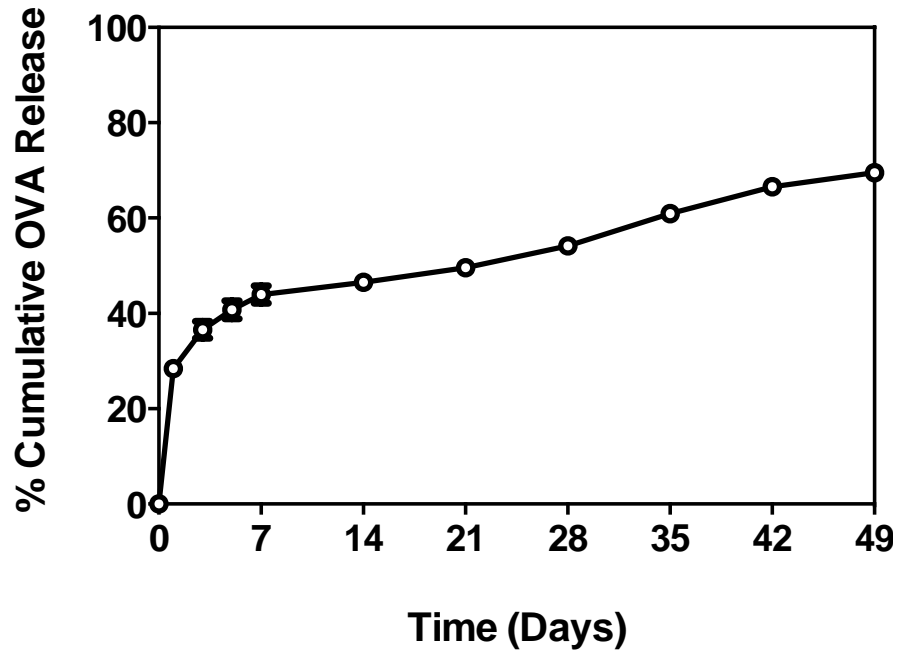


Figure 2-3. Cumulative ovalbumin released as a function of time by self-encapsulating PLGA microspheres. *In vitro* release was conducted in PBS (pH 7.4) at 37°C. Data represent mean \pm SEM ($n = 3$).

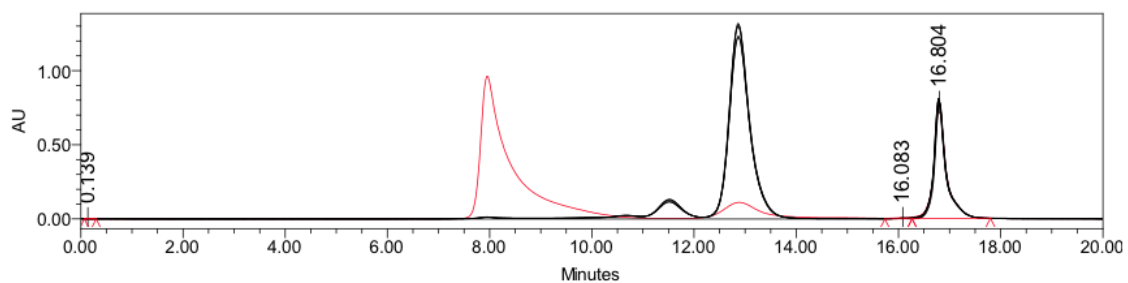


Figure 2-4. HP-SEC chromatograms of ovalbumin incubated in MOPS buffer at different temperatures. The overlaid black curves represent ovalbumin incubated at the temperatures of the self-encapsulation protocol individually and as one process (4°C for 24 hours, 25°C for 24 hours, 42°C for 48 hours, and 4°C (24 hours) + 25°C (24 hours) + 42°C (48 hours)). The red curve represents ovalbumin treated at 80°C for 24 hours to denature the protein.

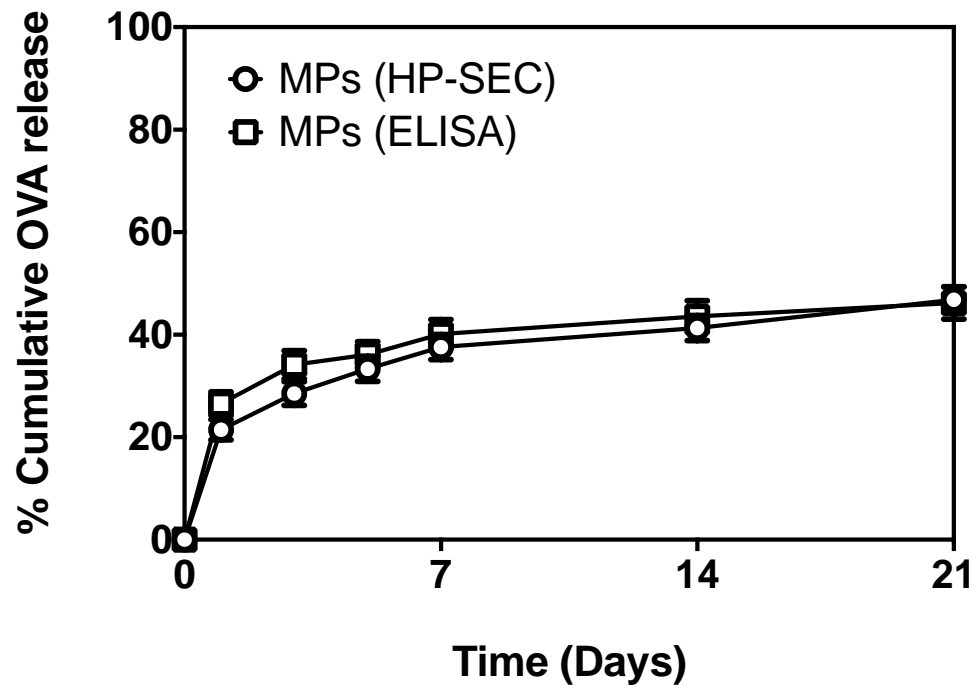


Figure 2-5. Plot of OVA released over a 21-day period from microspheres as measured by HP-SEC (●) and ELISA (□). ELISA analysis was performed to specifically measure the antigenic OVA present in the release samples.

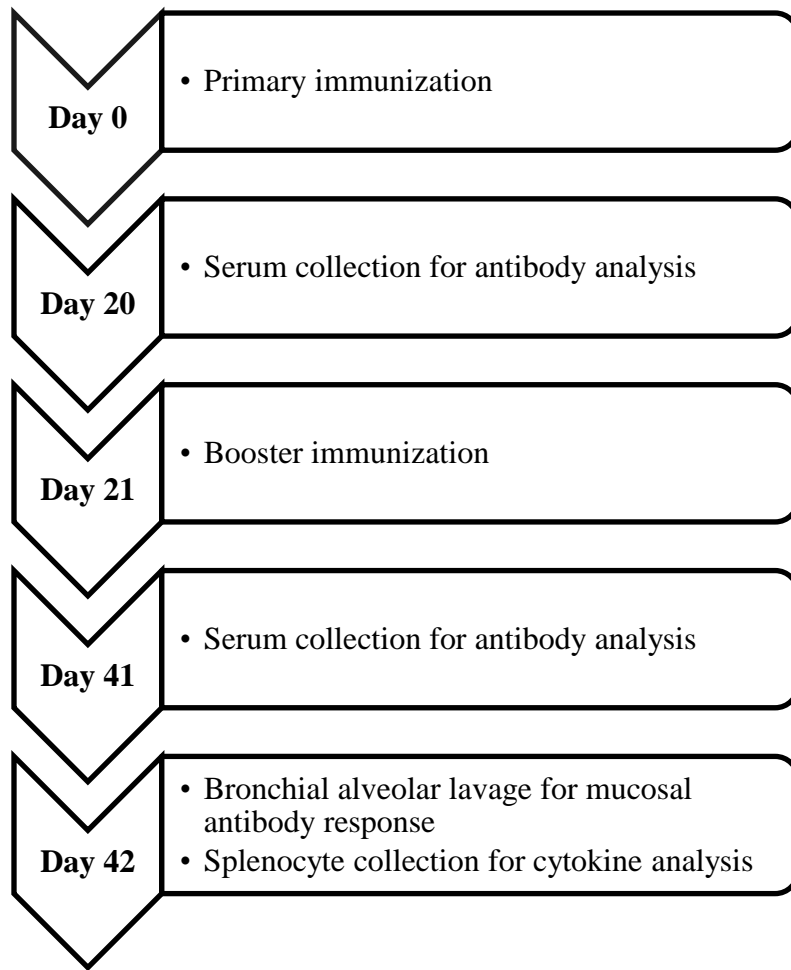


Figure 2-6. Timeline for the intranasal immunization study.

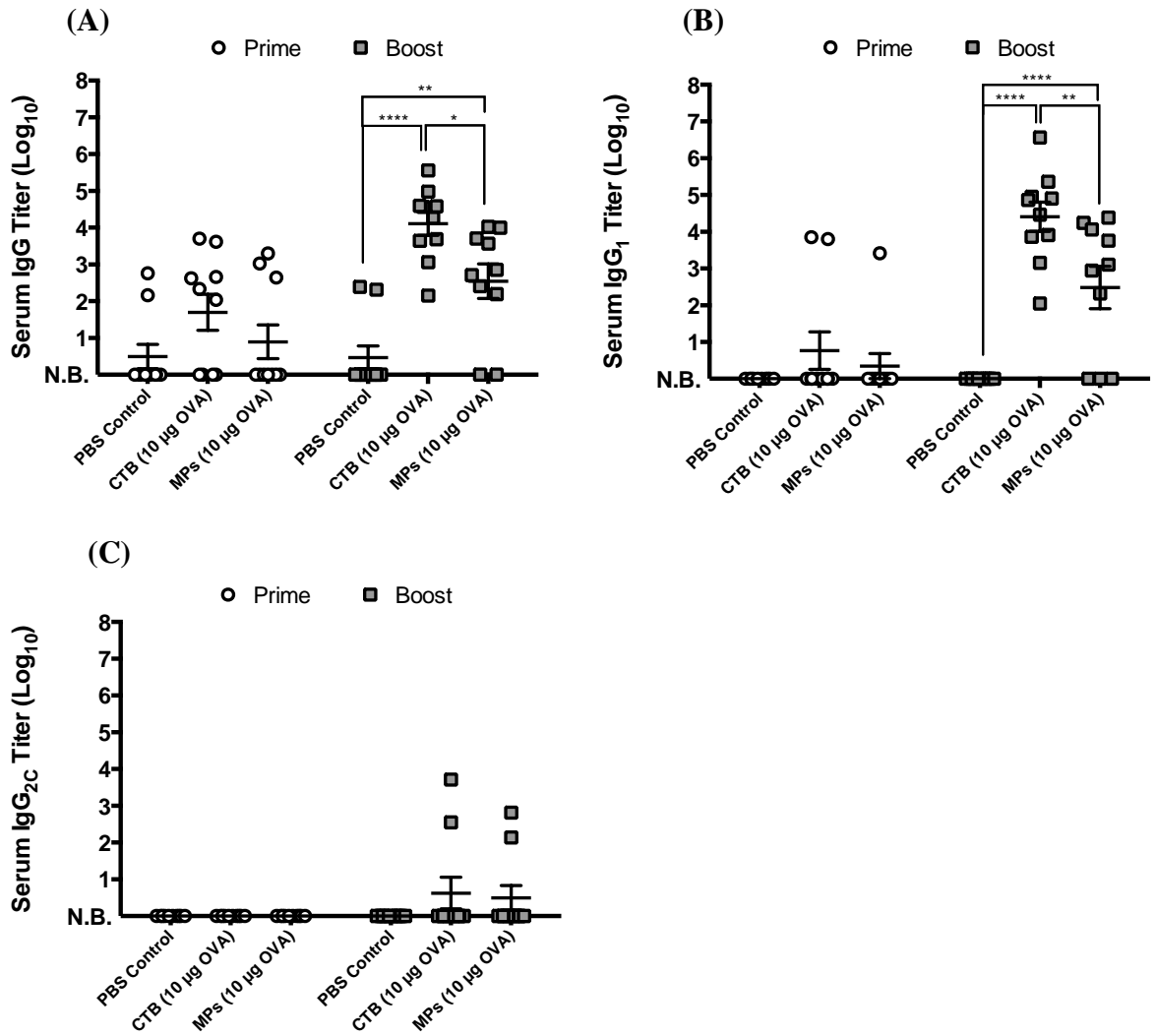


Figure 2-7. Serum anti-OVA antibody titers for groups on days 20 (prime response) and 41 (boost response). **(A)** IgG, **(B)** IgG₁, and **(C)** IgG_{2C}. Data were fit using a 4-parameter curve, and titers were calculated by solving for the inverse dilution factor resulting in an absorbance value of 0.5. Data represent mean ± SEM ($n = 10$). All groups were compared using two-way ANOVA followed by Bonferroni's post-test ($*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$, and $****p \leq 0.0001$). N.B. = no binding detected.

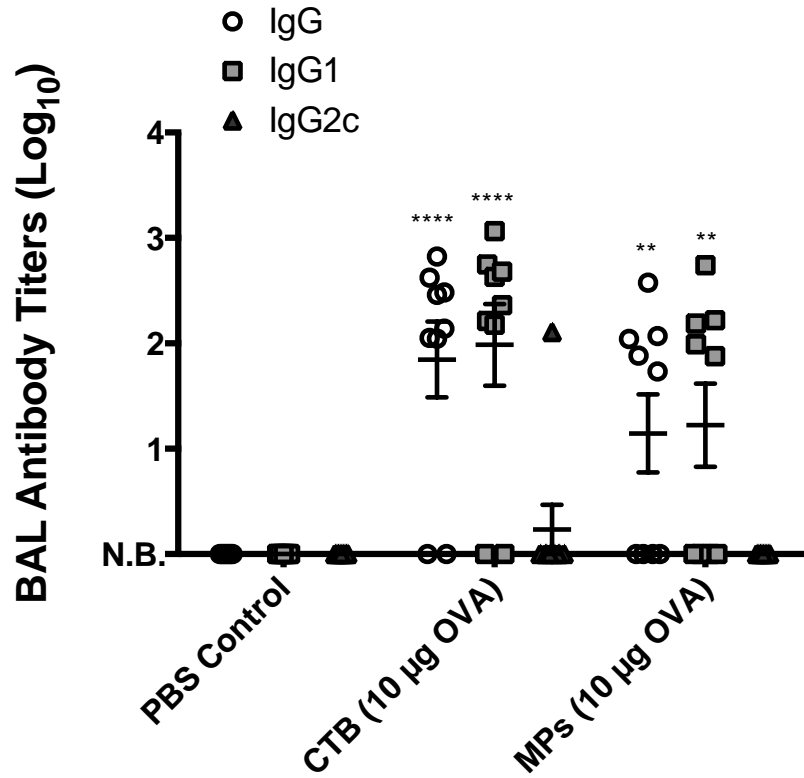


Figure 2-8. Anti-OVA antibody titers from bronchial alveolar lavage (BAL) samples collected from groups on day 42. Data were fit using a 4-parameter curve, and titers were calculated by solving for the inverse dilution factor resulting in an absorbance value of 0.5. Data represent mean \pm SEM ($n = 9$). All groups were compared using two-way ANOVA followed by Bonferroni's post-test. Statistical significance shown is in relation to the corresponding PBS control ($*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$, and $****p \leq 0.0001$). N.B. = no binding detected.

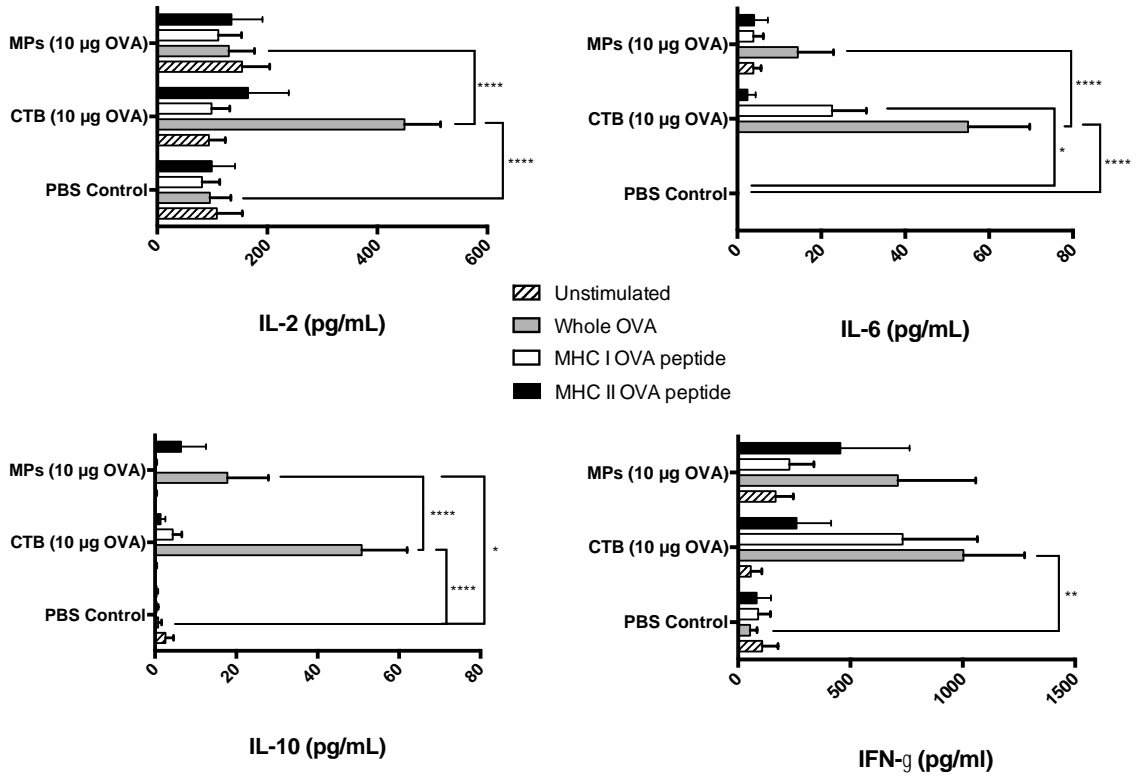


Figure 2-9. Analysis of cytokines produced from mouse splenocytes restimulated *ex vivo* with whole OVA protein and MHCI or MHCII OVA peptide. Splenocytes from intranasally immunized mice were collected three weeks post booster immunization. Data represent mean \pm SEM ($n = 5$). All groups were compared using two-way ANOVA followed by Bonferroni's post-test ($*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$, and $****p \leq 0.0001$).

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Chapter 3

Self-Encapsulating PLGA Microspheres for Delivery to Antigen-Presenting Cells and Immune Response Following Subcutaneous Immunization

3.1 Abstract

Antigen internalization and processing by antigen-presenting cells (APCs) is necessary for the induction of the adaptive immune response. Hence, antigen delivery directly to APCs is a major focus of vaccine development in an effort to promote a stronger immune response. Poly(lactic-*co*-glycolic acid) (PLGA) nano- and microspheres have been explored for this purpose due to their particulate nature and ability to be internalized by APCs. In previous work, we described the development of a formulation of self-encapsulating PLGA microspheres for vaccine delivery. Self-encapsulation is a method developed for the remote loading of antigen into pre-made PLGA microspheres, which has shown the ability to improve the maintenance of antigenicity of encapsulated protein. Here, we expand upon the formulation for delivery of vaccine antigens. We investigated the internalization of the microsphere formulation by dendritic cells, and then studied the type and magnitude of the immune response generated by the microspheres after subcutaneous immunization in mice. Our results show successful uptake of the formulation, with a median particle diameter of $7.05 \pm 0.31 \mu\text{m}$, by a

murine dendritic cell line. Subcutaneous immunization of mice with ovalbumin-loaded microspheres induced an ovalbumin-specific CD8⁺ T cell response and IgG subclass titers indicative of a mixed Th1/Th2 response. The formulation was found to perform better than Alhydrogel® at stimulating a cellular response and producing anti-ovalbumin IgG_{2C} titers. Additionally, mice given a single-dose of microspheres (containing twice the amount of antigen) generated a stronger CD8⁺ T cell response and comparable antibody response to two doses of Alhydrogel®. These results suggest that this formulation of self-encapsulating microspheres has potential for further development for vaccine delivery.

3.2 Introduction

The goal of vaccination is to induce a specific, protective immune response and long-term immunity [1]. Generation of the appropriate type of immune response for the disease or pathogen at hand is essential for vaccine efficacy. For example, an antibody or Th2-based response is more effective against extracellular pathogens, whereas a cellular or Th1-type response is generally desired against cancer and intracellular pathogens [2]. In reality, the immune response against a particular disease or pathogen may be a mixture of these responses, with one being more predominant [3].

The method of antigen presentation by a vaccine has a significant effect on the type of response that will ensue. Antigen-processing cells (APCs) are the link between innate and adaptive immunity and are a major target for vaccine delivery. APCs process exogenous (e.g. bacterial antigens) and endogenous antigen (e.g. tumor and viral products) and display it on the cell surface to be recognized by other immune cells. Exogenous antigens are processed and presented on the cell surface in context of major histocompatibility complex (MHC) class I molecules for recognition by CD8⁺ T cells,

resulting in a cell-based response ([4], [5]). On the other hand, endogenous antigens are presented with MHC class II molecules and are recognized by CD4⁺ T cells, which results in a humoral response ([4], [5]). Some APCs, such as dendritic cells, can also undergo cross-presentation, whereby exogenous antigen is presented with MHC class I [4]. However, the exact mechanism of cross-presentation is not fully understood.

Understanding the importance of antigen-APC interaction, much research has gone into improving the uptake of antigen by APCs for immune processing. In particular, polymeric micro/nanoparticle antigen delivery systems have been an intense area of focus due to their ability to (1) act as a depot to prolong the exposure of the antigen to the immune system, (2) be internalized by APCs for direct delivery of the antigen, and (3) potentially induce cross-presentation of antigen [6]. Poly(lactic-*co*-glycolic acid) has been the polymer of choice due to its biodegradable and biocompatible nature and ability for tunable, controlled release of antigen [7].

One notable challenge of PLGA particles is the potential for antigen instability, which can occur during particle production and antigen release, amongst other stages of the particle lifetime ([8], [9]). Traditional methods for loading the antigen into the particles can expose the antigen to detrimental conditions, including shear stress and oil-water interfaces ([8],[10]). As a result, these conditions may lead to protein aggregation and unfolding, potentially causing significant loss of protein antigenicity ([11], [12]).

Our group has developed a method for protein loading into PLGA microspheres that bypasses many of the challenges of traditional techniques ([8], [10]). This method, termed “self-encapsulation,” loads antigen by simple mixing of pre-made microspheres in an aqueous solution of protein. These microspheres contain an interconnecting pore

network and a protein-trapping agent (e.g. aluminum or calcium adjuvant gel) that is accessible through the pores. During mixing, the antigen diffuses into the pores and binds to the trapping agent. We have found that addition of a protein-trapping agent within the microspheres improves encapsulation efficiency compared to microspheres without a trapping agent ([10], [8]). Subsequent heating of the system above the glass-transition temperature (T_g) of the polymer causes pore closure, sealing the antigen inside the microspheres. We have shown improved antigenicity of tetanus toxoid released from self-healing microspheres over 28 days compared to that loaded by traditional techniques [8].

Here we expand upon previous work in which we developed a self-encapsulating formulation of PLGA microspheres for vaccine delivery (see Chapter 2). The goal of this investigation was to study the ability of the microspheres to be internalized by dendritic cells, which are known as “professional” APCs as they are the most potent activators of T cells [13]. In addition, we studied the immune response generated by the microsphere formulation after subcutaneous administration in mice using ovalbumin as a model antigen.

3.3 Materials and Methods

3.3.1 Materials

PLGA 50:50 (i. v. = 0.60 dL/g, M_w = 53.4 kDa, ester terminated) was purchased from Lactel (Durect Corporation, USA). Poly(vinyl alcohol) (PVA) (88% hydrolyzed, M_w = 25 kDa) was purchased from Polysciences Inc. (USA). Ovalbumin grade V, rhodamine 6G, and Triton-X 100 were purchased from Sigma (USA). Alpha-MEM, L-glutamine, penicillin/streptomycin, fetal bovine serum (FBS), recombinant mouse

granulocyte-macrophage colony-stimulating factor (GM-CSF), DAPI, Alexa Fluor 647-phalloidin, and ACK lysis buffer were purchased from Invitrogen (USA). Paraformaldehyde 40% was purchased from Electron Microscopy Sciences (USA). Alhydrogel® and Endofit OVA for immunization studies were purchased from Invivogen (USA). Biotinylated goat anti-mouse IgG, IgG₁, and IgG_{2C} were purchased from Southern Biotech (USA). All other reagents and solvents were purchased from commercial suppliers and were of analytical grade or higher.

3.3.2 Preparation of CaHPO₄ Adjuvant Gel

CaHPO₄ adjuvant gel was prepared as described in [14]. Equal parts Na₂HPO₄ and CaCl₂ were rapidly mixed together, and the pH immediately adjusted to 6.8-7.0 with NaOH. The resulting precipitate was washed 5 times with sterile 0.9% NaCl to remove excess phosphate and then resuspended in 0.9% NaCl to the desired concentration.

3.3.3 Preparation of Self-Encapsulating PLGA Microspheres

Porous self-encapsulating PLGA microspheres were prepared by the w/o/w double emulsion-solvent evaporation technique. Calcium phosphate adjuvant gel (CaHPO₄) was included as a protein-trapping agent and trehalose as a porosigen. The inner water phase contained calcium phosphate gel (28 mg/mL) and trehalose (28 mg/mL) in 25 mM succinate buffer (pH 4.0). One hundred microliters of inner water phase was added to 1 mL of 50 mg/mL PLGA in methylene chloride. The mixture was sonicated at 50% amplitude for 1 minute using a Sonics Vibra-Cell VC130 Ultrasonic Processor (Sonics & Materials Inc., USA) to form the first emulsion. Four milliliters of 5% (w/v) PVA solution (used as an emulsion stabilizer) was added to the primary w/o

emulsion and the mixture was vortexed (Genie 2, Scientific Industries Inc., USA) for 30 seconds to produce the w/o/w double emulsion. The w/o/w emulsion was poured into 100 mL of chilled 0.5% (w/v) PVA under rapid stirring and hardened at room temperature for 6 hours. Microspheres were passed through a 10- μ m mesh sieve and the filtrate was collected and centrifuged (7000 rpm for 5 minutes). The microspheres in the pellet were washed repeatedly with double-distilled (dd) H₂O and then lyophilized (FreeZone 2.5, Labconco, USA).

3.3.4 Determination of CaHPO₄ Loading by ICP-OES

Loading of CaHPO₄ in PLGA microspheres was determined by inductively coupled plasma-optical emission spectrometry (ICP-OES). Briefly, approximately 10 mg of microspheres were added to 1 mL acetone, vortexed, and then centrifuged at 7000 rpm for 5 minutes. The supernatant polymer solution was removed and the pellet was washed twice more with 1 mL acetone. The resulting residue was dried in a fume hood to allow for evaporation of the acetone. One milliliter of 30% HCl was added to each sample to dissolve the residue. The samples were then diluted with ddH₂O to bring the theoretical concentration of Ca ion in the samples to between 1 and 15 ppm. The concentration of Ca ion was then analyzed by ICP-OES (Perkin-Elmer Optima 2000 DV with Winlab software). Each sample was measured three times and analysis was done in triplicate.

3.3.5 Scanning Electron Microscopy

The surface morphology of microspheres was examined using a Hitachi S3200N scanning electron microscope (SEM) (Hitachi, Japan). Briefly, lyophilized microspheres were fixed on a brass stub using double-sided carbon adhesive tape. The sample was

made electrically conductive by coating with a thin layer of gold for 120 seconds at 40 W under vacuum. Images were taken at an excitation voltage of 8.0 kV. EDAX® software was used to obtain the final image.

3.3.6 Microsphere Size and Zeta Potential Analysis

The volume median diameter of the microspheres was measured using a Mastersizer 2000 (Malvern Instruments Ltd, UK). Ten milligrams of lyophilized microspheres were suspended in 5 mL 0.5% PVA solution and briefly vortexed. Six measurements were performed per sample at a stir speed of 2000 rpm and sampling time of 15 seconds. Zeta potential was measured with a Zetasizer Nano ZSP (Malvern Instruments Ltd, UK). Lyophilized microspheres were suspended in ddH₂O (0.1% mass) and placed in a disposable folded capillary zeta cell (Malvern Instruments Ltd, UK). Each sample was measured six times.

3.3.7 Active Self-Encapsulation of Ovalbumin by PLGA Microspheres

Active encapsulation of ovalbumin (OVA) by PLGA microspheres was carried out in two phases: an initial incubation of the porous microspheres in protein solution at a temperature below the polymer T_g for protein sorption onto the CaHPO₄ gel, and then incubation at a temperature above the T_g for the polymer pores to heal. Briefly, approximately 20 mg of lyophilized microspheres were incubated in an OVA solution (0.5 mg/mL OVA in 0.4 mL 10 mM MOPS buffer, pH 7.4) at 4°C for 24 hours and then 25°C for 24 hours ($T < T_g$), followed by incubation at 42°C for 48 hours ($T > T_g$). Incubation was performed under constant agitation using a rigged rotator (Glas-Col, USA).

3.3.8 Determination of Protein Loading and Encapsulation Efficiency

After self-encapsulation of the protein, the microsphere samples were centrifuged at 7000 rpm for 5 minutes. The supernatant was passed through a low protein-binding Durapore (PVDF) membrane-based syringe filter unit (Millipore Corporation, USA) and the filtrate collected. The microspheres were then washed once with ddH₂O and the rinse filtered and collected. A modified Bradford assay was used to determine OVA concentration in the filtrates. Coomassie Plus® reagent (Thermo Fisher Scientific, USA) was added to the appropriate volume of standard OVA solution or filtrate sample in a 96-well plate (Nunc, Thermo Scientific, USA). After 10 minutes, the absorbance was read at 595 nm using a Dynex II MRX microplate reader (Dynex Technology Inc., USA). The mass of OVA encapsulated by the microspheres was calculated by subtracting the mass of OVA in the filtrates from the mass of OVA in the initial loading solution. Percent w/w loading and encapsulation efficiency (EE) were quantified with the following formulas:

$$\% \text{ w/w loading: } \frac{\text{mass of OVA encapsulated by the microspheres}}{\text{mass of microspheres in loading sample} + \text{mass of OVA encapsulated}} \times 100$$

$$\% \text{ EE: } \frac{\text{mass of OVA encapsulated by the microspheres}}{\text{mass of OVA in initial loading solution}} \times 100$$

3.3.9 Evaluation of the *In Vitro* Release of Ovalbumin from Self-Encapsulating Microspheres

Approximately 20 mg of OVA-encapsulated microspheres were incubated in 0.5 mL PBS, pH 7.4, at 37 °C under constant agitation (240 rpm/min). At different incubation times (1, 3, 5, and 7 days, and then every 7 days until day 49), the mixture was centrifuged at 7000 rpm for 5 minutes and the supernatant was collected through a low protein-binding Durapore (PVDF) membrane-based syringe filter unit (Millipore Corporation, USA). Fresh release media (0.5 mL) was then added to the sample and the microspheres were resuspended to continue the release study. The OVA content in the supernatants was analyzed by size exclusion-high performance liquid chromatography (SE-HPLC).

3.3.10 Size Exclusion-High Performance Liquid Chromatography (SE-HPLC) of Ovalbumin

SE-HPLC was performed using a TSKgel G3000SWxl column (Tosoh Bioscience, USA) on a Waters HPLC system (Waters, USA). The mobile phase consisted of PBS, pH 7.4, at a flow rate of 0.7 mL/min and injection volume of 50 µL. Protein detection by UV was done at 210 and 280 nm.

3.3.11 Cell Lines and Tissue Culture

JAWSII dendritic cell line, obtained from the bone marrow of C57BL/6 mice, was purchased from ATCC (USA, ATCC no. CRL-11904). Cells were cultured in complete alpha-MEM media supplemented with 2 mM L-glutamine, 100 µg/mL streptomycin, 100

U/mL penicillin, 20% FBS, and 5 ng/mL GM-CSF. Cells were maintained in a humidified incubator at 37°C and 5% CO₂.

3.3.12 Confocal Microscopy of Microsphere Internalization by Dendritic Cells

JAWSII cells were treated with microspheres, and the internalization of the microspheres by the cells was visualized using confocal microscopy. Briefly, microspheres were made fluorescent by addition of rhodamine 6G to the oil phase during microsphere production to dye the polymer. Cells were plated in complete alpha-MEM at 2×10^5 cells/dish in a 35 mm culture dish with a glass coverslip on the base (MatTek Corporation, USA) and maintained in a humidified incubator at 37°C and 5% CO₂. After overnight incubation, the media was aspirated from the dish to remove non-adherent cells. The cells were then incubated for 24 hours with 0.1 mg/mL fluorescent microspheres. After incubation, media was once again aspirated to remove non-internalized microspheres. The cells were fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton-X solution. The actin filaments were stained with Alexa Fluor 647-phalloidin and the nucleus stained with DAPI. A Nikon A-1 spectral confocal microscope with NIS Elements software (Nikon Instruments) was used to view the sample.

3.3.13 Flow Cytometry Analysis of Microsphere Internalization

JAWSII cells were plated in a 12-well culture plate (Corning Inc., USA) at 1.5×10^5 cells/well in complete alpha-MEM. After overnight incubation at 37°C and 5% CO₂, media was removed from the wells and fresh media was added containing different doses of rhodamine 6G microspheres (25, 50, or 100 µg/well). After 6 or 24 hours of incubation, the cells were washed with PBS and collected. The cells were fixed with 4%

paraformaldehyde and permeabilized with a BD Perm/Wash buffer (BD Biosciences, USA). Actin filaments were stained with a phalloidin-iFluor 405 dye (AAT Bioquest, Inc., USA) and analyzed with the Amnis ImageStream^x Mark II imaging flow cytometer (EMD Millipore Corporation, USA).

3.3.14 Immunization Study

Female C57BL/6 mice, 6-7 weeks old, were purchased from Harlan Laboratories, Inc. and handled according to the University of Michigan Institutional Animal Care guidelines. Mice (5 mice per group) were immunized subcutaneously at the tail base with 100 μ L of sterile PBS, Alhydrogel[®] or CaHPO₄ adjuvant gel co-administered with OVA (100 μ g gel + 10 μ g OVA), or microspheres loaded with OVA (10 or 20 μ g OVA). A booster dose was given three weeks later on day 21 after primary immunization to all groups except for the group receiving microspheres at a dose of 20 μ g OVA (only prime injection given). On days 20 and 42, blood samples were collected by submandibular bleed for analysis of serum antibody titers. Blood was collected into Microvette 500 Z-Gel serum collection tubes (Sarstedt, Germany) and centrifuged at 10,000 x g for 5 minutes to separate the serum. Serum was stored at -80°C until analysis.

3.3.15 Measurement of Antibody Titers

Serum samples were sent to the Immunology Core at the University of Michigan Cancer Center for ELISA analysis. The assays were performed using a standard ELISA protocol. Briefly, OVA (10 μ g/mL in carbonate/bicarbonate buffer) was used to coat 384-well plates overnight at 4°C. Plates were washed and residual binding sites blocked with 0.2% casein in Tris-buffered saline (TBS) for at least one hour. The same buffer

was used for sample dilutions. Ig detection antibodies were minimally diluted to the manufacturer's suggested working range. ELISA analysis was used to measure IgG, IgG₁, and IgG_{2C} response.

3.3.16 CD8⁺ T Cell Tetramer Staining

Seven days following booster immunization, blood was collected by submandibular bleed and collected in a BD Microtainer tube with dipotassium EDTA additive to prevent clotting (BD Biosciences, USA). Red blood cells were lysed with ACK lysis buffer and washed to isolate the peripheral blood mononuclear cells (PBMCs). The Fc receptor was blocked with CD16/32 (eBioscience Inc., USA). Cells were incubated with anti-CD8 α and T-Select H-2Kb OVA Tetramer-SIINFEKL-PE (MBL, Japan), followed by staining with DAPI (Sigma-Aldrich, USA) to discriminate live from dead cells. A Beckman Coulter CyAn 5 flow cytometer (Beckman Coulter Inc., USA) was used to analyze the samples. Data was processed using FlowJo (FlowJo, USA).

3.3.17 Statistical Analysis

Statistical analyses were carried out using GraphPad Prism 6.0g software. One-way ANOVA or two-way ANOVA with Bonferroni's post-test were used to compare multiple groups. Values are reported as mean \pm SEM (standard error of the mean).

3.4 Results and Discussion

3.4.1 Microsphere Preparation and Characterization

Microspheres were prepared as described in Chapter 2. Briefly, the formulation was made using a standard double emulsion-solvent evaporation technique. Trehalose

was included in the inner water phase to act as a porosigen to create the interconnecting pore network that is necessary for protein diffusion into the microspheres during self-encapsulation [10]. The microsphere morphology and size, CaHPO₄ content, encapsulation of ovalbumin, and release profile were examined. The results were previously presented in Chapter 2. A brief summary of the results are provided in Table 1 and discussed in the following sections.

3.4.1.1 Formulation Morphology and Size

The size of the microspheres is of particular importance for promoting internalization by APCs. From a number of studies that have been conducted, it has been demonstrated that microspheres of a size less than 10 µm are more efficiently internalized by APCs compared to larger particles ([15], [16], [17], [18] [19], [20]). The formulation parameters (including polymer concentration and energy of agitation) were refined to produce porous, spherical microspheres of the desired size range. Sonication was found to be preferable to homogenization for forming the primary emulsion, which was expected since greater agitation is known to generate smaller particles [21]. The median microsphere diameter for this formulation was found to be 7.05 ± 0.31 µm, with a zeta potential of -21.9 ± 2.1 mV (Table 1).

3.4.1.2 Incorporation of CaHPO₄ Gel and Encapsulation of Ovalbumin

CaHPO₄ adjuvant gel was used as the protein-trapping agent for the formulation. Calcium phosphate adjuvant is a natural constituent of the body and, hence, is well-tolerated and readily resorbed [14]. The CaHPO₄ gel was prepared by a rapid mixing method as described by Gupta *et al.* [14]. The gel was added to the inner water phase to

be incorporated into the microspheres during production. Using ICP-OES, the w/w loading of CaHPO₄ within the microspheres was determined to be 2.2 ± 0.1 % (Table 1).

The self-encapsulating ability of the microsphere formulation was tested by loading ovalbumin as a model antigen. The w/w loading was found to be 0.60 ± 0.05 %, with an encapsulation efficiency of 55.6 ± 6.0 % (Table 1). The w/w % loading for the formulation was within the expected range based upon the loading of the incorporated CaHPO₄ gel within the microspheres and the loading capacity of the gel for the protein (see Chapter 2). The microsphere morphology prior to self-encapsulation was viewed by scanning electron microscopy (SEM) and was confirmed to be spherical with visible pores (Figure 1). Successful healing of polymer pores after self-encapsulation was confirmed by SEM (Figure 1).

3.4.1.3 Sustained Release of Model Antigen

The % cumulative release of ovalbumin from the microspheres is summarized in Table 1. The formulation produced a moderate burst release of 28.4 ± 1.5 %, with sustained release of protein over 6 weeks. Antigenicity of the protein was maintained during release, as confirmed by ELISA (see Chapter 2).

3.4.2 Internalization of Microspheres by Dendritic Cells

Antigen uptake and processing by APCs is necessary for presentation to other immune cells and induction of the adaptive immune response. Dendritic cells can be considered key APCs and are important for the generation of a primary immune response, i.e. the response upon first encounter of the immune system to the antigen, and subsequent immunological memory [22]. Depending on type of antigen, uptake by

dendritic cells can happen by receptor-mediated endocytosis, macropinocytosis and phagocytosis [23].

Herein, we studied the ability of the microsphere formulation to be internalized by dendritic cells for direct antigen delivery. Dendritic cells are known to internalize PLGA particulates via phagocytosis [4]. To evaluate this interaction, we used confocal microscopy to visualize internalized microspheres and flow cytometry to measure particle uptake.

3.4.2.1 Visualization of Microsphere Uptake Using Confocal Microscopy

To view the uptake of the microsphere formulation by dendritic cells, fluorescent microspheres were incubated with JAWSII cells, a murine bone marrow-derived dendritic cell line. Microspheres were made fluorescent by the addition of rhodamine 6G to the polymer phase during production. The microspheres were then incubated with JAWSII cells for 24 hours at 37°C. To visualize cellular components, the nucleus was stained with DAPI and the actin filaments stained with Alexa Fluor 647-phalloidin. Samples were observed with a confocal microscope (Figure 2).

As shown in Figure 2, the dendritic cells successfully internalized one or more microspheres over the 24-hour period. Both larger and smaller microspheres within the size distribution were taken up by the cells. Orthogonal images confirmed that microspheres were within the cells and not attached to the cell surface, as evidenced by the surrounding actin filaments.

3.4.2.2 Measurement of Particle Uptake by Flow Cytometry

Microsphere internalization was additionally explored by flow cytometry to measure particle uptake. JAWSII cells were incubated with three different concentrations of rhodamine 6G-labeled microspheres over 6 and 24 hours at 37°C. Cells were treated with phalloidin-iFluor 405 dye to stain the actin filaments. An Amnis ImageStream^x Mark II imaging flow cytometer was then used to quantify the percent of cells with associated rhodamine positivity (Figure 3a). Images obtained from the flow cytometer provided visualization of the counted cells and the associated microspheres (Figure 3b-e). From the results, a clear trend was observed in the data. As might be expected, the percent of microsphere-positive cells increased with both incubation time and microsphere concentration. This suggests that a longer incubation time and higher microsphere concentration promote microsphere-cell interaction, resulting in improved uptake.

3.4.3 Subcutaneous Immunization with Ovalbumin-Loaded Microspheres

Previously, we explored the immune response generated by the microsphere formulation in a proof-of-concept intranasal immunization study in mice (see Chapter 2). Here, we wanted to compare the response produced after subcutaneous administration of the microspheres. It has been shown that the route of administration plays a role in shaping the immune response, perhaps due to local cell types (e.g. different subsets of APCs) and stability of the microspheres by that administration method (e.g. particle agglomeration at the site of injection) [24].

The immunization study was designed to (1) determine the type and magnitude of the immune response induced by the microspheres, (2) compare the immune response

from the microspheres to that induced by commercial aluminum hydroxide adjuvant (Alhydrogel®), and (3) test the dose-sparing ability of the microspheres. C57BL/6 mice were immunized with Alhydrogel® or CaHPO₄ adjuvant gel co-administered with OVA (100 µg gel + 10 µg OVA), or microspheres loaded with OVA (10 or 20 µg OVA). All groups were given a prime dose on day 0 and a booster dose three weeks later (Figure 4), except for the microsphere group with 20 µg OVA. The latter group was given as a prime-only dose to test the duration of the immune response by single-dose administration.

Aluminum hydroxide was included in the study since it has become the reference for new adjuvants for use in humans [25]. It has been used in human vaccines since the 1920s and is one of the few widely-approved adjuvants ([26], [27]). The adjuvant is known to cause a more Th2-biased immune response, and is thought to act by prolonging antigen exposure to the immune system, improving antigen uptake by APCs, and increasing the expression and duration of MHCII complexes ([28], [26]).

3.4.3.1 CD8⁺ T Cell Response

On day 28, four weeks after prime dosing and one week following booster, peripheral blood mononuclear cells (PBMCs) were collected to measure the ovalbumin-specific CD8⁺ T cell response, a measure of the cellular response generated (Figure 5). Of the groups tested, Alhydrogel® and microspheres (both the 10 µg and 20 µg OVA doses) produced a significant CD8⁺ T cell response above that of the PBS control group. The group immunized with calcium phosphate adjuvant did not generate a cellular response above baseline.

The microsphere group with 10 μg of OVA produced the strongest ovalbumin-specific CD8^+ T cell population of the three groups, having a significantly higher cellular response compared to that produced by Alhydrogel®. In addition, the microsphere group given as prime-only (20 μg of OVA) generated a cellular response comparable to that of the Alhydrogel®, which was given to mice at both prime and boost. This data indicates that the microspheres are capable of inducing a significant antigen-specific cellular immune response compared to Alhydrogel®, which acted as a reference commercial adjuvant. Further, the microspheres demonstrate promising single-injection ability since microspheres given by one injection (with twice the amount of antigen) produced a similar cellular response to commercial adjuvant given by two separate injections.

3.4.3.2 Serum Antibody Titers

The serum anti-ovalbumin antibody response was analyzed following both prime and booster immunization of mice (Figure 6a-c). Total IgG and titers for IgG_1 , and $\text{IgG}_{2\text{C}}$ subclasses were measured. IgG subclass titers provide information about the polarization of the Th response, with IgG_1 associated with a Th2-type response and $\text{IgG}_{2\text{C}}$ associated with a Th1 response ([29], [30]).

All groups produced antibody titers significant from baseline after both prime and booster immunization, except for the post-prime $\text{IgG}_{2\text{C}}$ response (all groups at baseline). For the total IgG response, the Alhydrogel® and microsphere groups generated comparable post-prime and post-boost titers, though the post-boost response for the two-dose microsphere group was higher than that for the single-dose group. Similarly, the prime and booster IgG_1 titers were comparable for all groups, however the mean post-boost titer for the two-dose microsphere group was higher than that produced by

Alhydrogel®. Finally, the mean post-booster IgG_{2C} titer for the two-dose microsphere group was significantly higher than for the other groups. The mean titer for the single-dose of microspheres was comparable to the titers achieved by the Alhydrogel® and calcium phosphate groups.

Taking into account all of the antibody data, administration of two doses of the microspheres loaded with ovalbumin resulted in a mixed Th1/Th2 immune response that was stronger in comparison to two doses of ovalbumin co-administered with Alhydrogel®. Additionally, the Th1/Th2 response induced by the single-dose microsphere treatment was comparable to two doses of the Alhydrogel® adjuvant. This data suggests that the microspheres can induce a stronger Th1/Th2-associated response compared to Alhydrogel® when given by the same number of doses, or produce a comparable response when twice the amount of antigen is given by a single dose. Single-injection ability is desired for a vaccine due to potential improvements in convenience and patient compliance, particularly in low-resource settings.

3.5 Conclusions

In previous work, we developed and characterized a formulation of self-encapsulating microspheres for vaccine delivery (see Chapter 2). In this chapter, we expand upon that research by studying the ability of the microspheres to be internalized by antigen-presenting cells, for direct delivery of antigen to APCs, and investigate the type and magnitude of the immune response generated by the formulation following subcutaneous administration in mice. We compared immunization with our microsphere formulation to Alhydrogel® as a standard commercial adjuvant for human vaccines.

Our results show that the self-encapsulating microspheres, with a median diameter of 7 μm , are successfully internalized by a murine dendritic cell line and are capable of producing a mixed humoral and cellular immune response against ovalbumin following subcutaneous immunization. Two-dose immunization with the microspheres generated an improved CD8^+ T cell response and Th1/Th2-mixed response compared to two doses of Alhydrogel®. Further, a single dose of microspheres, containing twice the amount of antigen, produced a stronger cellular response and comparable antibody response to two doses of Alhydrogel®. In conclusion, this formulation demonstrates excellent potential for further development for vaccine delivery.

3.6 Figures

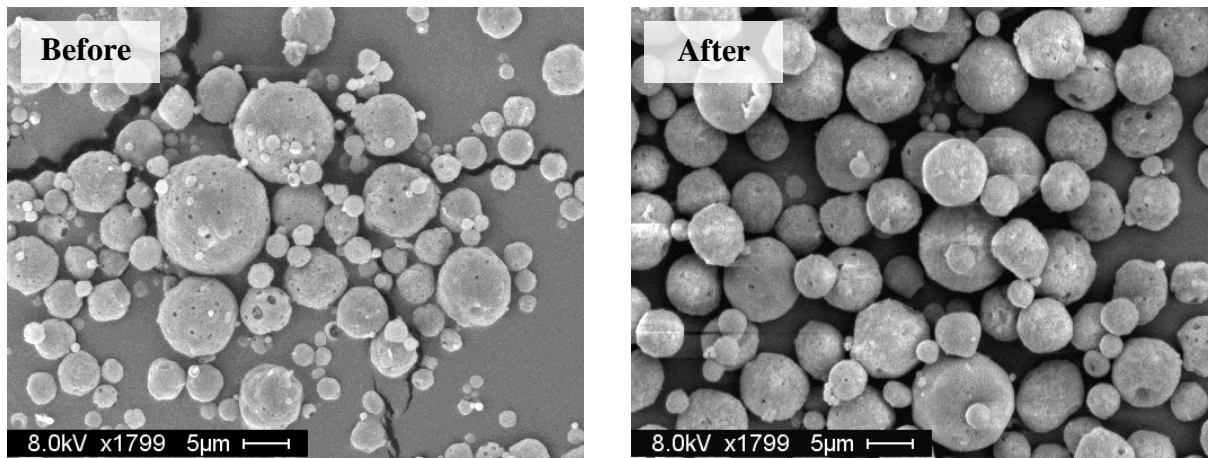


Figure 3-1. SEM images of self-encapsulating PLGA microspheres containing CaHPO_4 adjuvant gel as the protein-trapping agent. Microspheres are shown before (left) and after (right) self-encapsulation of ovalbumin and pore healing. Scale bars represent 5 μm .

Table 3-1. Summary of the properties of the self-encapsulating microsphere formulation. Data are shown as mean \pm SEM ($n = 3$).

Microsphere composition, size, and zeta potential		
	Measured CaHPO ₄ loading (wt. %)	2.2 \pm 0.1
	Volume median diameter (μ m)	7.05 \pm 0.31
	Zeta potential (mV)	-21.9 \pm 2.1
OVA loading by self-encapsulation		
	% (w/w) loading	0.60 \pm 0.05
	% Encapsulation efficiency	55.6 \pm 6.0
% Cumulative release of OVA from microspheres		
	1	28.4 \pm 1.5
Day	21	49.6 \pm 1.1
	28	54.2 \pm 0.1
	42	66.5 \pm 1.4

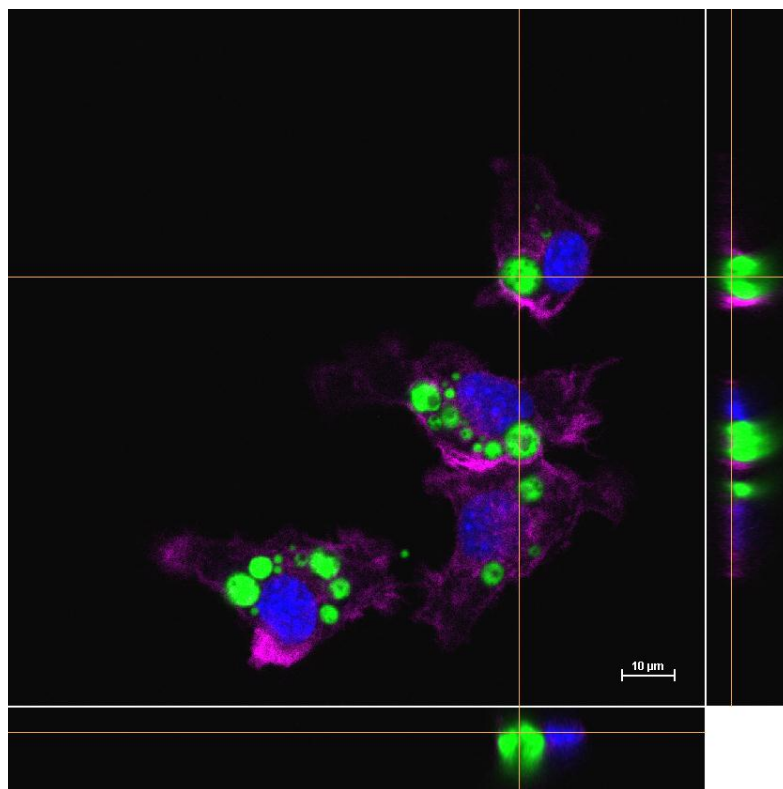


Figure 3-2. Confocal microscopy image showing healed rhodamine-labeled self-encapsulating microspheres (green) internalized by JAWSII dendritic cells after 24 hours of incubation. Actin filaments were stained with Alexa Fluor 647-phalloidin (violet) and nuclei were stained with DAPI (blue). Scale bar represents 10 μm .

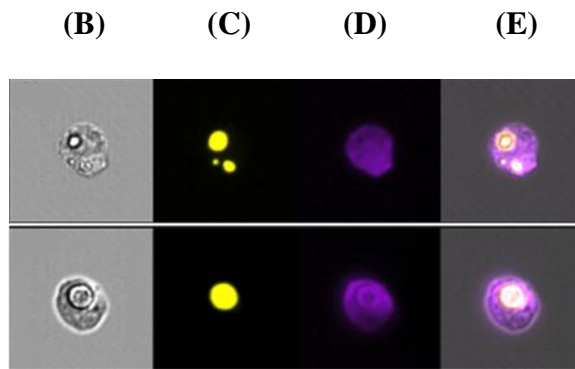
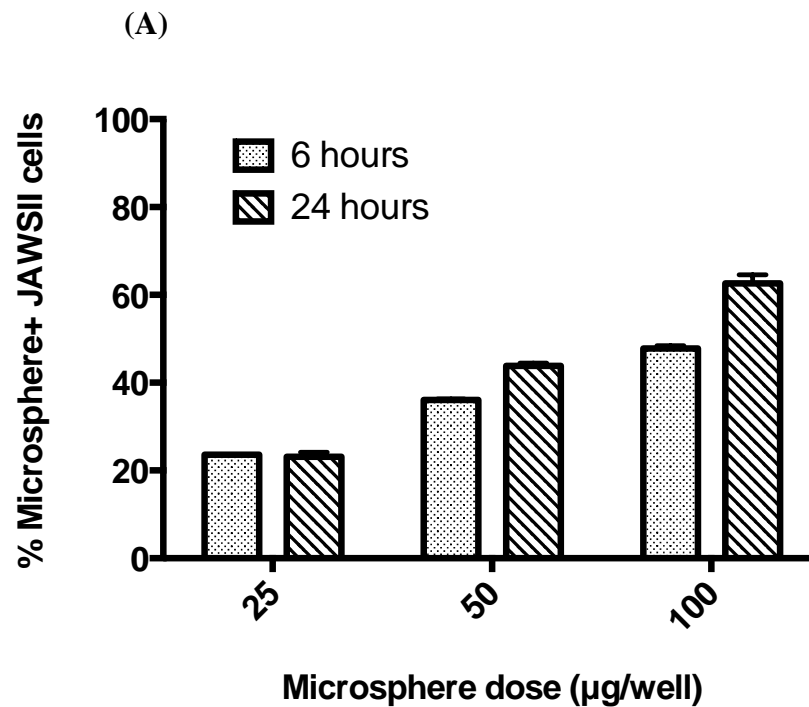


Figure 3-3. Internalization of microspheres by JAWSII dendritic cells over time. (A) Flow cytometry analysis (Amnis ImageStream^x Mark II) of JAWSII cells treated with different doses of rhodamine 6G-labeled microspheres over two incubation times. Columns show the percent of gated events containing cells with associated microspheres. Data represent mean \pm SEM ($n = 3$). (B-E) Representative images of those obtained by ImageStream^x analysis. (B) Bright-field image of the cell with associated microspheres; (C) and (D) fluorescent images of the microspheres (dye: rhodamine 6G) and JAWSII cells (stained with phalloidin-iFluor 405), respectively; and (E) overlay of images B-D.

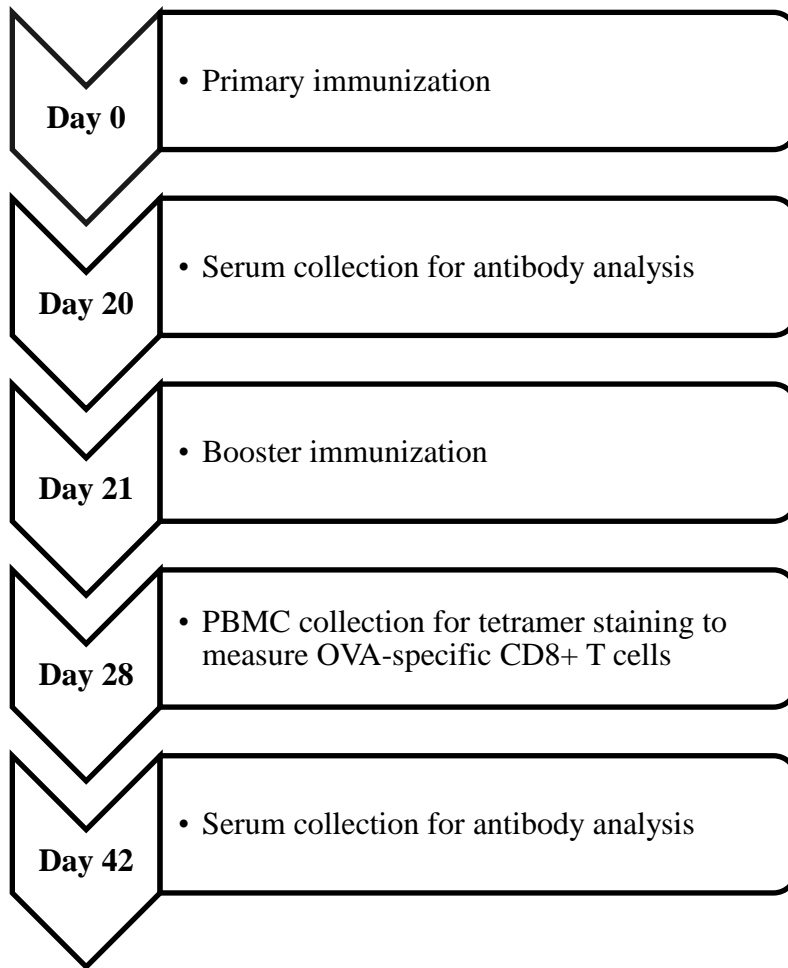


Figure 3-4. Timeline for the subcutaneous immunization study.

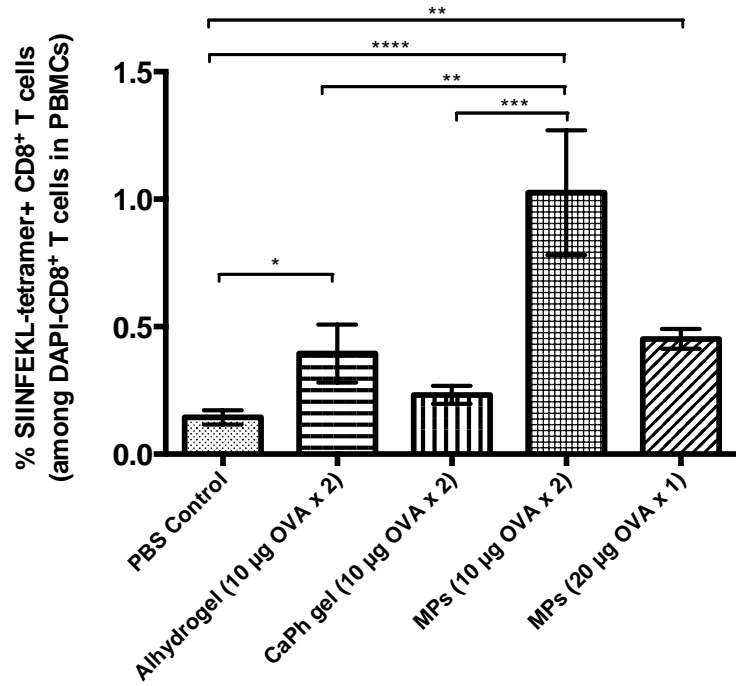


Figure 3-5. Plot of % SIINFEKL-tetramer+ among CD8⁺ T cells in PBMCs for the test groups on day 28. Groups given prime and booster doses are marked (x 2), while (x 1) denotes that only a prime injection was administered. Data represent mean \pm SEM ($n = 5$). All groups were compared using one-way ANOVA followed by Bonferroni's post-test ($*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$, and $****p \leq 0.0001$).

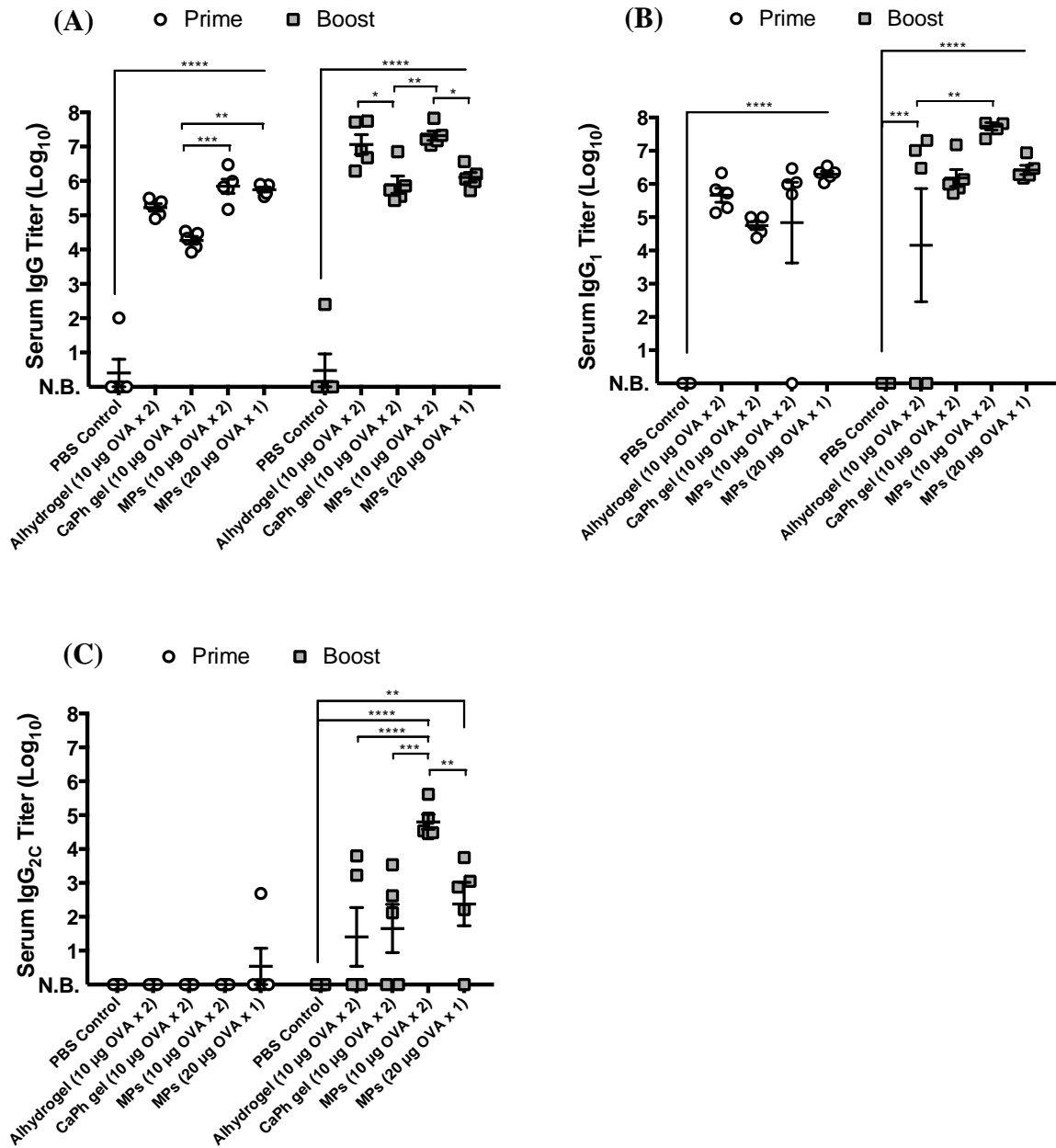


Figure 3-6. Serum anti-OVA antibody titers for groups on days 20 (prime response) and 42 (boost response). **(A)** IgG, **(B)** IgG₁, and **(C)** IgG_{2c}. Groups given prime and booster doses are marked (x 2), while (x 1) denotes that only a prime injection was administered. Data were fit using a 4-parameter curve, and titers were calculated by solving for the inverse dilution factor resulting in an absorbance value of 0.5. N.B. = no binding detected. Data represent mean \pm SEM ($n = 5$). All groups were compared using two-way ANOVA followed by Bonferroni's post-test ($*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$, and $****p \leq 0.0001$).

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Chapter 4

Conclusion

4.1 Significance

This thesis presents the first application of the self-encapsulation paradigm developed by our lab to the delivery of vaccine antigens in cell culture and mammalian subjects. Our previous work focused on the development of the self-encapsulation approach for conventionally larger, non-phagocytosable microspheres and the study of antigen stability after encapsulation by this process and subsequent release ([1], [2]). Confirming the ability of self-encapsulation to improve the antigenicity of loaded protein compared to traditional methods, we designed a formulation of self-encapsulating microspheres specifically for vaccine delivery that would promote delivery of antigen to antigen-presenting cells (APCs).

The results from this work illustrate a few key findings and support the thesis hypothesis. The microsphere formulation (1) provided sustained release of antigenic model protein over several weeks, far better than conventional microspheres prepared by solvent evaporation; (2) was successfully internalized by dendritic cells, the primary APCs for adaptive immunity; (3) was capable of inducing mixed Th1/Th2 immunity and an antigen-specific CD8⁺ T cell (cellular) response; (4) performed better than Alhydrogel® after subcutaneous delivery at achieving both cellular and humoral

immunity; and (5) has the potential for single-time injection. These findings address certain challenges of traditional encapsulation techniques and vaccine delivery.

The ability of the formulation to maintain protein antigenicity during release, particularly over multiple weeks, is a major advantage that is challenging for traditional encapsulation methods. As discussed, traditional techniques expose the antigen to destabilizing conditions, leading to aggregation and loss of structure and having a detrimental effect on the antigenicity ([2], [1], [3], [4]). This formulation demonstrates that it can maintain the antigenicity of the protein cargo and prolong its exposure over many weeks.

Current strategies in vaccine development are focusing on improving the potency of the immune response (e.g. induction of cellular immunity) and improving vaccine coverage (e.g. reducing dose burden) ([5], [6], [7]). This formulation shows promising results in regards to both challenges. The results achieved from the subcutaneous immunization study demonstrate a notable ability of the formulation to induce a CD8⁺ T cell response (i.e. cell-mediated response) and IgG subclass titers associated with a mixed Th1/Th2 response. In addition, microspheres administered by single-dose immunization performed comparably to commercial Alhydrogel® adjuvant given by two doses. This finding suggests the possibility of single-time injection, which is beneficial for convenience and patient compliance, particularly in resource-limited settings.

4.2 Future Directions

The self-encapsulating formulation developed through this research has shown potential as a vaccine delivery system. Further work should be conducted to go beyond a model antigen and apply the system to delivery of a clinically relevant antigen. For

example, one possibility is the recombinant hepatitis B surface antigen (HBsAg). The commercial hepatitis B vaccine, which consists of HBsAg adsorbed to aluminum adjuvant, requires three doses for efficacy [8]. The microsphere formulation could be tested for its single-time injection ability with the antigen in an attempt to minimize the doses required for protective immunity.

Additionally, improvements to the immune response could be explored by incorporation of immunostimulators to enhance and direct the immune response against the antigen. Immunostimulators, such as cytokines and TLR ligands, can affect the balance of Th1/Th2 immunity [9]. For example, bacterial monophosphoryl lipid A (MPLA) and CpG oligodeoxynucleotides (ODN) have been shown to induce improved Th1-type immunity ([10], [11], [12]). In future work, addition of an immunostimulator to the microspheres, to be co-delivered with the antigen, would be worth exploring for enhancement of the protective immune response.

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