BIODEGRADABLE POLYMERIC DELIVERY SYSTEMS FOR PROTEIN SUBUNIT VACCINES

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BIODEGRADABLE POLYMERIC DELIVERY SYSTEMS FOR PROTEIN SUBUNIT VACCINES

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To my beautiful and loving wife, Kasey,
for her encouragement, support, and prayers throughout this journey.

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LIST OF ABBREVIATIONS

AFM	Atomic force microscopy
APC	Antigen-presenting cell
CD	Cluster of differentiation
CDM	1,4-Cyclohexanedimethanol
CpG	Cytidine-Guanidine motif found in bacterial DNA
CTAB	Cetyltrimethylammonium bromide
CTL	Cytotoxic T lymphocyte
d.p.	Degree of polymerization
DAPI	4',6-Diamidino-2-phenylindole
DC	Dendritic cell
DCl	Deuterium chloride
DCM	Dichloromethane
DLS	Dynamic light scattering
DMF	Dimethylformamide
DMP	2,2-Dimethoxypropane
DMSO	Dimethylsulfoxide
DOODT	3,6-Dioxa-1,8-octanedithiol
DOTAP	1,2-dioleoyl-3-trimethylammonium-propane
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
FLT3	FMS-like tyrosine kinase 3
GPC	Gel permeation chromatography
IFN	Interferon
IHC	Immunohistochemistry
IL	Interleukin
IRF	Interferon regulatory factor
LPS	Lipopolysaccharide
Lys(Z)	ε-Benzyloxycarbonyl-*L*-lysine

MACS	Magnetic-activated cell sorter
MF59	A vaccine adjuvant
MHC	Major histocompatibility complex
MPL	Monophosphoryl lipid A
MyD88	Myeloid differentiation factor 88
NCA	*N*-carboxyanhydride
NMR	Nuclear magnetic resonance
OT-1	Transgenic mouse with expanded population of T cells specific to the ovalbumin peptide SIINFEKL
Ova	Ovalbumin (albumin from chicken egg)
PAGE	Polyacrylamide gel electrophoresis
PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate-buffered saline
PDTEA	Pyridyldithioethylacrylate
PEG	Poly(ethylene glycol)
PIC	Polyion complex
PK	Polyketal
PKN	Polyketal nanoparticle
PK3	Co-polyketal synthesized using 80% 1,4-cyclohexane dimethanol and 20% 1,5-pentanediol
PLA, PLGA	Poly(lactic acid), poly(lactic-*co*-glycolic acid)
PLL	Poly(*L*-lysine)
PLTP	Poly(*L*-lysine-dithiopyridyl)
Poly(I:C)	Poly(riboinosinic acid)-poly(ribocytidylic acid)
PPADK	poly(1,4-phenylene-acetonedimethylene ketal)
PVS	Poly(vinyl sulfate)
SDS	Sodium dodecyl sulfate
SEM	Scanning electron microscopy/microscope
SIINFEKL	Octapeptide with sequence Ser-Ile-Ile-Asn-Phe-Glu-Lys-Leu
SPDP	Succinimidyl-pyridyl-dithio-proprionate

T_H1	T-helper 1
THF	Tetrahydrofuran
TIR	Toll-IL-1 receptor
TRAM	TRIF-related adaptor molecule
TRIF	TIR domain containing adaptor inducing IFN-β
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor alpha

SUMMARY

Viral infections and cancer are major causes of death and disability worldwide, and new vaccine strategies are needed for preventive and therapeutic vaccines. While traditional vaccines derived from attenuated or inactivated viruses have been successful in immunizing populations against certain infectious diseases, there are currently no approved vaccines for certain pathogens such as HIV, hepatitis C virus, and malaria, or for therapeutic treatment of most forms of cancer. A promising strategy is the protein subunit vaccine, which is composed of purified protein antigens and immunostimulatory adjuvants. The most widely studied adjuvants are agonists for Toll-like receptors (TLRs), which are expressed in antigen-presenting cells (APCs) and can recognize pathogen-associated molecular patterns. Delivery of protein antigens and TLR agonists to APCs elicits cellular immunity through the generation of cytotoxic T lymphocytes. While many candidate delivery systems have been developed for protein/TLR vaccines, there is a need for further improvement with regard to the carrier materials and formulation methods, and through the utilization of new adjuvants, such as double-stranded (ds)RNA (a TLR3 agonist). The **long-term objective** of this research was to develop improved delivery vehicles for protein/TLR-based vaccines, guided by the **central hypothesis** that an effective vaccine delivery system would have stimulus-responsive degradation and release, biodegradability into excretable non-acidic degradation products, and the ability to incorporate various TLR-inducing adjuvants.

The first two specific aims were centered around developing biodegradable polymeric delivery systems for protein antigens and TLR agonists, as model vaccine delivery systems. In the first specific aim, we developed a cross-linked block copolymer

micelle for efficient encapsulation and retention of proteins, DNA, and RNA. The micelle-based delivery system consists of a block copolymer of poly(ethylene glycol) (PEG) and poly(L-lysine), cross-linked by dithiopyridyl side groups to provide transport stability and intracellular release. The focus of the second specific aim was to develop a pH-sensitive biodegradable polymer for fabricating microparticles encapsulating proteins/DNA/RNA and hydrophobic small molecules. Using a new polymerization method based on the acetal exchange reaction, we synthesized hydrophobic, linear polymers containing pH-sensitive ketal linkages in the backbone. This new polymer, termed a polyketal, has a combination of properties not found in existing drug delivery polymers, namely pH-sensitivity, biodegradability, ease of synthesis, and non-acidic degradation products. Also, the technique of hydrophobic ion pairing was utilized to enhance the encapsulation of ovalbumin, DNA, and RNA in the polyketal microparticles via a single emulsion method. In the third specific aim, we demonstrated that the micelle- and polyketal-based vaccine delivery systems enhanced the cross-priming of $CD8^+$ T cells using in vitro and in vivo immunological models. The model vaccines were composed of ovalbumin antigen and various TLR-inducing adjuvants including CpG-DNA, monophosphoryl lipid A, and dsRNA. The results demonstrate that the cross-linked micelles and polyketal microparticles have considerable potential as delivery systems for protein-based vaccines.

CHAPTER 1
INTRODUCTION

1.1 Motivation

Viral infections and cancer are major causes of death and disability worldwide, and new strategies are greatly needed for preventive and therapeutic vaccines. Treatment of these diseases requires vaccines that induce an adaptive immune response, specifically the generation of cytotoxic T lymphocytes which can eliminate virus-infected cells or tumors cells. Traditional vaccines derived from attenuated or inactivated viruses have been successful in immunizing populations against certain infectious diseases such as polio, measles, and rubella. However, there are currently no approved vaccines for certain pathogens such as HIV, hepatitis C virus, and malaria, or for therapeutic treatment of most forms of cancer (Kanzler 2007, Heit 2008). Also, there is growing concern with preparedness for potential outbreaks such as influenza due to the long lead time in vaccine manufacturing by current processes. There is thus a need for new vaccine strategies for preventive and therapeutic vaccines for cancers and infectious diseases.

A promising approach is the protein subunit vaccine, which is based on purified proteins derived from viruses or tumor cells (Bramwell 2005, Storni 2005). Protein subunit vaccines have the potential to elicit an adaptive immune response specific to the pathogen or cancer cell. Vaccines based on recombinant proteins can be manufactured on a large scale and would potentially have shorter lead times than current manufacturing processes such as egg-based cultures. Also, protein-based vaccines do not carry the risk of infection as with live attenuated virus vaccines. This is an important concern with

lethal viruses such as HIV. Furthermore, there is flexibility in developing multivalent vaccines to improve efficacy against multi-clade viruses, such as HIV. Multivalent vaccines could easily be formulated by combining proteins from different virus strains into a single vaccine or by tailoring the targeted strains to populations or individuals.

One important consideration with protein subunit vaccines is that proteins alone are weakly immunogenic and thus immunostimulatory adjuvants are required to boost their efficacy (Pashine 2005, Guy 2007). The only approved adjuvant in the U.S. is alum, which consists of aluminum salts such as aluminum hydroxide or aluminum phosphate (Gupta 1998). However, alum generally induces a T helper (T_H)2-biased humoral (antibody) immune response as opposed to T_H1-biased cellular (cytotoxic T cell) immunity (Lindblad 2004). Thus there is a need for new adjuvants which stimulate a T_H1 response. A widely studied class of immunostimulatory adjuvants being developed includes pathogen-derived molecules that are capable of stimulating innate immune responses through pathogen-associated molecular pattern (PAMP) recognition receptors such as the Toll-like receptors (TLRs) (Petrovsky 2004, Pashine 2005, Trinchieri 2007, Guy 2007).

TLRs are expressed in antigen-presenting cells (APCs) and recognize characteristic molecules from pathogens, such as bacterial lipopolysaccharide (LPS) (TLR4) and bacterial and viral nucleic acids (TLRs 3, 7, and 9), as well as synthetic analogs of pathogen-derived molecules (Akira 2001, Takeda 2003, Ishii 2007). Treatment of APCs with agonists for TLR 3, 4, 7, and 9 leads to secretion of inflammatory cytokines such as Type I interferons, tumor necrosis factor (TNF)-α, interleukin (IL)-6, and IL-12, as well as upregulation of costimulatory surface molecules

such as CD80 and CD86 (Verdijk 1999, Alexopoulou 2001, Loré 2003, Napolitani 2005, Gautier 2005). Also, the administration of protein antigens along with these TLR agonists to dendritic cells (DCs) enables the cross-priming of $CD8^+$ T lymphocytes and the generation of an adaptive immune response with a $CD4^+$ T helper 1 (T_H1) bias. (Schulz 2005, Heit 2007, Zhang 2007, Standley 2007, Hamdy 2007). Many formulations are in clinical development which utilize the T_H1-inducing properties of TLR agonists (Kanzler 2007).

A second major consideration with protein-based vaccines is the need for a delivery system which can target dendritic cells. Proteins and nucleic acid TLR agonists are charged molecules which are not membrane-permeable and thus are not taken up effectively by DCs. Considerable research has been reported on the development of microparticle-based vaccine delivery systems composed of poly(lactic-co-glycolic acid) (PLGA) and other polymers (Bramwell 2005, Jiang 2005, Storni 2005, Waeckerle-Man 2005, and Tamber 2005). These delivery systems typically target APCs via non-specific phagocytosis. Vaccine delivery systems have been developed to deliver antigens for various infectious diseases and toxins, including anthrax, clostridium botulinum, ricin toxin, Marburg virus, Ebola virus, and tetanus, in addition to model antigens (Bramwell 2005, Jiang 2005). Microparticles also provide an advantage in that they enable simultaneous delivery of antigen and adjuvant to APCs. Several studies have shown that injection of conjugated or co-encapsulated antigen/TLR ligand generates a stronger immune response than co-injection of separate antigen and TLR ligand (Cho 2000, Tafaghodi 2006, Kwon 2005b, Hamdy 2007, Heit 2007, Standley 2007, Zhang 2007). This is consistent with recent findings that the efficiency of major histocompatibility

complex (MHC) presentation of phagocytosed antigen is dependent on the presence of TLR ligand within the same phagosome (Blander 2006).

Based on the objective of delivering protein antigen and immunostimulatory adjuvants to APCs, several design requirements can be identified for a protein vaccine delivery system. The delivery vehicle should consist of a nano- or micro-scale particle which encapsulates protein antigen and adjuvants, and which is composed of biocompatible, degradable materials. The vaccine should be easy to formulate and the carrier material should provide storage stability for the vaccine components. It is also desirable to have a stimulus-responsive mechanism for intracellular release of the vaccine. Finally, the formulation must have the flexibility of incorporating a variety of TLR agonists with different chemical properties.

While many candidate delivery systems have been developed for protein/TLR vaccines, there is a need for further improvement with regard to the carrier materials and formulation methods, and through the utilization of new adjuvants. For example, many of the microparticle systems are composed of PLGA and other polyesters, which have acidic degradation products. This is a concern due to the potential for degradation of proteins or nucleic acids within the acidic microclimate inside degrading PLGA particles (Shenderova 1999, Fu 2000, Tamber 2005). Also, many drug delivery polymers, including PLGA, do not exhibit stimulus-responsive release mechanisms, such as pH- or glutathione-sensitivity, which can selectively deliver therapeutic molecules to target cells. Certain delivery systems, such as acetal-crosslinked hydrogels, exhibit pH-sensitivity, however they are not composed of biodegradable polymers (Kwon 2005a, Kwon 2005b, Standley 2007).

There is also a need to expand the repertoire of available adjuvants in order to maximize the potential of protein-based vaccines. Due to the variability in TLR expression among cell types and between species, different TLR agonists may be needed to target specific infectious diseases or cancers (Jarrossay 2001, Edwards 2003, Bagchi 2007, Naik 2008). For example, double-stranded (ds)RNA, a ligand for TLR3, is a strong inducer of a T_H1 immune response and has shown promise as a vaccine adjuvant (Sloat 2006, Sloat 2008, Trumpfheller 2008). TLR3 is an endosomal receptor, and thus delivery of dsRNA to the endosome would likely enhance engagement of TLR3 (Guy 2007). However, dsRNA has previously not been incorporated into microparticles, which are capable of endosomal delivery and thus could improve the efficacy of dsRNA as an adjuvant.

1.2 Research Objectives and Specific Aims

The **long-term objective** of this research was to develop improved delivery vehicles for protein-based vaccines. These improvements include new polymeric materials and formulation methods for encapsulating protein antigens and nucleic acid TLR agonists. This work was guided by the **central hypothesis** that an effective vaccine delivery system would have the properties of stimulus-responsive degradation and release, biodegradability into excretable non-acidic degradation products, and the ability to incorporate various TLR-inducing adjuvants.

The first two specific aims were centered around developing biodegradable polymeric delivery systems for protein and nucleic acid therapeutics. While the two delivery systems – cross-linked micelles and polyketal microparticles – have somewhat

different designs, both systems are capable of encapsulating proteins, DNA, and RNA. The third specific aim focuses on protein subunit vaccines, which is the primary application pursued in this thesis research. In this aim, both of the delivery systems were used to formulate model vaccines that were evaluated using in vitro and animal models in collaboration with researchers at the Emory Vaccine Center.

Specific Aim 1: Develop a cross-linked block copolymer micelle for efficient encapsulation and retention of proteins, DNA, and RNA, as a model delivery system for protein-based vaccines.

Hypothesis 1: Efficient encapsulation and retention of proteins, DNA, and RNA can be achieved by optimizing the parameters governing self-assembly and cross-linking of a polyionic complex micelle delivery system.

The micelle-based delivery system consists of a block copolymer of poly(ethylene glycol) (PEG) and poly(L-lysine), cross-linked by dithiopyridyl side groups to provide transport stability (Figure 1.1). The PEG-poly(L-lysine) (PLL) polyionic complex micelle is based on a design originally developed by the Kataoka laboratory (Harada 1995, Harada 1998, Kakizawa 1999). The micelles self-assemble via electrostatic interactions between the polylysine block and the protein or nucleic acid, forming the core of the micelle. The PLL block is modified with pyridyldithio groups to allow for cross-linking by a dithiol molecule. The disulfide cross-linking is designed to be cleaved in the intracellular reducing environment, enabling release of the biotherapeutics. An important development by the Murthy laboratory was the use of a Michael addition reaction to attach the pyridyldithio groups to the poly(Lys) chain, thereby retaining the

positive charge on the secondary amine. Micelle formulations were prepared using various proteins and nucleic acids. The micelles were initially developed for vaccine applications, in which a model protein antigen (ovalbumin) was encapsulated alone or with DNA- or RNA-based immunostimulatory agents. Another application was later pursued, enzyme therapy, in which the model enzyme catalase was encapsulated. The encapsulation of proteins, DNA, and RNA was confirmed by gel electrophoresis and filtration methods. The size and morphology of the micelles was confirmed by dynamic light scattering and atomic force microscopy. The efficacy of micelle-based vaccine formulations was tested in Specific Aim 3.

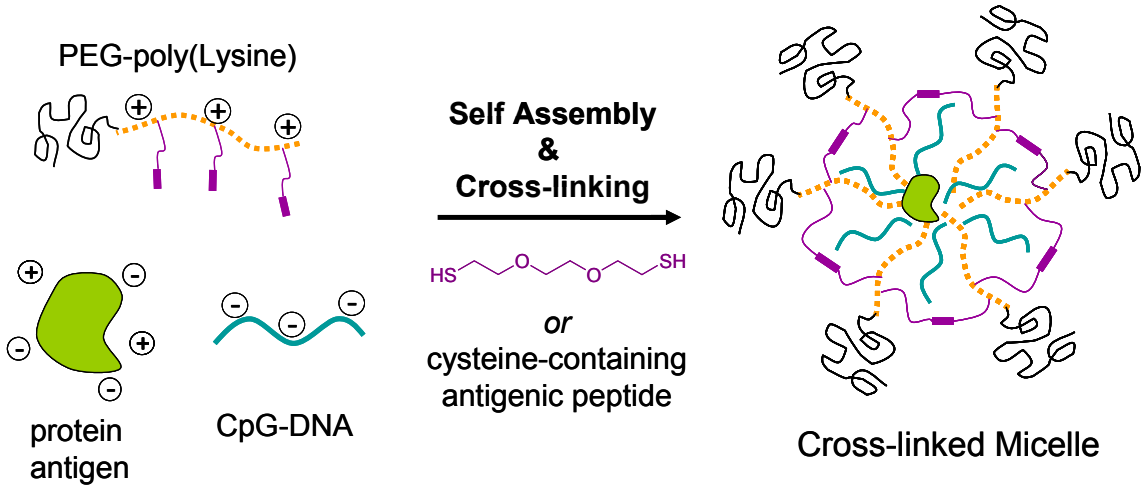


Figure 1.1 Cross-linked micelle delivery system for proteins and nucleic acids.

Specific Aim 2. Develop a pH-sensitive biodegradable polymer for fabricating microparticles encapsulating proteins/DNA/RNA and hydrophobic small molecules, as a model protein-based vaccine delivery vehicle.

Hypothesis 2: Using the acetal exchange reaction, a ketal-containing polymer can be synthesized that will exhibit pH-sensitive degradation into biocompatible products and will thus provide a platform for intralysosomal delivery of proteins/DNA/RNA.

A new polymerization method was developed, based on the acetal exchange reaction, which produced hydophobic, linear polymers containing pH-sensitive ketal linkages in the backbone (Figure 1.2). This new polymer, termed a polyketal, has a combination of properties not found in existing drug delivery polymers, namely pH-sensitivity, biodegradability, ease of synthesis, and non-acidic degradation products. Polyketals were used to fabricate pH-sensitive microparticles that are designed to release drugs or biotherapeutics in the acidic endosome of phagocytic cells. Additionally, the technique of hydrophobic ion pairing was utilized to enhance the encapsulation of ovalbumin, DNA, and RNA in the polyketal microparticles via a single emulsion method.

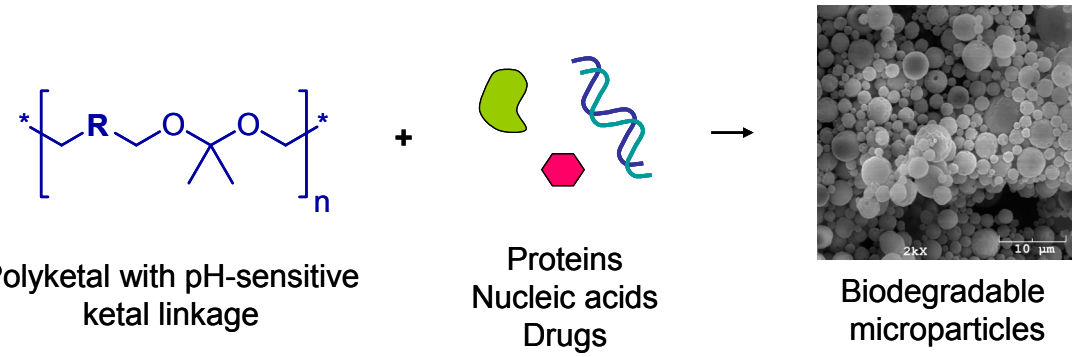


Figure 1.2. Polyketal-based delivery system for proteins, nucleic acids, and hydrophobic molecules.

The chemical structure of the polyketals was characterized by gel permeation chromatography (GPC) and nuclear magnetic resonance (NMR) spectroscopy, and pH-

sensitive degradation was demonstrated by NMR. Encapsulation of ovalbumin, DNA, and RNA was measured using standard fluorescent labeling or fluorescent staining techniques. Various polyketal microparticle-based vaccine formulations were evaluated for their efficacy in Specific Aim 3.

Specific Aim 3: Demonstrate the in vitro and in vivo efficacy of model vaccine formulations containing ovalbumin and immunostimulatory agents, using the cross-linked micelle and polyketal microparticle delivery systems.

Hypothesis 3: Encapsulation of ovalbumin and Toll-like receptor (TLR) agonists in a micelle or polyketal microparticles can enhance the cross-priming of cytotoxic T lymphocytes.

The efficacy of the two vaccine delivery systems (cross-linked micelles and polyketal nanoparticles) was evaluated using in vitro and in vivo methods. The model vaccine formulations contained ovalbumin plus one or more immunostimulatory agents, including agonists for TLRs 3, 4, and 9. In an in vitro cross-priming assay, murine splenic dendritic cells were treated with vaccine formulations and co-cultured with OT-1 splenocytes for 4 days. The expanded splenocytes were re-stimulated with ovalbumin peptide (SIINFEKL), and cross-priming was assayed by measuring the production of the cytokines interferon (IFN)-γ, tumor necrosis factor (TNF)-α, and interleukin (IL)-2. For the in vivo studies, mice were immunized with the model vaccine formulations and peripheral blood mononuclear cell (PBMC) samples were taken at later time points. Harvested cells were stimulated with SIINFEKL peptide and assayed for intracellular cytokine production.

1.3 Review of Relevant Literature

Mechanism of inducing cellular immunity

Traditional vaccines for viral infections have largely been developed using empirical methods and typically consist of live attenuated or inactivated viruses or purified virus proteins adjuvanted with aluminum-based compounds. Despite the decades of successful use, very little was known about vaccines' mechanism of action until recent years. Through the discovery of the Toll-like receptors (TLRs) and other receptors that recognize pathogen-associated molecular patterns (PAMPs), researchers have begun to elucidate the mechanisms by which pathogens (or vaccines derived form pathogens) induce adaptive immune responses (Akira 2001). This process begins with the uptake of a vaccine or pathogen by an antigen-presenting cell (APC). The APC processes the pathogen proteins by cleaving the proteins into peptide fragments and displaying the peptides on major histocompatibility (MHC) molecules on the cell surface. T lymphocytes with T cell receptors (TCRs) specific to the peptide antigen bind to the MHC-peptide complex, which constitutes the first signal in APC-T cell interaction. A second signal comes in the form of costimulatory molecules, such as B7-1 (CD80) and B7-2 (CD86), which are also displayed on the APC cell surface and engage T cell ligands.

The presence of only these two signals can lead to tolerance, and thus a third signal is required to fully activate the APC and induce a robust T cell response. This third signal consists of cytokines such as interleukin (IL-12) and type I interferons (IFN-α and IFN-β), which are induced by TLR activation (Mescher 2006). IFN-α and IFN-β mediate an antiviral response through the production of viral replication inhibitors, the

recruitment of inflammatory cells, and the upregulation of MHC protein expression (Guidotti 2001). The proinflammatory cytokine IL-12 provides a link between the innate and adaptive immune response, through the production of IFN-γ, which promotes recruitment and activation of inflammatory cells and upregulation of antigen processing and presentation. IL-12 also promotes a T helper 1 (Th1) bias, which leads to expansion of cytotoxic T lymphocytes (CTL) to mount an attack against intracellular pathogens (Trinchieri 2003).

Toll-like receptors (TLRs) as immunostimulatory adjuvants

The Toll-like receptors are a class of pathogen-recognition receptors within the innate immune system that detect the presence of bacteria and viruses in the extracellular space of endosomal compartments. Engagement of TLRs leads to production of IFN-α and IL-12 through two distinct pathways. All TLRs except TLR3 signal through a myeloid differentiation factor 88 (MyD88) pathway, whereas TLR3 and TLR4 signal through a MyD88-independent pathway. TLR3 and TLR4 signal through an adaptor termed the Toll-IL-1 receptor (TIR) domain containing adaptor inducing IFN-β (TRIF), through an intermediate TRIF-related adaptor molecule (TRAM). The MyD88 and TRIF pathways lead to the activation of nuclear factor (NF)-κB and IRF-3, which promote the expression of pro-inflammatory cytokines and chemokines (Pashine 2005).

Several of the TLRs are important in stimulating cellular immunity, by inducing a T_H1 bias in the $CD4^+$ T cell response. For example, TLR4 is a cell surface receptor that recognizes lipopolysaccharide (LPS), a component of bacterial cell wall. Monophosphoryl lipid A (MPL) is a synthetic TLR4 agonist that is being developed for

hepatitis B and human papillomavirus vaccines and to treat non-small-cell lung cancer, among other applications (Kanzler 2007). TLR9 recognizes unmethylated DNA containing "CpG" motifs, which is characteristic of bacterial DNA (Klinman 2004a, 2004b, 2006). CpG-DNA is administered in soluble form or conjugated to a protein antigen. Clinical application under development include vaccines for hepatitis B, anthrax, influenza, HIV, and melanoma (Kanzler 2007). Recently several groups have developed microparticles encapsulating CpG-DNA with protein antigens and have demonstrated enhancement of $CD8^+$ T cell responses (Kwon 2005b, Heit 2007, Standley 2007, Zhang 2007). TLR7 is an endosomal receptor that recognizes single-stranded viral RNA (Heil 2004, Pashine 2005). Synthetic TLR7 agonists such as Imiquimod have been developed which have more "drug-like" properties. Imiquimod has been approved for treatment of basal cell carcinoma and papilloma-induced genital warts and is under clinical development for other applications (Kanzler 2007).

TLR3 is an endosomal receptor that recognizes double-stranded (ds)RNA, including the dsRNA analog poly(inosinic acid)-poly(cytidylic acid) (poly(I:C)) (Alexopoulou 2001). Studies have shown that interaction of dsRNA with TLR3 depends on the acidification of the endosome (de Bouteiller 2005, Schulz 2005, Kumar 2005). Viral dsRNA and poly(I:C) induce maturation of dendritic cells (DCs), high IL-12 expression, effective MHC-I antigen presentation, and strong T cell proliferation (Verdijk 1999, Loré 2003, Schulz 2005). Poly(I:C) has been investigated as a potential adjuvant for intranasal influenza vaccines, therapeutic treatment of cervical cancer, and treatment of herpes simplex virus type 2 infection (Ichinohe 2005, Cui 2006, Herbst-Kralovetz 2006). While TLR3 was the first receptor identified for dsRNA, the cytosolic receptor

melanoma differentiation associated gene 5 (Mda5) is also stimulated by dsRNA (Gitlin 2005, Kato 2006).

The property of poly(I:C) as a pro-inflammatory agent makes it a potentially effective vaccine adjuvant; however, there may be concerns with the systemic inflammation resulting from high doses of soluble poly(I:C). For example, one study reported that intraperitoneal (i.p.) injection of poly(I:C) in mice at doses of 2 to 12 mg/kg resulted in sickness behavior, reduced body weight, and fever (Cunningham 2007). Another study showed that i.p. injection of 3 mg/kg of poly(I:C) in rats resulted in reduced running wheel activity and increased expression of IFN-α in the central nervous system (Katafuchi 2003). Similarly, i.p. injection of 5 mg/kg of poly(I:C) in mice was shown to reduce exercise capacity and increase levels of IFN-α and IFN-β in the bloodstream (Davis 1998). These findings suggest that targeted delivery of poly(I:C) would be needed in order to reduce the adjuvant dosage and limit systemic effects.

Co-delivery of antigen and adjuvants

Recently, many studies have focused on the co-delivery of antigen and TLR agonists to enhance Th1 responses and cross-priming of CTLs. For example, while CpG-DNA is often administered in soluble form, a few studies have demonstrated the advantages of co-delivery of CpG-DNA with antigen. The Raz laboratory conjugated ovalbumin (a tumor model antigen) to CpG ssDNA (TLR9 ligand) and showed that the conjugate was more effective at suppressing an ovalbumin-expressing tumor than treatment with Ova antigen or CpG DNA alone (Cho 2000). Tafaghodi et al. (2006) encapsulated tetanus toxoid (TT) with CpG-ODN in alginate microspheres for a vaccine

administered intranasally to rabbits, and showed improved IgA response in nasal lavages with TT/CpG versus TT microparticles or corresponding non-particle controls. Also, the Fréchet group has demonstrated that hydrogel nanoparticles containing ovalbumin and CpG-DNA stimulate DCs to produce higher levels of IL-12 and induce stronger $CD8^+$ T cell responses compared to Ova nanoparticle or free CpG DNA controls (Kwon 2005b, Standley 2007). TLR4 ligands have also been employed as adjuvants. For example, the Samuel laboratory has shown an effective in vivo T cell response in mice by co-encapsulating ovalbumin with MPL (a TRL4 ligand) in PLGA nanoparticles (Hamdy 2007). The common finding in these studies is that injection of conjugated or co-encapsulated antigen/TLR ligand generates a stronger immune response than co-injection of separate antigen and TLR ligand.

Other protein/DNA delivery systems

Various other delivery systems have been developed to deliver proteins and nucleic acids for vaccine applications. Uto et al. encapsulated ovalbumin in poly(γ-glutamic acid) (γ-PGA) nanoparticles and showed increased cytokine production, upregulation of costimulatory molecules, and enhanced antigen-specific B and T cell stimulation with Ova-nanoparticles. Also, listerolysin (LLO) peptide immobilized on the γ-PGA NPs protected mice from infection with *Listeria monocytogenes* bacteria (Uto 2007). A DNA vaccine formulation was prepared by complexing polyethylene amine (PEI) with plasmid (p)DNA and reacting it with PEG-NHS to form a PEGylated polyplex, which was encapsulated in PLGA microparticles. Oral delivery to rats resulted in plasmid DNA transgene expression of β-galactosidase in the spleen (Howard 2004).

Another DNA delivery vehicle consists of pDNA complexed with thiolated multi-arm PEG, crosslinked to form 300-800 nm size particles; the PEG nanogels exhibited glutathione-sensitive release of pDNA (Mok 2006). Mohamed et al. fabricated hollow dimpled PLGA microcapsules for pulmonary vaccine delivery. The low density 8 μm particles have a 3-4 μm aerodynamic diameter, which is suitable for delivery to the bronchus associated lymphoid tissue (BALT) and lung periphery (Mohamed 2006).

Polyionic Crosslinked (PIC) Micelles for Delivery of Charged Molecules

The formation of polyionic complex (PIC) micelles was first reported by Harada and Kataoka (1995), where stable complexes were produced from a stoichiometric mixture of poly(ethylene glycol)(PEG)-poly(Lys) and PEG-poly(Asp) in aqueous buffer. The complexes were formed through the electrostatic interactions between the poly(Lys) and poly(Asp) chains, with the PEG chains forming the corona, or shell, of the micelle. This concept was further developed through the addition of disulfide cross-linking to stabilize PIC micelles. Is this design, the micelles were composed of PEG-poly(Lys) and PEG-poly(Asp), where a portion of the lysine residues were modified through a reaction with SPDP and reduced to give free thiols (Kakizawa 1999). After complexation, the thiols were oxidized to form disulfide cross-links, which provide micelle stability even at high salt concentrations. The disulfide cross-linking was intended for intracellular delivery of charged biomolecules, based on evidence that a stronger reductive environment exists intracellularly (Huang 1998). Cross-linked micelles were also formed with thiolated PEG-poly(Lys) and antisense DNA, and were shown to be stable against a competing polyelectrolyte, poly(vinyl sulfate) (PVS), and yet release the DNA upon

treatment with PVS and glutathione (Kakizawa 2001, Miyata 2005). The PIC micelles have also been used for applications in delivering plasmid DNA and siRNA (Katayose 1997, Miyata 2005, Nishiyama 2006a, Nishiyama 2006b).

The Kataoka group also demonstrated that PIC micelles could be formed with PEG-poly(Asp) and proteins such as trypsin and lysozyme (Harada 1998, Harada 1999, Harada 2001, Jaturanpinyo 2004). The lysozyme micelles were shown to have greater enzyme activity than free lysozyme, which was attributed to attraction of the substrate by the PEG corona, which resulted in a decrease in the observed Michaelis constant (Harada 2001). Poly(Asp) micelles with trypsin were prepared using glutaraldehyde to cross-link the trypsin molecules within the core. The micelles were shown to have less trypsin degradation (more retention of enzyme activity) than free trypsin, supposedly due to immobilization of trypsin in the core, which prevented autolysis of the enzyme. (Jaturanpinyo 2004)

pH-Sensitive Vaccine Delivery Systems

The property of pH-sensitivity has been pursued in many drug delivery vehicles as a means of achieving intracellular release through degradation of the polymer in the acidic lysosomal compartment. The Frechet laboratory has developed polyacrylamide nanoparticles that contain acid-cleavable cross-linkers (Murthy 2003b, Standley 2004). The nanoparticles are prepared by inverse microemulsion polymerization of acrylamide monomers with a benzyl acetal bisacrylamide monomer. In one system, a trimethylammonium monomer were used to create cationic nanoparticles encapsulating ovalbumin, with CpG-DNA or IL-10 antisense DNA complexed to the surface of the

particles. The ovalbumin/CpG-DNA nanoparticles were shown to enhance IL-12 production in bone marrow-derived dendritic cells (BMDCs) versus ovalbumin nanoparticles or free CpG-DNA. Also, IL-10 antisense ODN-coated ovalbumin nanoparticles were shown to have a moderate reduction in IL-10 production as compared to free IL-10 antisense ODN. (Kwon 2005b) In another variation on the hydrogel design, CpG-DNA was derivatized with methacrylamide to be incorporated into the acetal crosslinked polyacrylamide nanoparticles. Nanoparticles containing ovalbumin and CpG-DNA enhanced stimulation of IL-12 and surface activation markers in BMDCs as compared to ovalbumin particles, and also induced strong ovalbumin-specific cytotoxic T cell responses in mice. (Standley 2007)

Hydrophobic Ion Pairing

The technique of hydrophobic ion paring has been developed by several research groups as means of altering the solubility of proteins and nucleic acids, through the stoichiometric pairing with oppositely charged surfactants (Hegg 1979, Powers 1993, Bromberg 1994, Meyer 1998, Patel 2004, Dai 2007). Various applications of ion pairing have been considered, such as making an enzyme available to a substrate that is soluble in a nonpolar solvent. Another application is in the encapsulation of charged molecules in polymeric microparticles. One group has utilized a microparticle fabrication method, precipitation with compressed antisolvent (PCA), which requires the drug molecules to be soluble in a nonpolar solvent (Falk 1997). They have demonstrated that plasmid DNA can be ion paired with DOTAP via a Bligh-Dyer extraction method, producing a DNA:DOTAP complex that is soluble in dichloromethane (DCM), and have shown high

encapsulation efficiencies (>70%) in poly(lactic acid) microspheres. (Patel 2004) It has also been shown that the anti-cancer drug cisplatin can be ion-paired with docusate sodium (aerosol OT, or AOT) to produce a compound with greater cytotoxicity toward cancer cells in vitro. This effect was attributed to the greater hydrophobicity and consequent increase in cell permeability of the ion-paired cisplatin. (Feng 2004).

CHAPTER 2

DEVELOPMENT OF CROSS-LINKED BLOCK COPOLYMER MICELLES

2.1 Introduction

In this chapter we discuss Specific Aim 1, which was to develop a **cross-linked micelle** delivery system for efficient encapsulation of proteins and nucleic acids. Polyion complex (PIC) micelles had originally been developed by the Kataoka laboratory and were based on self-assembly of a poly(ethylene glycol)(PEG)-poly(Lys) or PEG-poly(Asp) block copolymer with charged molecules such as DNA or proteins. The core of the micelle is formed via electrostatic interactions between the polylysine and DNA, or between the poly(Asp) and positively charged proteins such as lysozyme or trypsin, while the PEG chain occupies the shell, or corona, of the micelle. (Harada 1995, Kakizawa 1999, Kakizawa 2001). Various cross-linking schemes have been developed, such as disulfide linkages and aldehyde-amine reactions. The cross-linking provides for transport stability in serum or extracellular fluid, where the presence of competing charged molecules would cause disruption of the electrostatic interactions. Figure 2.1 shows a schematic of the PIC micelle self-assembly and cross-linking.

The aldehyde cross-linking of trypsin within the PIC micelle, as reviewed in Chapter 1, does not however generate a stimulus-responsive release mechanism for intracellular delivery. The disulfide cross-linking method used with thiolated PEG-poly(Lys), on the other hand, was effective at generating stable complexes with DNA and offers a means of intracellular release through cleavage by reducing agents such as

glutathione (Huang 1998). However, it may not securely encapsulate proteins, which generally have fewer charged groups available for electrostatic complexation. Furthermore, in the Kataoka design, the pyridyldithio cross-linking moiety was attached by reacting the PLL backbone with SPDP. The reaction of the lysine amine with the succinimidyl group produces an amide bond, which means one positive charge is lost for each cross-linking group added. Because the positive charges are needed for complexation, an improvement would be to use a reaction that would attach pyridyldithio groups without losing positive charges on the poly(Lys) chain.

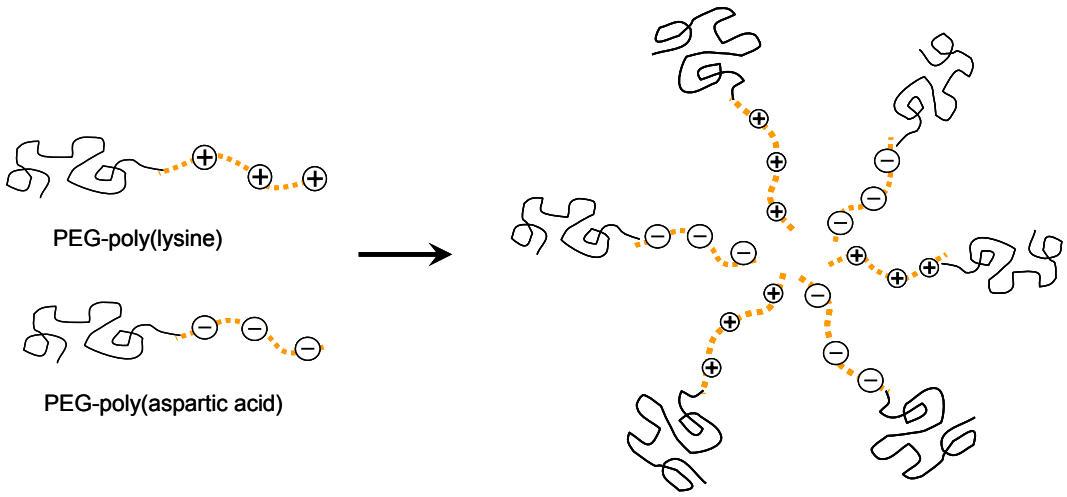


Figure 2.1 Schematic of polyion comlex (PIC) micelle self-assembly.

We hypothesized that efficient encapsulation and retention of proteins, DNA, and RNA could be achieved by optimizing the parameters governing the self-assembly (electrostatic complexation) and cross-linking of a PIC micelle delivery system. As a starting point for our PIC micelle design, we chose PEG-poly(Lys) as the block copolymer, which had previously been used to complex DNA. The initial goal of this

project was to deliver ovalbumin and CpG-DNA for a model vaccine. Therefore, PEG-poly(Lys) would be suitable to complex with CpG-DNA and ovalbumin, which has an isoelctric point of pH 5 and carries a net negative charge. In order to retain the positive charges on poly(Lys), we developed a new method of attaching the pyridyldithio group via a Michael addition, which generates a secondary amine. Thus, we can maximize the number of disulfide groups and positively charged amines, to optimize the complexation and cross-linking. An additional improvement was the conjugation of pyridyldithio groups to ovalbumin to covalently tether the protein to the PEG-poly(Lys) in the core of the micelle.

For vaccine applications, we developed micelles containing peptide- and protein-based antigens. These vaccine formulations are designed to deliver antigen and immunostimulatory adjuvants to phagocytic antigen-presenting cells (APCs) to induce activation of the APCs and cross-priming of cytotoxic T lymphocytes (CTLs). The original micelle developed in the Murthy laboratory was the peptide-crosslinked micelle (PCM), in work led by Jihua Hao (Hao 2006). In the PCMs, the antigenic peptide SIINFEKL was modified with two cysteine residues and served as the cross-linking agent, while immunostimulatory CpG-DNA was the polyionic complexing agent in the core of the micelle. The PCMs were shown to be stable in a solution of poly(vinyl sulfate) (PVS), which is intended to represent the conditions in serum or extracellular fluid, where the presence of competing amphiphilic molecules would cause disruption of the electrostatic interactions. It was also shown that the disulfide cross-linking bonds can be cleaved by reducing agents such as glutathione (GSH), which is found in higher intracellular concentrations versus in the bloodstream (Huang 1998). Additionally, the

PCMs enhanced the uptake of FITC-labeled peptide antigen by dendritic cells (Hao 2006).

In our subsequent work with protein-loaded micelles, we encapsulated the whole protein antigen ovalbumin along with immunostimulatory nucleic acids (CpG-DNA and poly(I:C)). We demonstrated retention of CpG-DNA and poly(I:C) by agarose gel and retention of ovalbumin by SDS-PAGE. We also showed that covalent tethering of ovalbumin via a pyridyldithio group resulted in better protein retention than untethered ovalbumin. The efficacy of the ovalbumin-based model vaccine formulations was tested in vitro using primary murine dendritic cells and OT-1 splenocytes, and in vivo in mice, as described in Chapter 4.

2.2 Experimental Methods

Synthesis of PEG-poly(lysine-dithiopyridyl) (PEG-PLTP) copolymer.

Synthesis of PEG-Polylysine Block Copolymer.

The block copolymer backbone, consisting of a 5000 molecular weight PEG chain and approximately 10 to 20 lysine repeats, was synthesized by the methods developed by the Kataoka laboratories (Harada 1995, Kakizawa 1999). Briefly, the *N*-carboxyanhydride (NCA) of ε-benzyloxycarbonyl-*L*-lysine (Lys(Z)-NCA) was generated by reacting Lys(Z)-OH (10 g, 35.7 mmol) with triphosgene (5.3 g, 17.8 mmol) for 4 hours at 50°C in dry THF. The product was recrystallized three times in cold hexanes, with a 72% yield. The poly(ethylene glycol)-*block*-poly(*L*-lysine(Z)) (PEG-PLL(Z)) copolymer was synthesized via a ring-opening polymerization of α-methoxy-ω-amino-PEG (1.0 g, 0.2 mmol) with Lys(Z)-NCA (3.66 g, 12 mmol) for 20 hours at 40°C in

anhydrous DMF. The product was precipitated in cold ethyl ether, vacuum filtered, and vacuum dried to yield 1.5 g of PEG-PLL(Z) block copolymer. From the ^{1}H NMR spectrum, the degree of polymerization (d.p.) was calculated to be 30.8. Deprotection of the ε-benzyloxycarbonyl group was carried out by mixing PEG-PLL(Z) with trifluoroacetic acid, then a mixture of anisole and methanesulfonic acid, followed by ether/water extraction. The aqueous phase was dialyzed against a 1000 molecular weight cut-off membrane, and lyophilized to yield 543 mg of PEG-PLL. After deprotection, the d.p. was calculated to be 13.7 based on the ^{1}H NMR spectrum. The change in d.p. during the deprotection step was attributed to low molecular weight polylysine chains that were initiated by water molecules in the ring-opening polymerization reaction, and were subsequently removed during dialysis.

Synthesis of Side Chain Monomer Pyridyldithio-Ethylacrylate

Synthesis of pyridyldithioethylacrylate is described by Hao and Murthy (2006). Briefly, mercaptoethanol (0.886 g, 11.36 mmol) was reacted with 2,2-dithiodipyridine (5 g, 22.7 mmol) and acetic acid (1.36 g, 22.7 mmol) in 15 mL of methanol for 1 hour at room temperature. The 2-(2-pyridinyldithio)-ethanol product was purified by silica gel chromatography with 79.5% yield. Next, 2-(2-pyridinyldithio)-ethanol (1.6 g, 8.56 mmol) was dissolved in 16 mL of dichloromethane (DCM) with triethylamine (1.297 g, 12.84 mmol). Acryloyl chloride (1.156 g, 12.84 mmol) was added in a dropwise manner to initiate the reaction. After 1 hour, the product was purified by DCM/brine extraction (2 times), dried with sodium sulfate, and purified by silica gel chromatography, for a 45% yield of pyridyldithioethylacrylate (PDTEA).

Synthesis of Final Copolymer PEG-PLTP

The pyridyldithioethylacrylate (PDTEA) side chain monomer was conjugated to the PEG-PLL backbone via a Michael addition reaction, which retains the positive charge on the lysine amine. Briefly, PEG-PLTP (250 mg, 36.8 µmol) was dissolved in 6 mL of DMF, and PDTEA (133 mg, 552 µmol) was added with an additional 4 mL of DMF. Triethylamine (154 µL, 1.104 mmol) was added, and the reaction was run at 30°C overnight. The reaction mixture was precipitated in 500 mL of cold ethyl ether, vacuum filtered, and vaccum dried to yield 179 mg of polymer. The final polymer is termed poly(ethylene glycol)-*block*-poly(*L*-lysine-*N*-pyridyldithioethylethanoate) (PEG-PLTP) (Figure 2.2).

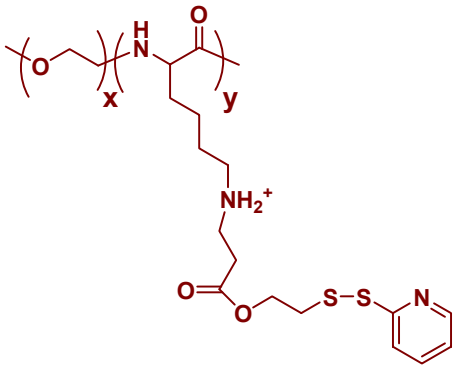


Figure 2.2. PEG-poly(lysine-dithiopyridyl) (PEG-PLTP) block copolymer.

Encapsulation of proteins, and nucleic acids in cross-linked copolymer micelles.

The block copolymer PEG-PLTP was used to prepare polyionic complex micelles containing proteins and nucleic acids, including ovalbumin, catalase, CpG-DNA, and poly(I:C) (Figure 2.3). The micelles were prepared by first combining solutions of FITC-labeled ovalbumin, CpG-DNA, and/or poly(I:C), and vortexing to ensure thorough mixing. Next, a 20 mg/mL solution of the PEG-poly(*L*-lysine-dithiopyridyl) (PEG-

PLTP) copolymer was added to form the micelles. Alternatively, PEG-PLTP was mixed with either FITC-labeled ovalbumin or FITC-labeled catalase. The mixtures were left to stand at room temperature for 30 to 60 minutes to allow self-assembly of the micelles. Next, 3,6-dioxa-1,8-octanedithiol (DOODT) was added in a step-wise manner to crosslink the dithiopyridyl side chains through a disulfide exchange reaction. The micelles were allowed to stand for 20 minutes following each addition of DOODT to complete the disulfide exchange.

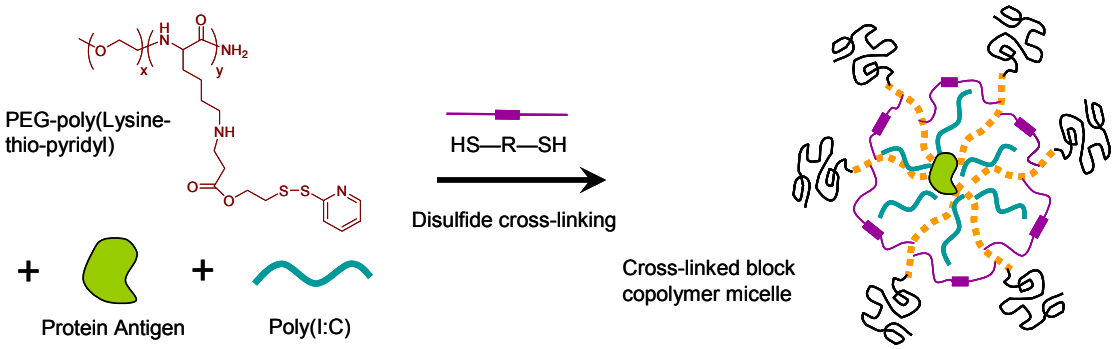


Figure 2.3. Cross-linked copolymer micelle encapsulating protein antigen and Poly(I:C).

The step-wise cross-linking method was developed to ensure that the cross-linking efficiency was as close as possible to 100%. The amount of cross-linker thiols must be equimolar to the calibrated dithiopyridyl content of the PEG-PLTP polymer; if the amount of cross-linker is too high or too low, there will potentially be less than 100% cross-linking. In a typical four-stage cross-linking, we add 70% of the stoichiometric amount of dithiol, followed by 15%, 10%, and 10%, at 20 minute intervals. This method is preferred to simply adding an excess of DOODT, because that would result in some DOODT molecules reacting with a dithiopyridyl only at one end and not generating a

cross-link. The stepwise method ensures that the cross-linking will be between 95-100%, assuming a +/-5% inaccuracy in the DOODT stoichiometry.

The dithiopyridyl content of the PEG-PLTP solution was calibrated prior to micelle preparation, by reacting the polymer with dithiothreitol (DTT) and measuring the production of thiopyridone by the increase in absorbance at 342 nm. In a similar manner, the micelle cross-linking can be monitored by reacting a small sample with DTT to determine if all of the dithiopyridyl groups have been converted.

Covalent linkage of protein within micelle.

While the Ova/CpG/poly(I:C) micelles showed high encapsulation and retention of the nucleic acids in agarose gels, it was found that the FITC-Ova was not completely retained when the micelles were subjected to SDS-PAGE (data not shown). Thus, we modified the protein-based micelle by first reacting the ovalbumin with Sulfo-LC-SPDP, a commercially available reagent based on succinimidyl-pyridyl-dithio-proprionate (SPDP) (Pierce). Briefly, a 6.0 mg/mL solution of ovalbumin (15 mg, 0.34 μmol) was reacted with Sulfo-LC-SPDP (2.15 mg, 4.08 μmol) and purified by PD-10 column. This gave the Ova approximately 5 or 6 pyridyldithio groups per protein molecule, so that the Ova was covalently tethered to the PEG-PLL-dithiopyridyl polymer during the cross-linking step.

2.3 Results

Characterization of Protein-Loaded Micelles

Micelles Containing FITC-Ovalbumin, CpG-DNA, and Poly(I:C)

Micelle-based vaccine formulations were prepared with concentrations of 10 mg/mL PEG-poly(L-lysine-dithiopyridyl), 0.5 mg/mL FITC-Ova, 1 mg/mL CpG ssDNA, and/or 1 mg/mL poly(I:C). An agarose gel stained with ethidium bromide shows that CpG-DNA and Poly(I:C) are retained in the micelles (lanes 6, 7, and 8) (Figure 2.4). FITC-ovalbumin is retained in the FITC-Ova (lane 5) and FITC-Ova/poly(I:C) (lane 7) micelles, but is retained to a lesser extent in the FITC-Ova/CpG-DNA (lane 6) and FITC-Ova/CpG-DNA/poly(I:C) (lane 8) micelles.

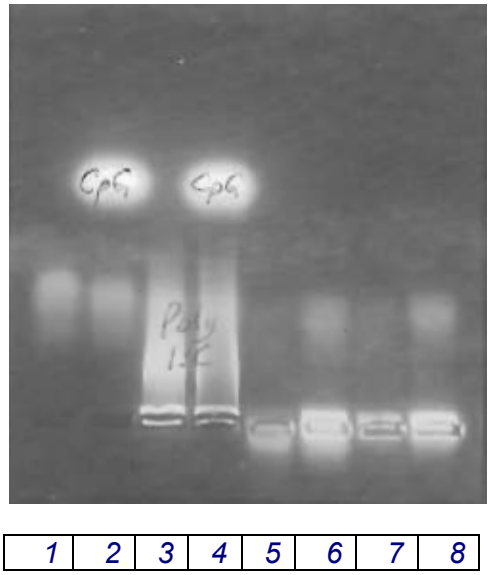


Figure 2.4. Agarose gel of cross-linked copolymer micelles with ovalbumin, CpG DNA, and poly(I:C) (9-25-05 batch). Lanes: (1) free Ova, (2) free Ova/CpG, (3) free Ova/poly(I:C), (4) free Ova/CpG/poly(I:C), (5) micelle with Ova, (6) micelle with Ova/CpG, (7) micelle with Ova/Poly(I:C), (8) micelle with Ova/CpG/Poly(I:C).

Micelles Containing SPDP-FITC-Ovalbumin and CpG-DNA

The micelles made with Sulfo-LC-SPDP-modified FITC-Ova showed virtually 100% retention of the Ova as determined by SDS-PAGE (Figure 2.5), as well as a significant degree of CpG DNA retention in agarose gel electrophoresis (Figure 2.6). The size and shape of the micelles were characterized by dynamic light scattering (DLS) (Figure 2.7) and atomic force microscopy (AFM) (Figure 2.8). The micelles had an effective diameter of 130 nm, with clusters in the size distribution at 45 nm and 190 nm. AFM images show a spherical shape, and measurement of the half-peak widths ranged from 50 nm to 150 nm.

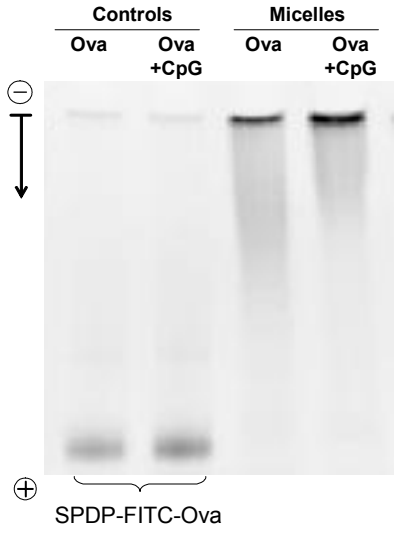


Figure 2.5. SDS-PAGE (4-20%) of SPDP-Ova/CpG micelles, prepared with 0.5 mg/mL FITC-Ova-SPDP, 0.5 mg/mL CpG DNA, and 10 mg/mL PEG-poly(Lys-Thio-Pyridyl).

Figure 2.6. Agarose gel of SPDP-Ova/CpG micelles, prepared with 0.5 mg/mL FITC-Ova-SPDP, 0.5 mg/mL CpG DNA, and 10 mg/mL PEG-poly(Lys-Thio-Pyridyl).

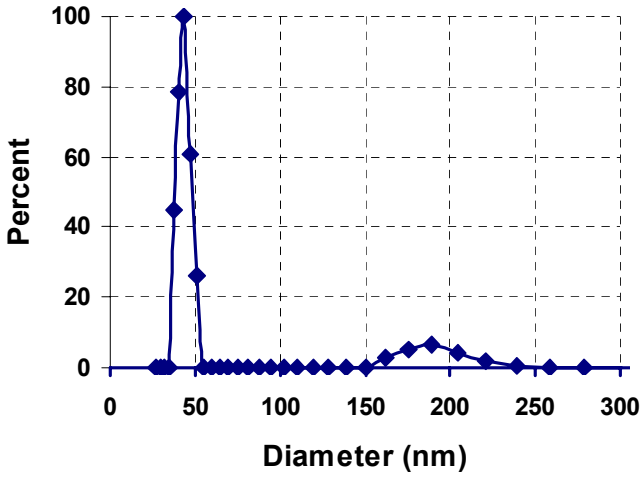
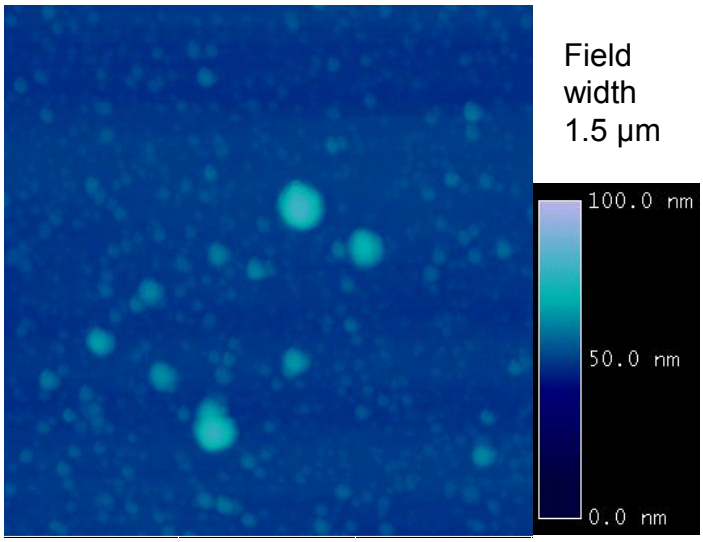


Figure 2.7. Characterization of SPDP-Ova/CpG micelles by dynamic light scattering (DLS) size distribution by volume (effective diameter 130 nm).



Courtesy of Catherine Santai (Hud lab, Ga. Tech)

Figure 2.8. Characterization of SPDP-Ova/CpG micelles by atomic force microscopy (AFM); approximate diameter = 50 to 150 nm. *(Courtesy of Catherine Santai (Hud lab, Georgia Tech)*

Micelles containing catalase

Cross-linked micelles were prepared with Sulfo-LC-SPDP-conjugated catalase, with 1:20 and 1:40 ratios of catalase to PEG-PLTP. Both samples showed a high degree of encapsulation and retention in an SDS-PAGE assay (Figure 2.9).

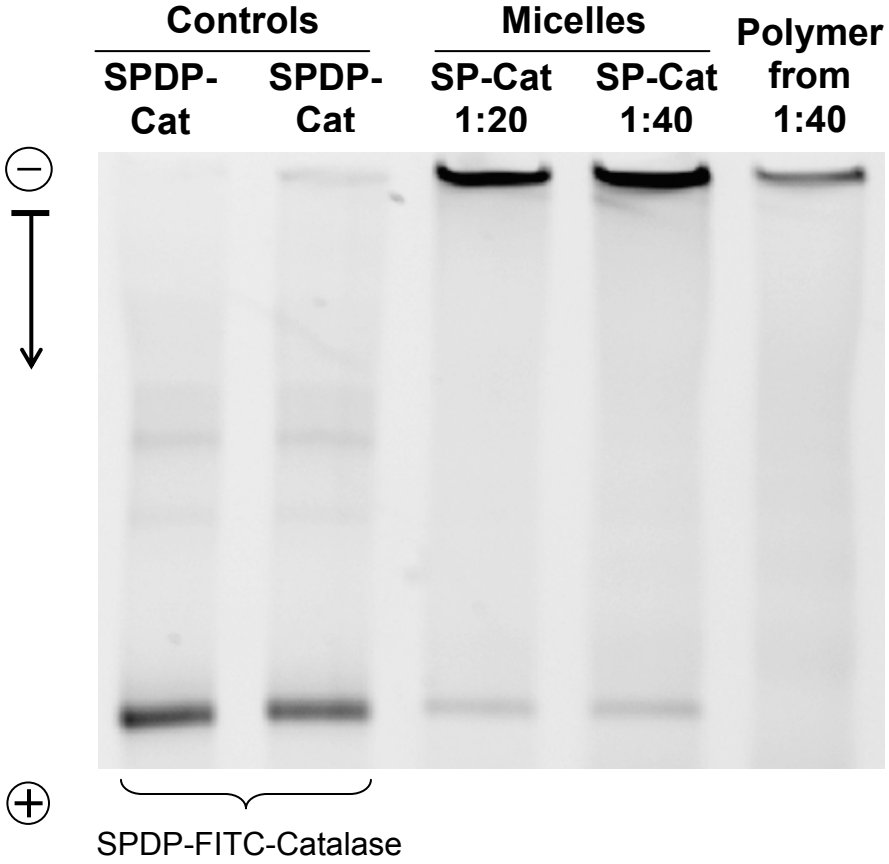


Figure 2.9. SDS-PAGE (4-20%) of SPDP-FITC-Catalase micelles, prepared with 0.25 or 0.50 mg/mL SPDP-FITC-Catalase and 10 mg/mL PEG-poly(Lys-Thio-Pyridyl).

2.4 Discussion

The objective of this research was to develop an improved polyion complex (PIC) micelle delivery system for proteins and nucleic acids. The PIC micelle concept had first been demonstrated by the Kataoka laboratory as stable, self-assembled complexes between PEG-poly(Lys) and PEG-poly(Asp). They later developed the PIC micelle as a delivery system for charged molecules, e.g., by complexing PEG-poly(Lys) with DNA and by complexing PEG-poly(Asp) with positively charged proteins such as trypsin and lysozyme. Cross-linking schemes based on disulfide exchange reactions or aldehyde-amine reactions were used to provide stability of the micelles against competing electrolytes or under dilute conditions. In this project, we have focused on improving the chemistry involved in the cross-linking schemes, to generate a PIC micelle capable of efficient encapsulation and retention of a protein and/or nucleic acid. In the context of developing a protein subunit vaccine, the cargo molecules were ovalbumin, CpG-DNA, and poly(I:C). A secondary application was to deliver a therapeutic enzyme, for which we chose catalase as a candidate enzyme because of its applicability to treating inflammatory conditions (a focus area in the Murthy laboratory).

We used the PEG-poly(Lys) block copolymer as our platform for developing an improved micelle design. The first modification was to use a Michael addition reaction to attach the pyridyldithio (PDT) cross-linking monomer to the PEG-poly(Lys) backbone. The Michael addition reaction was shown to proceed nearly quantitatively in DMF, resulting in a high number of PDT groups and retaining the positively charged amines. A second change was to use a dithiol molecule to cross-link the PDT groups directly, in contrast to the reported method of reducing the PDT groups and allowing open air

oxidation to proceed over 3 days. The direct cross-linking of PDT has the advantages of being more rapid and is easily monitored by the 342 nm absorbance of thiopyridone.

While this design was capable of a moderate degree of protein retention, an additional modification was introduced to covalently tether the protein in the core of the micelle, by conjugating multiple SPDP molecules to each protein molecule. This method was shown to improve the retention of ovalbumin in SDS-PAGE assays. It would be especially useful for proteins having a low charge density or neutral isoelectric point, in which case the electrostatic complexation and poly(Lys) core cross-linking would be insufficient to retain a globular protein. Based on agarose gel assays, however, the covalent tethering does not appear to be as necessary for oligo- and polynucleotides, which are more linear and have a higher charge density than proteins, and thus form strong complexes with the poly(Lys) chain.

In summary, these improvements have resulted in a cross-linked micelle design that has efficient encapsulation and retention of ovalbumin, CpG-DNA, and poly(I:C), as well as the enzyme catalase. The PEG-PLTP block copolymer is generated through a six-step convergent synthesis, using standard reaction mechanisms and commercially available reagents. PEG-PLTP is stored in dry form and is reconstituted prior to micelle formation. Preparation of cross-linked micelles requires about 3 to 4 hours and can be done under sterile conditions. Furthermore, the micelles have the advantage that small scale batches can be efficiently prepared. The developments reported here demonstrate that the cross-linked micelle is a promising delivery system for protein and nucleic acid therapeutics, with potential applications in vaccines or enzyme-based therapy.

CHAPTER 3

DEVELOPMENT OF POLYKETAL MICROPARTICLES

3.1 Introduction

Objective

The overall objective of **Specific Aim 2** was to develop a pH-sensitive biodegradable polymer which could be used to fabricate micro- or nano-sized particles for intracellular delivery of biotherapeutics and organic drugs. Various polymers have been employed in making colloid nanoparticles, with poly(lactic-glycolic acid) (PLGA) being the most commonly used due to its excellent biocompatibility profile and commercial availability. However, there are concerns over the highly acidic microclimate within degrading PLGA particles that may complicate the use of PLGA in delivering functional proteins and nucleic acids (Fu 2000). Furthermore, numerous medical applications, such as targeting the acidic environment of lysosomes and tumors, require drug delivery systems that undergo rapid, pH-sensitive degradation. Several pH-responsive delivery systems have been reported for delivery of proteins and DNA (Murthy 2003b, Kwon 2005a, Kwon 2005b, Standley 2007). These systems are ketal-crosslinked hydrogels generated by free-radical polymerization and thus contain non-degradable carbon backbone polymer chains. Another family of pH-sensitive polymers, the poly(ortho ester)s, have lengthy synthetic routes and generate acidic degradation products (Wang 2004). Therefore, there is a need for new materials for intracellular delivery of proteins and nucleic acids, that have the necessary combination of properties,

namely pH-sensitivity, biodegradability, ease of synthesis, and non-acidic degradation products.

We **hypothesized** that by using the acetal exchange reaction, a ketal-containing polymer could be synthesized that would exhibit pH-sensitive degradation and thus provide a platform for intralysosomal delivery of proteins/DNA/RNA. This new pH-sensitive polymer would be synthesized in a one-step reaction and would have material properties needed for fabricating microparticles. For example, the polymer would be sufficiently hydrophobic to be soluble in chloroform or dichloromethane, in order that the polymer could be used to fabricate nano- or microparticles using oil-in-water emulsion methods. Another requirement was that the polymer would degrade into low molecular weight, water-soluble compounds, so that the degradation products could be excreted from the circulation. Finally, it was desirable to develop a polymer which would not generate acidic degradation products, as with polyester-based materials such as PLGA.

Development of the Acetal-Exchange Polymerization

In order to meet the requirements of pH-sensitivity and degradation into low molecular weight products, we decided to construct a polymer containing acid-labile acetal or ketal linkages in the polymer backbone. Acetals and ketals are functional groups that are cleaved by acid-catalyzed hydrolysis and are commonly used in drug delivery polymers where intracellular, phagolysosomal release is desired. We initially attempted to synthesize a ketal-containing molecule with an amine group on each end. The ketals are a subset of acetals in which neither substituent is a hydrogen. We synthesized the precursor to the diamine ketal molecule, 2,2-di-(phthalimidoethoxy)

propane using the acetal exchange reaction (Sheppard 1989). The diamine ketal molecule would be reacted with a molecule containing an acyl chloride group on either end. The two molecules, designated A-A and B-B, would polymerize in a step-growth manner through the reaction of the amine with the acyl chloride, thus producing a linear polymer with a ketal and amide groups in the backbone.

A preliminary trial of the step-growth polymerization using 1,5-diaminopentane and terephthaloyl chloride resulted in a polyamide product which was not soluble in dichloromethane and chloroform and thus would not be suitable for the oil-in-water method of microparticle formation. However, in our work with synthesizing the diamine ketal precursor molecule, we gained familiarity with the use of the acetal exchange reaction to generate ketal intermediates. The acetal exchange reaction is an equilibrium reaction which can be driven forward by removal of one of the products (Lorette 1960). In the case where 2,2-dimethoxypropane is the starting ketal, the byproduct will be methanol, which can be removed by distillation. We hypothesized that a ketal-containing polymer could be generated by directly reacting a diol (a molecule containing two alcohol groups) with 2,2-dimethoxypropane at a high temperature where the methanol byproduct would be distilled off. This reaction would generate mixed ketal intermediates, which can combine with each other or with the starting materials in a step-growth manner to form oligomer chains and eventually a polymer (Figure 3.1). The acetal exchange method avoids the introduction of a amide bond and thus can produce a hydrophobic polymer. The development of the acetal exchange polymerization led to the synthesis of various ketal-containing polymers, termed “polyketals”, which could be used to fabricate microparticles via oil-in-water emulsion methods.

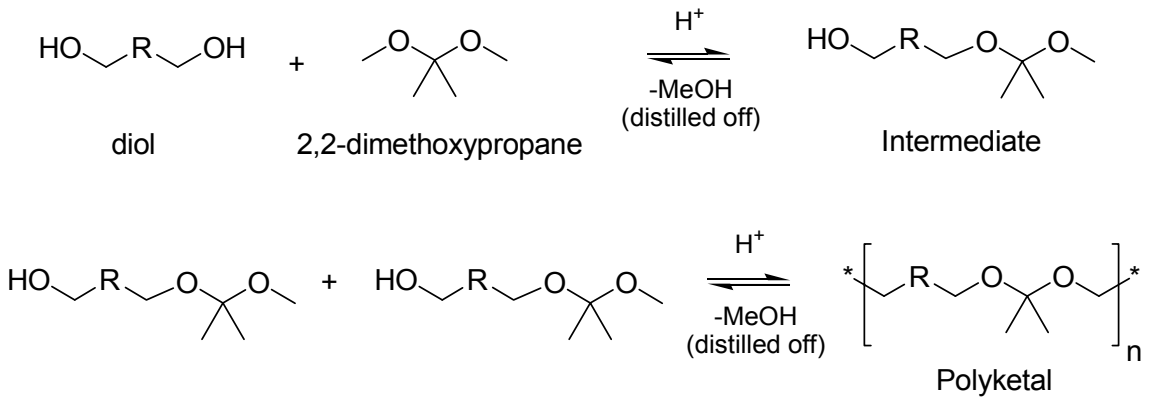
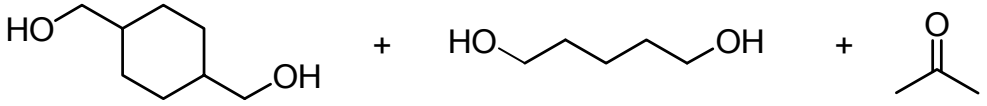


Figure 3.1. Synthesis of a polyketal via the acetal exchange reaction.

Development of Co-Polyketals Based on Cyclohexanedimethanol

We initially used the diol 1,4-benzenedimethanol to generate the prototype polyketal poly(1,4-phenylene-acetonedimethylene ketal) (PPADK) (Heffernan 2005). Because the hydrolysis of the ketal linkage produces a molecule of acetone and two alcohol groups, the degradation of PPADK were simply acetone and the original diol, 1,4-benzenedimethanol. One concern with 1,4-benzenedimethanol as a degradation product was that there was no data available on the compound's toxicity. An alternate diol compound was considered, namely 1,4-cyclohexanedimethanol (CDM), which has a known biocompatibility profile. In work led by Stephen Yang of the Murthy laboratory, a solid polyketal (PCADK) was synthesized with CDM; however, its degradation rates were much slower than PPADK (Lee 2007). Other polyketals were synthesized with 1,4-butanediol, 1,5-pentanediol, and 1,8-octanediol; these polymers, however, were liquids at room temperature and thus were not suitable for microparticle formation. Next, copolymers of CDM and the various short-chain diols were synthesized, resulting in solid co-polyketals with a range of degradation rates corresponding (inversely) to the

hydrophobicity of the monomers. One of these is an 80:20 copolymer of CDM and 1,4-pentanediol, termed PK3, which is suitable for microparticle fabrication and has relatively fast degradation kinetics (Yang 2008). PK3 has a hydrolysis half-life on the order of 1 to 2 days and has material properties which are suitable for microparticle fabrication. One significant property of PK3 is the biocompatibility of its degradation products. As shown in Figure 3.2, the degradation products of PK3 have relatively high lethal dose values. For the purpose of comparison, a typical in vivo application of the polyketal microparticles would require 50 mg/kg of polyketal; thus the amount of the degradation products would be much less than the reported LD_{50} values.



LD_{50} = 3200 mg/kg (Rat – oral) LD_{50} = 1600 mg/kg (Mouse – oral)	LD_{50} = 6300 mg/kg (Rabbit – oral) LD_{50} = 20000 mg/kg (Rabbit - skin)	LD_{50} = 5800 mg/kg (Rat – oral) LD_{50} = 7426 mg/kg (Guinea pig – dermal)

Figure 3.2. Toxicity of degradation products of co-polyketal PK3. (Sigma-Aldrich MSDS)

Hydrophobic Ion Pairing

In this chapter we also describe the development of the hydrophobic ion pairing (HIP) technique for encapsulation of proteins and nucleic acids in polyketal microparticles. We established the need for the ion pairing technique based on preliminary findings that proteins such as ovalbumin could not be encapsulated with high

efficiency using the water-oil-water double emulsion method. These findings may have been due to insufficient polymer chain length and entanglement which allows escape of the protein from the inner aqueous phase to the outer aqueous phase. The hydrophobic ion pairing technique has been reported in the literature for the extraction of water-soluble biotherapeutics (DNA and proteins) into an organic solvent (Hegg 1979, Powers 1993, Bromberg 1994, Meyer 1998, Patel 2004). The basic procedure involves the pairing of a polar lipid or surfactant molecule with a (charged) DNA, RNA, or protein molecule, with an equimolar ratio of opposite charges (Figure 3.3). This cancels the charges and produces a complex with a hydrophobic character. The resulting hydrophobic complex can be co-dissolved with the polyketal in an organic solvent, or a mixture of solvents, enabling microparticles to be fabricated by the oil-in-water single emulsion method. Because the hydrophobic complex partitions to the oil phase, this

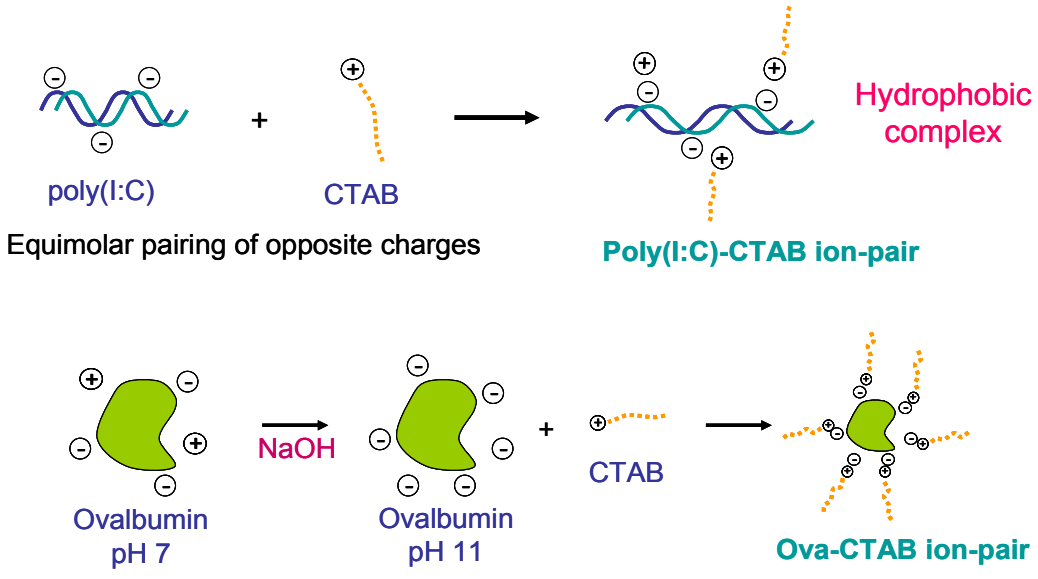


Figure 3.3. Hydrophobic ion pairing of poly(I:C) to CTAB (top) and ovalbumin to CTAB (bottom).

method has the potential to generate higher encapsulation efficiencies than the double emulsion method. Several water-soluble compounds were encapsulated in polyketals using the HIP/single emulsion process, including ovalbumin, single-stranded oligo DNA, poly(I:C) (dsRNA), and siRNA.

3.2 Experimental Methods

Synthesis of Polyketals via the Acetal Exchange Reaction

As described in the Introduction, we developed a novel polymerization strategy based on the acetal exchange reaction, which generated a linear polymer with ketal groups in the backbone. This synthesis uses a step-growth acetal exchange polymerization of a diol with 2,2-dimethoxypropane, yielding a polyketal (Figure 3.4). The reaction was based on a published protocol for the acetal-exchange reaction between N-(2-hydroxy ethyl) phthalimide and 2,2-dimethoxypropane (Sheppard 1989). The first polyketal synthesized in the Murthy laboratory was poly(1,4-phenylene-acetonedimethylene ketal) (PPADK) (Heffernan 2005). Polyketals based on 1,4-cyclohexanedimethanol as the primary diol were later synthesized (Lee 2007, Yang 2008).

Synthesis of poly(1,4-phenylene-acetonedimethylene ketal) (PPADK)

A schematic of the synthesis of PPADK is shown in Figure 3.4. PPADK was synthesized in a 25 mL two-necked flask, connected to a short-path distilling head. 1,4-Benzenedimethanol (1.0 g, 7.3 mmol) dissolved in 10 mL of warm ethyl acetate was added to 10 mL of distilled benzene kept at 100°C. Re-crystallized *p*-toluenesulfonic

acid (5.5 mg, 0.029 mmol) dissolved in 550 µL of ethyl acetate was then added. After allowing the ethyl acetate to distill off, distilled 2,2-dimethoxypropane (DMP, 900 µL, 7.4 mmol) was added to initiate the reaction. Five additional doses of DMP were added via a metering funnel, with each dose consisting of 2 mL of benzene plus 300 to 500 µL of 2,2-dimethoxypropane. Each dose was added over a 30 to 40 min period with a 30 min interval in between. The total duration of the reaction was 7 h. The reaction was stopped with the addition of 100 µL of triethylamine and was precipitated in cold hexanes. The crude product was vacuum filtered, rinsed with ether and hexanes, and vacuum dried to yield 600 mg of white solid product (48% yield). (Heffernan 2005)

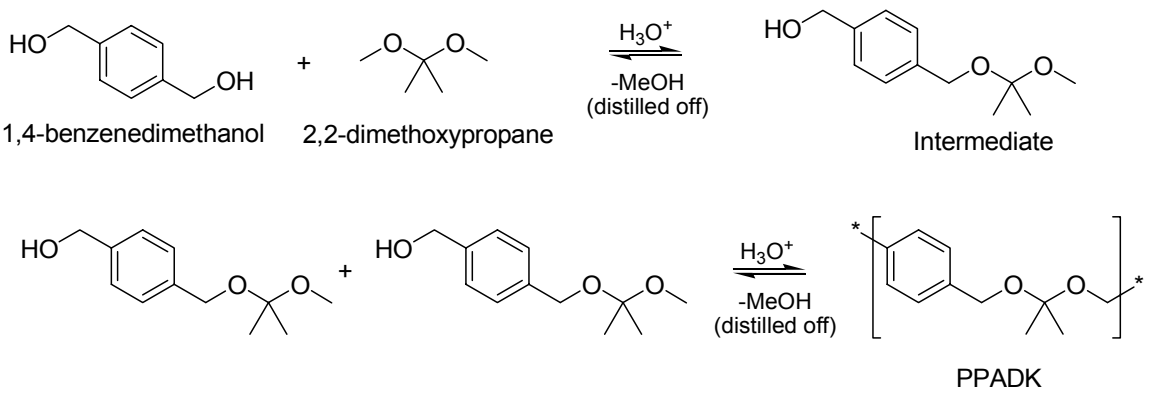


Figure 3.4. Synthesis of the polyketal, poly(1,4,-phenylene-acetone dimethylene ketal)(PPADK), via the acetal exchange reaction. (Heffernan 2005)

Synthesis of Co-polyketal Based on 1,4-Cyclohexanedimethanol and 1,5-Pentanediol (PK3)

The co-polyketal PK3 was developed by Stephen Yang of the Murthy laboratory, using the acetal exchange polymerization (Yang 2008). Two diols are reacted with 2,2-dimethoxypropane to create a copolymer, as shown in Figure 3.5. A typical synthesis of

PK3 is carried out in a 25 mL two-necked flask connected to a short-path distilling head. 1,4-Cyclohexanedimethanol (5.0 g, 34.7 mmol) and 1,5-pentanediol (0.903 g, 8.67 mmol) were dissolved in 30 mL of distilled benzene at 100°C. Re-crystallized p-toluenesulfonic acid (3.5 mg, 0.0197 mmol) dissolved in 3.5 mL of ethyl acetate was then added. Distilled 2,2-dimethoxypropane (DMP, 5.33 mL, 43.3 mmol) was added to initiate the reaction. Additional doses of DMP (2.5 mL, 20.3 mmol) and benzene (5 mL) were subsequently added to the reaction every hour for 6 hours via a metering funnel at slow drip rate to compensate for DMP and benzene that had distilled off. After 20 hours, the reaction was stopped with the addition of 2 mL of triethylamine. The polymer was isolated by precipitation in cold hexanes (-20°C) followed by vacuum filtration.

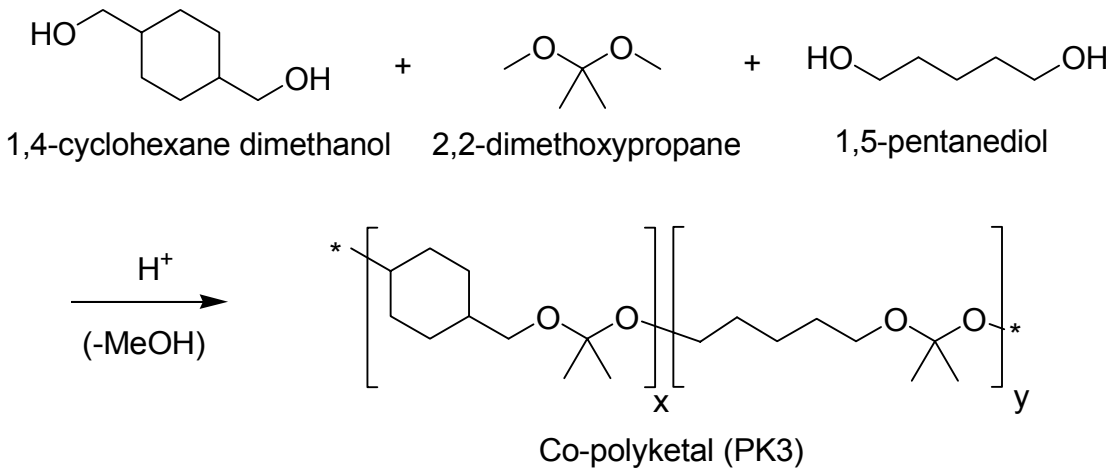


Figure 3.5. Synthesis of the co-polyketal PK3 via the acetal exchange polymerization. (Yang 2008)

Characterization of Polyketals PPADK and PK3

The polyketals were characterized by gel permeation chromatography (GPC) and ^{1}H nuclear magnetic resonance (NMR) spectroscopy. The GPC measurements were run in tetrahydrofuran (THF) using a Shodex KF-803 column with a Shimadzu SPD-10A

UV-Visible detector. Samples were analyzed against polystyrene standards (M_w = 1060; 2970; 10,680) to calculate molecular weight, using Shimadzu software. The ^{1}H NMR spectra of the polyketals were run in $CDCl_3$ using a Varian Mercury Vx 400 machine. A small amount of triethylamine was added to the polyketal samples to prevent hydrolysis during the analysis.

pH-Dependent Hydrolysis of PPADK

The hydrolysis rates of PPADK were measured at lysosomal pH (5.0) and physiological pH (7.4). To measure the hydrolysis rates, PPADK was ground into a fine powder and added to deuterated solutions at pH 7.4 (phosphate buffer), pH 5.0 (acetate buffer), and pH 1.0 (DCl) at a concentration of 10 mg/mL. Dioxane was added to each sample as a reference molecule. Next, the suspensions were stirred at 37°C and data points were taken at 3, 24, 48, and 72 hours. Each suspension was centrifuged for 4 minutes at 1800*g*, and a sample of the supernatant was analyzed by ^{1}H NMR. The NMR spectra contained peaks for 1,4-benzenedimethanol (7.24 and 4.47 ppm) and acetone (2.05 ppm). The average of the two 1,4-benzenedimethanol (BDM) peak integrals were used to determine the percentage of phenylene groups present as BDM; any phenylene groups present in the (water insoluble) polymer would not be present in the supernatant. The eight dioxane protons served as a reference to normalize the BDM levels between the various samples. The hydrolysis half-lives were determined by subtracting the percent hydrolysis curves from 100% and fitting the difference to an exponential decay function. (Heffernan 2005)

Fabrication of polyketal nano- and microparticles using the oil-in-water emulsion method.

Method 1: Preparation of PPADK nanoparticles

Polyketal nanoparticles were made using a single oil-in-water emulsion/solvent evaporation method. Typically, 50 mg of PPADK dissolved in 1 mL of $CHCl_3$ was added to 5 mL of pH 9 buffer (10 mM $NaHCO_3$) containing 0.2 to 1% poly(vinyl alcohol) (31–50 kDa) as the emulsifier. The oil-water mixture was shaken briefly and then sonicated for 2 to 3 minutes at 40 watts using a Branson Sonifier 250 to form a fine oil-in-water emulsion. The emulsion was stirred under nitrogen flow for at least 3 hours to evaporate the solvent and produce a nanoparticle suspension. The nanoparticles were centrifuged at 4°C for 10 minutes at 5000*g* and washed with deionized water to prepare samples for scanning electron microscopy. Particle sizes were analyzed by dynamic light scattering using a Brookhaven 90Plus particle sizer. (Heffernan 2005)

Method 2: Preparation of Polyketal Microparticles Containing Ova:CTAB and poly(I:C):CTAB Complexes

Microparticles were fabricated using an oil-in-water emulsion, solvent evaporation method. The oil phase contained 50 mg of PK3 dissolved in 400 μL of chloroform, plus 0.8 mg of Ova:CTAB in 400 μL of 1:1 chlorform-DMSO and 0.9 mg of poly(I:C):CTAB in 150 μL of 4:1 chloroform-methanol. Control batches included Ova:CTAB particles, poly(I:C):CTAB particles, or empty particles. The total volume of the organic phase was made up to 1.2 mL with chloroform and homogenized in 15 mL of 5% PVA solution at for 2 minutes 24000 rpm. The resulting emulsion was poured into

85 mL of 1% PVA solution and stirred for 4 hours to evaporate the chloroform. The PVA solutions were buffered with 8 mM sodium phosphate (pH 8) to prevent hydrolysis of the acid-sensitive polyketal. The microparticles were collected by centrifugation at 10000 *g*, washed once with OmniPur water, resuspended in 5 mL OmniPur water, then lyophilized overnight. Particle yields were 25% to 70% of the starting polyketal mass. The size and morphology of the microparticles were characterized by dynamic light scattering (DLS) and scanning electron microscopy (SEM). DLS samples were prepared by re-suspending the lyophilized particles in water and vortexing and bath sonicating for a few minutes. The SEM samples were prepared either by spreading dry microparticles onto sticky carbon tape or by drying a drop of re-suspended particles on the metal SEM mounting stub.

In vivo delivery of polyketal nanoparticles to murine macrophages.

In collaboration with Dr. Robert Pierce at the University of Rochester, we investigated the delivery of polyketal nanoparticles (PKNs) to liver macrophage cells. PPADK particles were prepared by Method 1 as described above, except that fluorescein was dissolved along with the polyketal in the chloroform phase. The attempted loading was 1% fluorescein by mass. A volume of 200 μL of (10 mg/mL) fluorescein-containing PKNs was injected into the tail vein of mice. The mice were sacrificed after 1 hour. Frozen sections of liver and spleen were fixed in 4% paraformaldehyde, stained with the macrophage marker F4/80, and analyzed by fluorescent microscopy and immunohistochemistry (IHC).

Hydrophobic Ion Pairing

The hydrophobic ion pairing technique was used to convert various nucleic acid and protein molecules to hydrophobic complexes that could be dissolved in organic solvents or mixtures of solvents. For oligonucleotides, such as 20-base single-stranded (ss)DNA or 21 base-pair short interfering (si)RNA, we used a water-dichloromethane (DCM)-methanol procedure with the surfactant 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP). For ovalbumin, the water-DCM-methanol method resulted in aggregation, so a purely aqueous method was developed, using the surfactants DOTAP, docusate sodium, and cetyltrimethylammonium bromide (CTAB). Similarly, poly(I:C) could not be ion-paired by the water-DCM-methanol method, so the aqueous method with CTAB was utilized.

Hydrophobic Ion Pairing of Oligonucleotides (CpG-DNA and siRNA) by the Water-Dichloromethane-Methanol Procedure

The oligonucleotides CpG-DNA and siRNA were each ion paired with 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP). This method was adapted from a reported procedure for ion pairing plasmid DNA for encapsulation in microparticles (Patel 2004). In a typical procedure, a solution of DNA or RNA (0.8 mg) in 1 mL TE buffer was combined with a solution of DOTAP (1.9 mg) in 1 mL dichloromethane (DCM) and 2.1 mL of methanol. This mixture created a Bligh & Dyer monophase, which was allowed to stand for 5 minutes. Next, we added 1 mL DCM and 1 mL water to bring about phase separation, and the two-phase mixture was vortexed for 1 minute. The mixture was then centrifuged at 1200 *g* for 5 minutes at 20°C. The organic phase

was immediately isolated using a syringe, and the nucleic acid content was analyzed by UV absorbance at 260 nm. This process resulted in nearly 100% extraction of DNA or RNA into the organic phase; however, the actual yield (70 to 80%) was limited by the volume of the DCM phase that could be isolated. Scaling down the process by one-half resulted in slightly lower percent yield due to losses in the isolation step.

Hydrophobic Ion Pairing of Ovalbumin with CTAB

Ovalbumin was first converted to the stabilized form (*S*-ovalbumin) by heating a 10 mg/mL ovalbumin solution to 55°C for 20 hours in 100 mM, pH 10 sodium phosphate buffer, followed by desalting in a PD-10 column. The *S*-ovalbumin solution was diluted to 1 mg/mL in OmniPur water and adjusted to pH 11 by addition of NaOH. Next, 10 mL (10 mg) of *S*-ovalbumin solution (4°C) was added to 364 μL of cetyltrimethylammonium bromide (CTAB) solution (3.64 mg), resulting in a precipitate which was collected by centrifugation at 20000*g* for 20 minutes at 5°C. The lyophilized *S*-Ova:CTAB ion-pair complex was dissolved in 1:1 chloroform-DMSO, at an approximate concentration of 1 to 2 mg/mL as determined by UV absorbance at 280 nm. The ratio of CTAB cations to ovalbumin anions was 0.7:1.

Hydrophobic Ion Pairing of Poly(I:C) to CTAB.

Poly(I:C) was also ion-paired with CTAB using a similar protocol at a molar charge ratio (+/-) of 1.32 to 1. Poly(I:C) was initially dissolved in IDTE pH 7.5 buffer at a concentration of 2.26 mg/mL, as determined by the UV absorbance at 260 nm (50 μg/mL/A.U., per supplier's literature). The poly(I:C) solution was diluted to 0.1 mg/mL

in OmniPur nuclease-free water and kept at 4°C. Next, 10 mL of poly(I:C) solution (1 mg) was combined with 1 mL of CTAB solution (1.52 mg), resulting in a precipitate which was collected by centrifugation at 20000*g* for 20 minutes at 5°C. The lyophilized poly(I:C):CTAB complex was dissolved in 4:1 chloroform-methanol at a concentration of 6 mg/mL as determined by absorbance at 260 nm.

Characterization of Protein and Nucleic Acid Loading in Polyketal Microparticles

For measuring the encapsulation levels of protein, DNA, or RNA, a sample of microparticles (approximately 5 mg) was first digested in 200 μL of 0.01 M HCl (with 0.25% SDS) over a 5 to 10 minute period. The sample was vortexed and bath sonicated as needed to ensure uniform digestion. This resulted in a slightly hazy solution which was neutralized with 400 μL of 0.05 M pH 9.5 sodium bicarbonate buffer. At this point, different methods were used to analyze loading, depending on the type of molecules being analyzed.

For ovalbumin, we used a fluorescamine assay in a 96-well plate, with 3 wells per particle digest sample. Ovalbumin standard dilutions were prepared in the same neutralized solution containing HCl, SDS, and sodium bicarbonate. The plate was read with a Bio-Tek plate reader using KC4 software.

To measure Poly(I:C) content, the samples and poly(I:C) standards were diluted in IDTE pH 7.5 buffer and stained with SYBR Green I or OliGreen nucleic acid dye. The samples were analyzed using a Shimadzu RF-5301PC spectrofluorophotometer with an excitation of 494 nm for SYBR Green I and 480 nm for OliGreen.

For siRNA-loaded microparticles, the digest mixture was diluted in half and the absorbance peak at 260 nm was recorded using a Shimadzu UV-1700 spectrophotometer. The absorbance difference between a plain polyketal microparticle sample and the siRNA particle sample was multiplied by the reported extinction coefficient for siRNA to obtain the concentration of siRNA in the sample.

MTS Cell Viability Assay

RAW264.7 macrophage cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% FBS and Pen/Strep/Fungizone per ATCC's protocol. Cells were seeded overnight in a 96-well plate at 15,000 cells/150 μL/well. Empty polyketal microparticles and particles containing Ova, poly(I:C), or Ova+poly(I:C) were re-suspended in complete medium with vortexing and bath sonication. Microparticle suspensions and solutions of Ova, poly(I:C), and CTAB were diluted in complete medium, and 50 μL of each dilution was added to triplicate wells. After 5 hours, Cell Titer 96 MTS reagent was added at 30 μL per well, and the cells were incubated. After 1 hour incubation, the difference in absorbance at 490 nm and 650 nm was recorded using a Bio-Tek Synergy™ HT plate reader with KC4 software. Measurements were corrected for background (medium only) and expressed as cell viability relative to untreated cells.

3.3 Results

Synthesis of Polyketals

Using the acetal exchange polymerization method developed in the Murthy laboratory, the prototype polyketal PPADK was synthesized and purified. Following the precipitation and drying steps, 600 mg of white solid product was recovered (48% yield). Analysis of polyketal batches by GPC indicated weight-average molecular weights ranging from M_w = 2290 to 4000, with a polydispersity index of 1.4 to 1.6 (Figure 3.6). The corresponding degree of polymerization ranges from 12.9 to 22.5 repeating units.

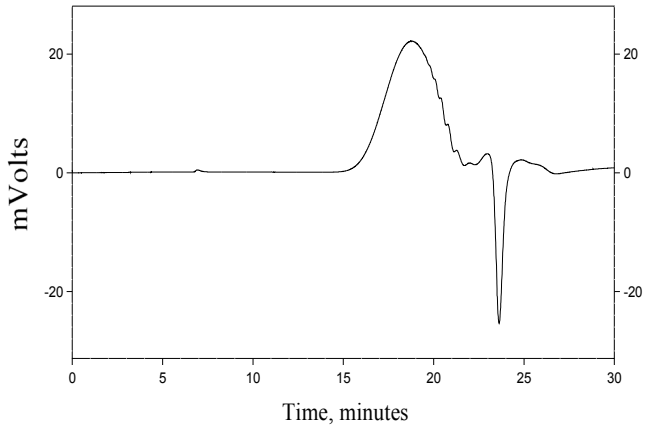


Figure 3.6. GPC trace of PPADK in THF (Shimadzu SCL-10A). (Heffernan 2005).

The ^{1}H NMR spectrum (Figure 3.7) shows repeating unit peaks at 7.3 ppm (4b), 4.5 ppm (4c), and 1.5 ppm (6a). The peaks at 2.5 ppm and 1.0 ppm are due to the triethylamine added to prevent ketal hydrolysis. The NMR spectrum confirms that the repeating unit of PPADK contains a ketal group (‘6a’). Together, the GPC and ^{1}H NMR data provide evidence for the successful synthesis of PPADK. (Heffernan 2005)

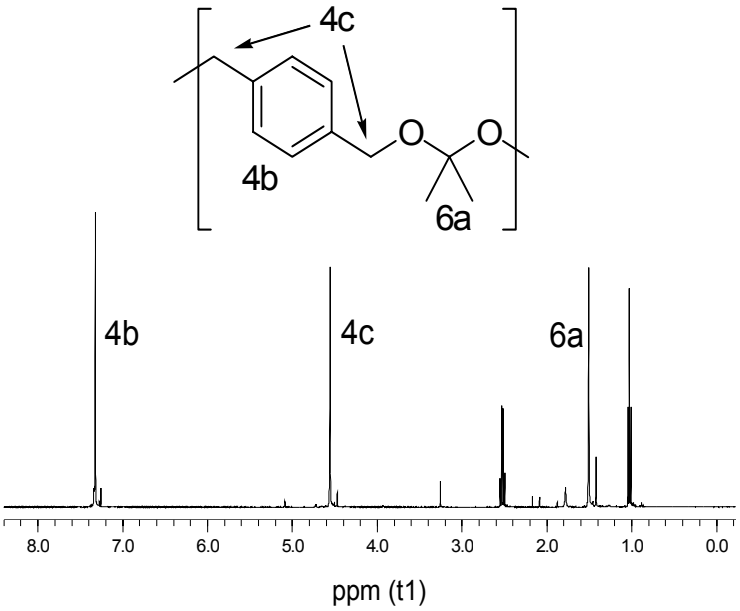


Figure 3.7. ^{1}H NMR spectrum of PPADK in $CDCl_3$ (Heffernan 2005).

pH-dependent hydrolysis of PPADK

The percent hydrolysis of PPADK is plotted over a 3-day time course at pH 1.0, pH 5.0, and pH 7.4 (Figure 3.8). The calculated exponential decay half-lives for the hydrolysis of PPADK are 102 hours at pH 7.4 and 35 hours at pH 5.0. The pH 1.0 sample was completely hydrolyzed by the first time point of 3 hours and thus did not have a calculated half-life. The PPADK hydrolysis data show a 3-fold rate increase from pH 7.4 to 5.0, which is significantly less than the 250-fold rate increase observed for a water-soluble ketal in going from pH 7.4 to 5.0 (Kwon 2005a) . It is hypothesized that the lower pH sensitivity of PPADK is due to its water insolubility, which limits the diffusion of water and creates a rate-limiting step that is pH-independent.

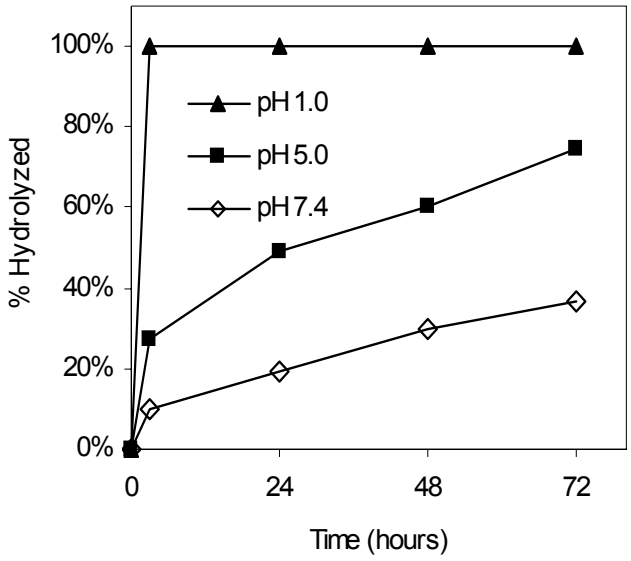


Figure 3.8. Hydrolysis kinetics of PPADK. (Heffernan 2005)

Release of Rhodamine B from polyketal microparticles

The release kinetics of Rhodamine B from PCADK microparticles at room temperature are shown in Figure 3.9. The particles were made by single emulsion and their diameter was approximately 5 μm by SEM. The amount of rhodamine B released was determined by measuring the fluorescence of the supernatant following centrifugation. The calculated half-lives were 1 day at pH 1.0, 5 days at pH 4.5, and greater than 15 days at pH 7.4.

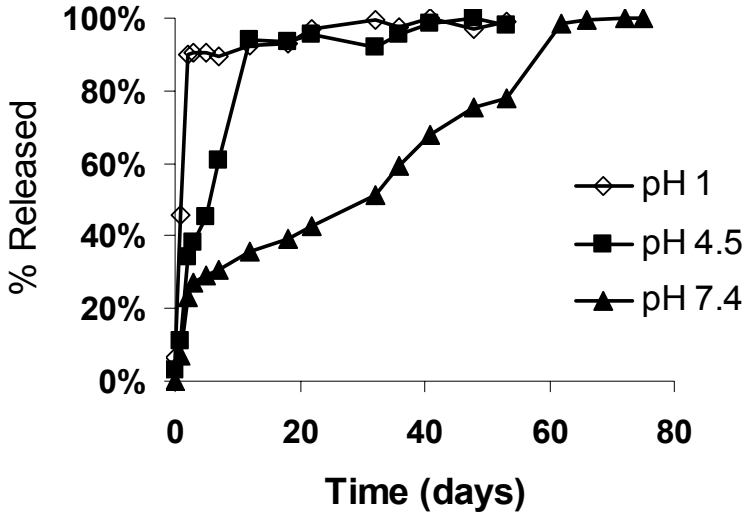


Figure 3.9. Release of rhodamine B from PCADK microparticles at room temperature.

In vivo delivery of polyketal nanoparticles to murine macrophages.

In collaboration with Dr. Robert Pierce at the University of Rochester, PPADK particles (effective diameter 256 nm by DLS) containing 1% (nominal) fluorescein by weight were administered intravenously to mice, and liver histology was examined 1 hour post-injection. Fluorescent microscopy of the liver sections indicates that the PKNs are abundantly present throughout the liver, residing mainly in the Kupffer cells (data not shown). Anti-FITC IHC confirms the presence of fluorescein-PKNs within the cytoplasm of a Kupffer cell lining a liver sinusoid (Figure 3.10). Control "empty" PKNs were also administered and show virtually none of the punctate fluorescence characteristic of the fluorescein-PKNs. Occasional green autofluorescent globules were identified within Kupffer cells of mice treated with either control PKNs or PBS (data not

shown). These likely represent scavenged cell debris. Similarly, the splenic macrophages contained FITC-PKNs (data not shown).

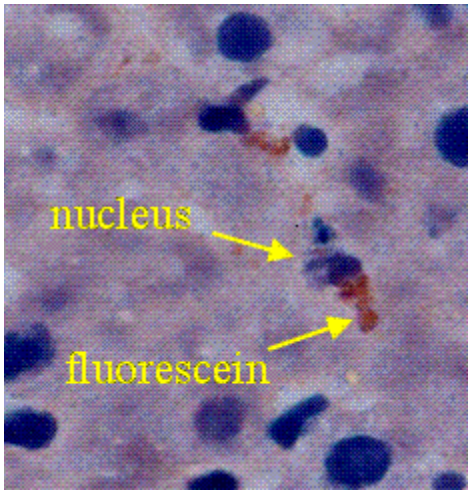


Figure 3.10. Release of fluorescein from PPADK nanoparticles. Dark red = anti-FITC staining; Blue = DAPI staining (nuclei).

Hydrophobic Ion Pairing of Proteins and Nucleic Acids

We used the technique of hydrophobic ion pairing to convert several water-soluble molecules – CpG-DNA, siRNA, ovalbumin and poly(I:C) – into hydrophobic complexes that were soluble in organic solvents or mixtures of solvents. The most efficient ion pairing was achieved with the oligonucleotides (CpG-DNA and siRNA) using the water-DCM-methanol procedure. This method typically results in nearly complete extraction of the nucleic acid into the DCM phase, with the yield being limited by the amount of the DCM phase that could be isolated. Typical yields were 70-80% based on UV absorbance measurements.

Initial attempts to ion pair DOTAP to either poly(I:C) or ovalbumin were unsuccessful due to aggregation at the DCM-water interface. Thus, a purely aqueous method was developed, and we were successful in ion pairing ovalbumin to three different surfactants: docusate sodium, DOTAP, and CTAB. In the case of docusate sodium, which is negatively charged, the ovalbumin solution was adjusted to pH 4, whereas with DOTAP and CTAB the ovalbumin solution was adjusted to pH 11. In all three cases, the resulting ion-pair complexes were soluble in a chloroform-DMSO mixture. Because the objective of this study was to co-encapsulate ovalbumin and poly(I:C) in a microparticle, it was essential that the respective ion-paired complexes be compatible in solution. It was observed that mixing of 1) an ovalbumin:docusate ion-pair dissolved in DMSO-chloroform and 2) a CpG-DNA:DOTAP ion-pair dissolved in DCM resulted in slight precipitation, from which it was concluded that ion-paired compounds would only be compatible in solution if the surfactants were of the same charge. Ovalbumin was thus ion-paired with one of the cationic lipids (CTAB), so that it would be compatible with poly(I:C) paired to a cationic lipid. One issue with the ion-pairing of ovalbumin was the formation of aggregates that could not be dissolved in the organic solvents. This resulted only about 10 to 15% of the ovalbumin being recovered after the lyophilization step. Because aggregation is related to the stability of the protein, it was speculated that converting ovalbumin to the stabilized form, *S*-ovalbumin, would resulted in a higher yield when ion paired with CTAB. CTAB was ion-paired with *S*-ovalbumin (at pH 11) with a yield of 15 to 20%. The mass ratio was 0.364 to 1, which corresponds to a calculated charge ratio of 0.7 moles of CTAB cations to 1 mole of ovalbumin anions.

The ion pairing of poly(I:C) to CTAB was more efficient, with yields of 90% using the aqueous method.

Characterization of Polyketal Microparticles

Microparticles were fabricated using the oil-in-water single emulsion methods described above. Various small hydrophobic molecules, such as dyes or inhibitors, have been encapsulated by dissolving the molecule with the polyketal in the oil phase. Initial attempts at using a double emulsion method to encapsulate water-soluble proteins, however, resulted in low encapsulation levels. Thus, the hydrophobic ion pairing technique was used to form hydrophobic complexes of the proteins or nucleic acids with a surfactant. The ion-paired complexes were efficiently incorporated into polyketal microparticles using a single emulsion method. Figure 3.11 shows scanning electron microscope (SEM) images of representative polyketal microparticle batches. Typical loading levels of PK3 microparticles were 20 μg/mg ovalbumin and 10 μg/mg poly(I:C).

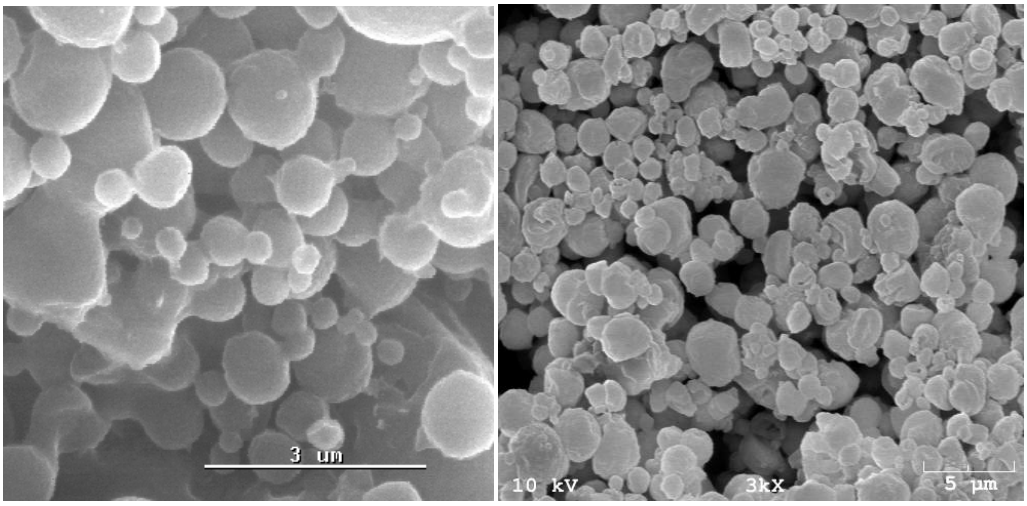


Figure 3.11. Scanning electron microscope images of microparticles fabricated with PPADK (left) and PK3/Ova/poly(I:C) (right).

Cell toxicity of polyketal microparticles

An MTS cell viability assay was performed to measure cytotoxicity of polyketal microparticles containing ion-paired complexes. RAW264.7 cells treated with microparticles containing Ova:CTAB and/or poly(I:C):CTAB complexes had approximately 50% cell viability at a concentration range of 0.05 to 0.25 mg/mL. Plain polyketal microparticles were less toxic, with 70% cell viability at 1.25 mg/mL. (Figure 3.12). The corresponding concentrations of soluble ovalbumin and poly(I:C) were not toxic to RAW264.7 cells, however the ion-pairing surfactant CTAB showed significant toxicity at 0.8 µg/mL (Figure 3.13).

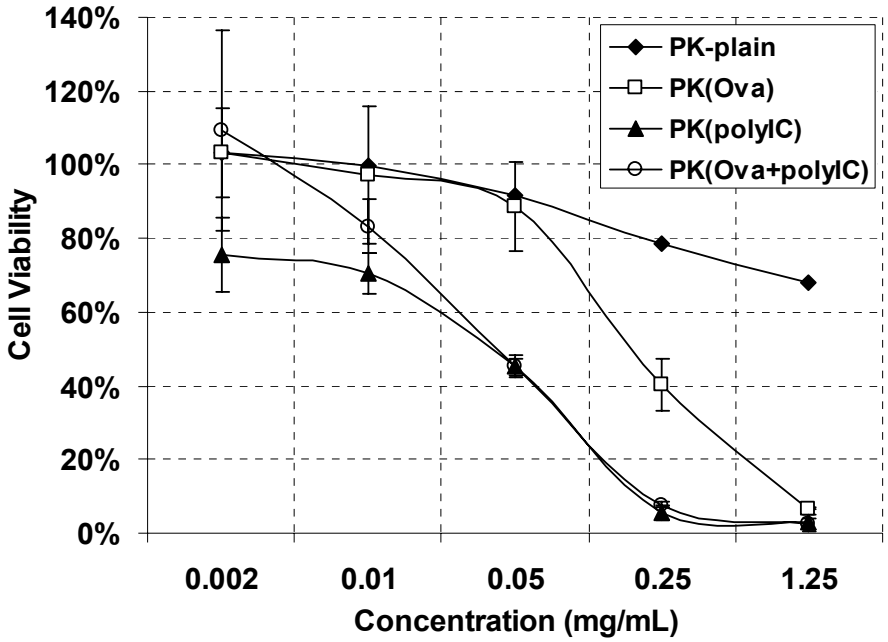


Figure 3.12 MTS cell viability assay for polyketal microparticles containing ovalbumin and/or poly(I:C).

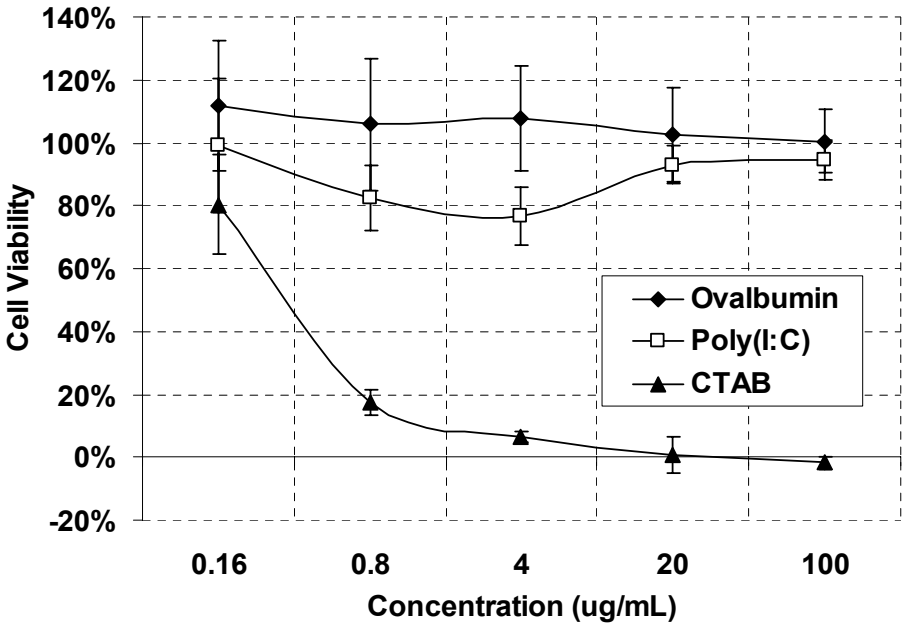


Figure 3.13. MTS cell viability assay for soluble ovalbumin, poly(I:C), and CTAB.

3.4 Discussion

In this study, the acetal exchange reaction has been shown to be an effective method of synthesizing a linear, hydrophobic polymer with pH-sensitive ketal linkages in the polymer backbone. The acetal exchange polymerization uses the ketal as the joining group between 2,2-dimethoxypropane and a diol, and this method allows flexibility in the selection of the diol to achieve the desired combination of material properties and degradation products. The incorporation of a pH-sensitive ketal linkage produces a polymer that is relatively stable at physiological pH (7.4) yet degrades more rapidly at acidic pH (5.0). This property make polyketal microparticles useful as an intracellular delivery vehicle designed to undergo accelerated degradation and release in the acidic lysosomes of phagocytic cells. Also, because the ketal linkage is contained in the backbone of the polymer, the polyketal will degrade into small molecules that are excretable. This is a significant advantage over ketal cross-linked hydrogels that contain non-biodegradable carbon backbone polymers.

Another important property of polyketals is that they do not have acidic degradation products. This is in contrast to polyesters such as poly(lactic-co-glycolic acid) (PLGA), polyanhydrides, and poly(ortho ester)s. One area of concern with polyester-based particles is that the acidic microenvironment within degrading particles can be deleterious to proteins or nucleic acids being delivered (Tamber 2005). The pH within degrading PLGA microparticles has been estimated to reach levels as low as pH 2 (Fu 2000, Shenderova 1999). This has necessitated the inclusion of buffer salts in PLGA microparticle formulations or the modification of proteins (such as PEGylation) prior to

encapsulation, in order to protect acid-labile proteins from hydrolysis (van de Weert 2000, Mok 2007). A second area of concern is the release of acid within cells or into the surrounding tissue, which can lead to an inflammatory response. The absence of acidic degradation products in polyketals is particularly important in delivering drugs or biotherapeutics to treat inflammatory diseases, because the generation of acid would itself cause inflammation. This is also a consideration with pulmonary vaccines, where the release of acid from degrading microparticles might induce lung inflammation (Armstrong 1996, Segal 2007). In other vaccine applications, this property allows the inflammatory response to be controlled by specific immunostimulatory adjuvants and localized to the targeted antigen-presenting cells, which offers a potential advantage over polyester-based biomaterials such as PLGA.

The cell toxicity of polyketal microparticles was measured with cultured RAW 264.7 macrophages, using an MTS cell viability assay. The toxicity was greater with the ion-paired ovalbumin and/or poly(I:C) formulations as compared to the plain polyketal particles. The higher cytotoxicity of the ovalbumin/poly(I:C) particles is potentially due to the ion-pairing surfactant, CTAB, contained in the particles. Based on the stoichiometry of the ion pairing procedures and the measured loading of ovalbumin and poly(I:C) in the microparticles, the combined Ova/poly(I:C) particles are estimated to have 22.5 μg/mg of CTAB. Thus the particle dosage of 0.05 mg/mL, which exhibited approximately 50% toxicity, contained 1.13 μg/mL of CTAB. The toxicity data for soluble CTAB showed a drop in cell viability at 0.8 μg/mL. This suggests that a significant proportion of the ion-paired CTAB was released from the microparticles during the 5 hour incubation with cells. This data for CTAB toxicity also falls within the

range reported for other cell lines treated with CTAB-containing liposomes, micellar solutions, cationic vesicles, and nanoparticles (Mirska 2005, Cortesi 1996, Kuo 2005, Delie 2001).

In this study we have employed the technique of **hydrophobic ion pairing** to enable encapsulation of water-soluble components such as ovalbumin and poly(I:C) via a single emulsion, solvent evaporation method. This method produced polyketal microparticles with loading levels in the range of 1% to 2% (wt/wt), which could not be achieved by the water-oil-water double emulsion method. Hydrophobic ion pairing has been used by several groups to encapsulate nucleic acids and proteins in microparticles, with improved loading levels or release profiles (Choi 2006, Yoo 2004, Fu 2003, Patel 2004, Delie 2001). In the present study, we have achieved the first hydrophobic ion pairing of the double-stranded RNA analog poly(I:C) and the first co-encapsulation of ion-paired ovalbumin and poly(I:C) in a microparticle. The methods developed in our study can potentially be used for other protein antigens and TLR ligands for vaccines, as well as delivery of nucleic acids such as plasmid DNA, mRNA, and siRNA.

In conclusion, we have developed a new family of pH-sensitive polymers, termed polyketals, which degrade into water-soluble, non-acidic products. Polyketals are linear hydrophobic polymers that can be fabricated into microparticles using solvent evaporation methods. Polyketals contain acid-labile ketal linkages in the backbone and are synthesized by a step-growth polymerization based on the acetal exchange reaction. Polyketals exhibit pH-sensitivity and thus the microparticles are designed to be stable at physiological pH yet release the vaccine components within the acidic phagolysosomes of dendritic cells. Additionally, an adaptation of the hydrophobic ion-pairing technique

was used to improve the encapsulation efficiency of water soluble molecules. Ovalbumin and the double-stranded RNA analog poly(I:C) were each ion-paired to the surfactant CTAB to produce hydrophobic complexes which were encapsulated in biodegradable polyketal microparticles. The ion-paired polyketal formulation represents a significant contribution to the field of microparticle vaccine delivery systems, and the methods developed here can be applied to other areas such as intracellular delivery of enzymes, plasmid DNA, or interfering RNA.

CHAPTER 4

EFFICACY OF MICROPARTICLE VACCINES CONTAINING OVALBUMIN AND IMMUNOSTIMULATORY AGENTS

4.1 Introduction

In **Specific Aim 3**, the objective was to demonstrate the efficacy of model vaccine formulations based on the PEG-polylysine cross-linked micelles and polyketal microparticles, using *in vitro* and *in vivo* protocols. The cross-linked micelles and polyketal microparticle vaccines are designed to deliver protein antigen and TLR-inducing adjuvants to the phagolysosomes of dendritic cells, where the vaccine components will induce activation of DCs and cross-priming of cytotoxic T lymphocytes. The adjuvants consisted of TLR agonists including CpG-DNA, MPL, and poly(I:C). These adjuvants are capable of stimulating the innate immune response within antigen-presenting cells (APCs), leading to secretion of inflammatory cytokines such as Type I interferons, tumor necrosis factor (TNF)-α, and interleukin (IL)-6, IL-10, and IL-12, as well as upregulation of costimulatory surface molecules such as CD80 and CD86 (Verdijk 1999, Alexopoulou 2001, Loré 2003, Napolitani 2005, Gautier 2005).

Our working **hypothesis** was that encapsulation of antigen and TLR agonists in a vaccine delivery formulation would enhance the cross-priming of CTLs, in comparison to administration of soluble antigen and adjuvant. To test this hypothesis, we used an *in vitro* dendritic cell-OT1 splenocyte co-culture system and an *in vivo* immune response model. The experimental protocols are designed to measure the ability of vaccine-treated antigen-presenting cells (APCs) to cross-prime cytotoxic T lymphocytes (CTLs). In the

in vitro test, murine splenic dendritic cells were treated with vaccine formulations and co-cultured with splenocytes from an OT-1 mouse. The OT-1 mouse strain expresses a transgenic T-cell receptor that recognizes the H-2Kb-restricted ovalbumin peptide SIINFEKL presented by major histocompatibility complex (MHC) class I molecules. Following the co-culture, the expanded T cells were re-stimulated with the SIINFEKL peptide along with a Golgi block to induce production and accumulation of cytokines within the cells. We used an anti-$CD8^+$ surface stain to identify cytotoxic T lymphocytes (CTLs) and intracellular cytokine staining for IFNγ, TNFα, and IL-2 to measure T cell activation and differentiation.

These experiments were conducted in collaboration with Dr. Bali Pulendran's laboratory at the Emory Vaccine Center. Dr. Marcin Kwissa and Dr. Sudhir Kasturi made significant contributions to the in vitro and in vivo experiments presented in this chapter.

4.2 Experimental Methods

Preparation of Murine Splenic Dendritic Cells and OT-1 Splenocytes

Splenic dendritic cells were expanded by daily intraperitoneal injections of C57B16 mice with Flt-3 ligand (20 μg/mouse) for 8 days. Flt-3 ligand was provided by Dr. Robert Mittler at the Emory Vaccine Center. On day 9, the mice were sacrificed and splenocytes were harvested, pooled, and frozen. Dendritic cells were isolated from thawed splenocytes using a MACS cell sorter with anti-CD11c magnetic beads, prior to cross-priming experiments. OT-1 splenocytes were obtained by harvesting total splenocytes from a C57BL/6-Tg (OT-1) RAG<tm1Mom> mouse. All animal work was

performed under protocols approved by the Institutional Animal Care and Use Committee at Emory University.

Dendritic Cell Cross-Priming of $CD8^+$ T Cells

Dendritic cell-$CD8^+$ T cell cross-priming experiments were conducted with model vaccine formulations based on cross-linked copolymer micelles and polyketal microparticles. A schematic of the in vitro protocol is shown in Figure 4.1. In this procedure, Flt-3 $CD11c^+$ dendritic cells at 1.5 x 10^5 cells/well were treated with vaccine formulation in RPMI medium in a 96-well U-bottom plate. The micelle formulations were added at concentrations ranging from 0.1 to 1 μg/mL ovalbumin, 0.1 to 2 μg/mL of CpG-DNA, and/or 0.1 to 2 μg/mL of poly(I:C). Polyketal and polyketal/PLGA hybrid microparticles were added at concentrations ranging from 0.005 to 1 μg/mL of ovalbumin. Control wells were treated with corresponding concentrations of soluble ovalbumin and adjuvants. After 4 or 5 hours of incubation at 37°C, cells were washed twice with PBS, and co-cultured with OT-1 splenocytes for 88 hours at 37°C. In one variation, the OT-1 splenocytes were sorted for $CD8^+$ cells before adding to the DCs. Cells were then given fresh culture medium containing 5 μg/mL Brefeldin A and 1 μg/mL ovalbumin-specific MHC class I-restricted peptide (SIINFEKL) and cultured for 5 hours at 37°C. Cells were then stained and analyzed by flow cytometry as described below.

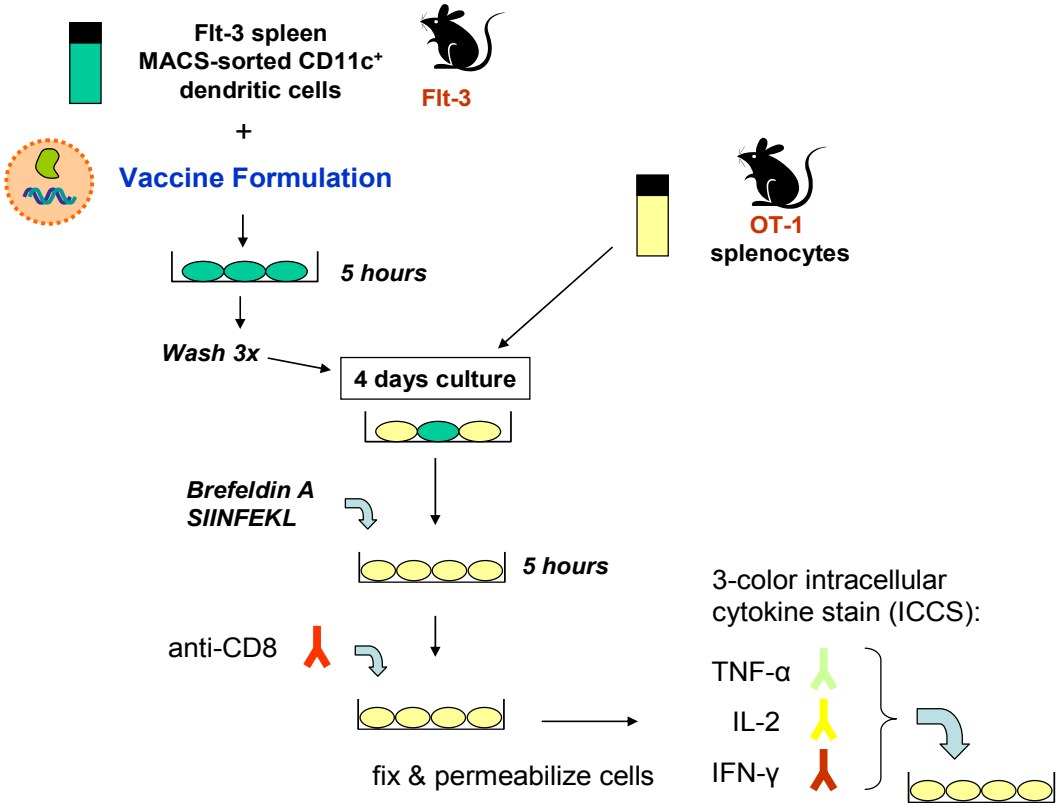


Figure 4.1 In vitro protocol for dendritic cell cross-priming of OT-1 splenocytes.

In Vivo Vaccine Efficacy of Micelle-Based Vaccine

The *in vivo* efficacy of micelle-based vaccine formulations was evaluated using a prime-boost study or a single-injection study in B6 mice. Mice were vaccinated with micelles containing 2 to 20 μg of ovalbumin and 2 to 20 μg of CpG-DNA. For the prime-boost study, injections were performed on days 0 and 36, and blood was collected on days 6, 12, 28, 42, 48 and 58. PBMCs were isolated using Histopaque®-1077 Hybri-Max™ solution and restimulated with SIINFEKL peptide for 6 hours and stained as described below. For the single injection study, mice were injected on day 0 and sacrificed on day 7, at which time PBMCs were isolated.

In Vivo Vaccine Efficacy of Polyketal-Based Vaccine

The *in vivo* efficacy of polyketal-based vaccine formulations was evaluated using a single-injection, 7 day study in B6 mice. Mice were vaccinated with polyketal or polyketal/PLGA hybrid microparticles containing 20 to 50 μg of ovalbumin and 10 to 25 μg of adjuvant, including MPL, CFA, and poly(I:C). On Day 7, blood was collected, the mice were sacrificed, and spleens and draining lymph nodes were harvested. Peripheral blood mononuclear cells (PBMCs) were isolated using Histopaque®-1077 Hybri-Max™ solution. Spleen cells and lymph node cells were recovered by gentle grinding in a plastic strainer.

Intracellular Cytokine Staining and Flow Cytometry

Intracellular cytokine staining and surface CD8a staining was performed following manufacturer's protocol. Cells were centrifuged and resuspended in 100 μL of FACS staining buffer (PBS + 5% FBS) containing a 1:200 dilution of Fc block (Anti CD16/32, 2.4G2) and a 1:66 dilution of anti-CD8 antibody and stained for 30 min at 4°C. Cells were washed twice with 150 μL of FACS staining buffer and then fixed and permeabilized using BD Cytofix/Cytoperm solution (BD) for 15 min at 4°C. Cells were washed twice with Perm Wash buffer and stained for IFNγ, IL-2, and TNFα for 30 min at 4°C. Cells were washed twice with Perm Wash buffer and once with FACS staining buffer and resuspended in 250 μL of FACS staining buffer. Flow cytometry was performed on a FACSCalibur™ system and analyzed using FlowJo software. Cells were

gated on forward/side scattering (live cells) and CD8a (surface stain), and populations were analyzed for intracellular cytokines IFNγ, IL-2, and TNFα (Figure 4.2)

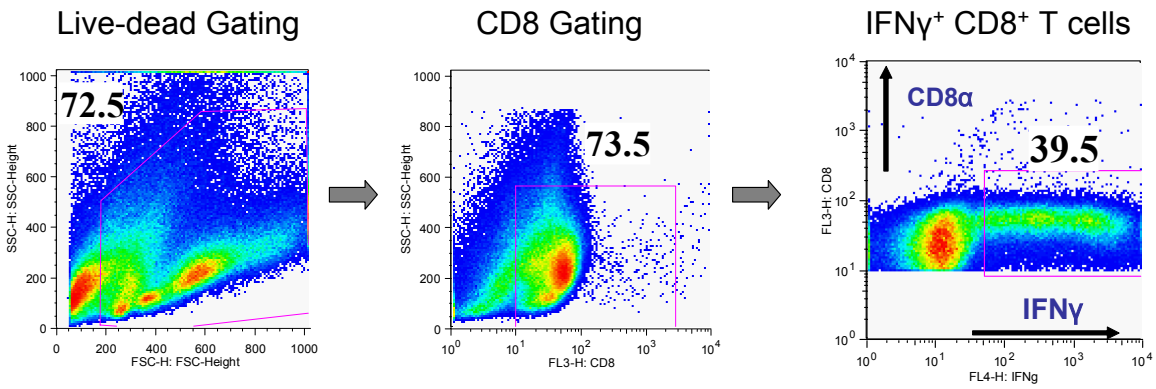


Figure 4.2 Flow cytometry gating for surface CD8 and intracellular cytokines.

4.3 Results

Efficacy of Cross-Linked Micelle Vaccine Containing Ovalbumin and CpG-DNA (June 2005 batch)

In Vitro Cross-Priming of $CD8^+$ Splenocytes by Ova/CpG Micelles

The in vitro cross-priming experiment was conducted with micelles containing 0.5 mg/mL each of ovalbumin and CpG-DNA. In one experiment, Flt3-expanded dendritic cells were pulsed with the micelle vaccine and then co-cultured with total OT-1 splenoctyes. At a dosage of 1 μg/mL, the micelles enhanced the cross-priming of $CD8^+$ T cells, as measured by the percentage of IFNγ-producing $CD8^+$ T cells (Figure 4.3). Also, the adjuvant CpG-DNA boosted the efficacy of the micelle formulation at this dosage.

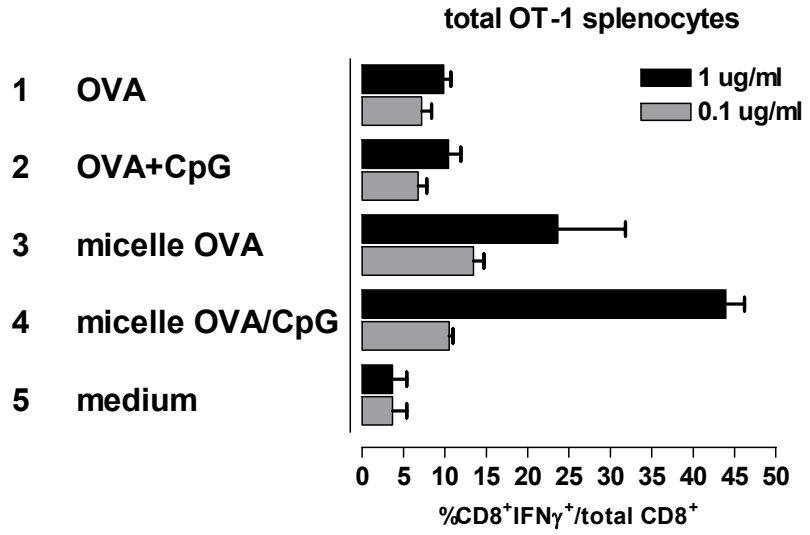


Figure 4.3. In vitro cross-priming of $CD8^+$ T cells by micelles containing ovalbumin and CpG-DNA. Vaccine-pulsed dendritic cells were co-cultured with total OT-1 splenocytes.

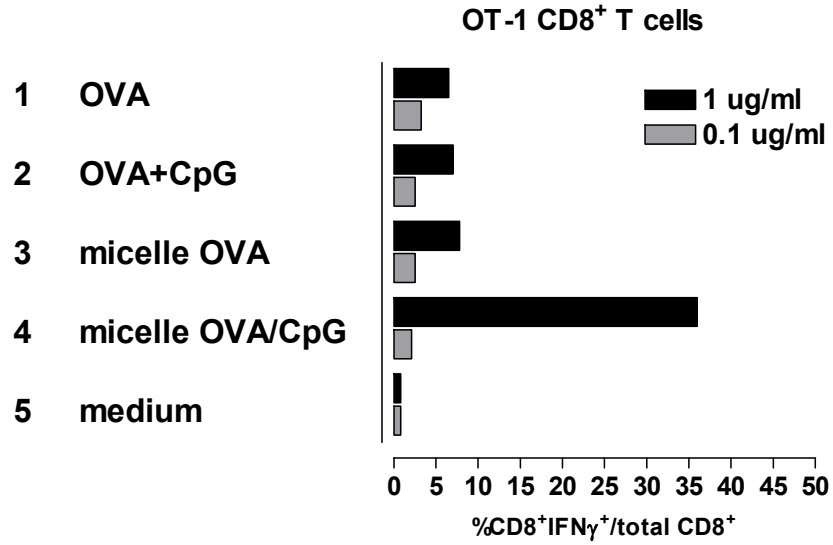


Figure 4.4. In vitro cross-priming of CD8+ T cells by micelles containing ovalbumin and CpG-DNA. Vaccine-pulsed dendritic cells were co-cultured with $CD8^+$ OT-1 splenocytes.

In a separate experiment, the vaccine-pulsed DC were co-cultured with $CD8^+$ sorted OT-1 splenocytes; i.e., the cell population was devoid of $CD4^+$ helper T cells. As shown in Figure 4.4, the micelle formulation containing ovalbumin and CpG-DNA induced a strong $CD8^+$ T cell response at the 1 μg/mL dosage. This suggests that the vaccine formulation bypasses the requirement for $CD4^+$ T cells in stimulating a $CD8^+$ T cell reponse.

In Vivo Prime-Boost Vaccination with Ova/CpG Micelles

A 58-day prime-boost vaccination study was conducted with the June 2005 batch of ovalbumin/CpG-DNA micelles. The results in Figure 4.5 show a strong $CD8^+$ T cell response at 6 days after the prime injection and 6 to 12 days following the boost injection. The fact that the boost response was longer-lasting than the prime response

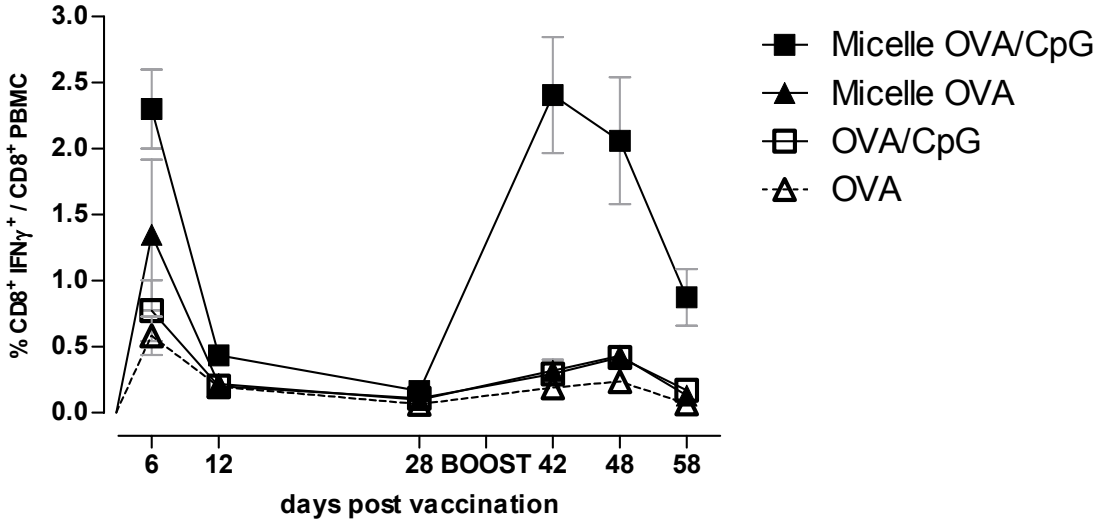


Figure 4.5. In vivo prime-boost vaccination by micelles containing ovalbumin and CpG-DNA. Mice were vaccinated on Day 0 and Day 36.

suggests that memory T cells were generated by the first injection. The micelle(Ova/CpG) vaccine formulation had enhanced efficacy in comparison to the soluble Ova/CpG control. Interestingly, the micelle(Ova) formulation had a decreased boost response, which suggests tolerance due to the lack of an immunostimulatory TLR agonist.

Efficacy of Cross-Linked Micelle Vaccine Containing Ovalbumin, CpG-DNA, and Poly(I:C) (Sept 2005 batch)

In vitro cross-priming of $CD8^+$ splenocytes by Ova/CpG/poly(I:C) micelles

Micelles containing ovalbumin, CpG-DNA, and poly(I:C) were evaluated using an in vitro DC-OT1 co-culture assay. The co-culture used either total OT-1 splenocytes or $CD8^+$ sorted OT-1 splenocytes. Figure 4.6 shows the number of $CD8^+$ cells that were positive for intracellular IFNγ, as a percentage of total $CD8^+$ cells. In the control groups, the addition of either soluble TLR agonist CpG-DNA or poly(I:C) enhances IFNγ production as compared to Ova alone. The Ova/CpG micelle showed an increased response versus soluble Ova/CpG in stimulating $CD8^+$ purified OT-1 splenocytes.

In vivo cross-priming of $CD8^+$ splenocytes by Ova/CpG micelles

Because the efficacy of the Sept. 2005 batch was not a strong as the June 2005 batch, the two batches were compared side-by-side using a 7 day in vivo assay. In this experiment, the Sept. 2005 batch in general had a weaker response for in vivo activation of PBMCs (Figure 4.7). The large variability between micelle batches eventually led us to switch to polyketal microparticle formulations as the vaccine delivery system.

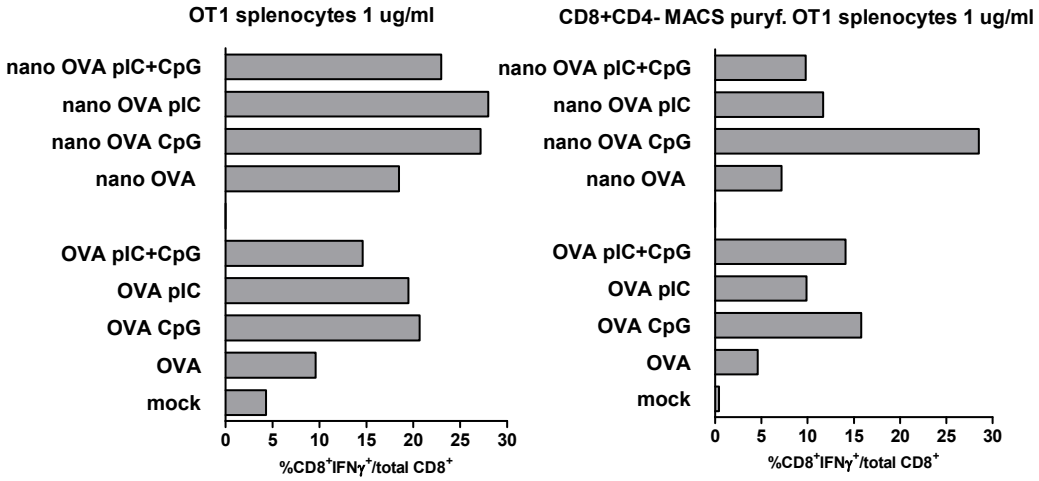


Figure 4.6. In vitro cross-priming of CD8+ T cells by micelles containing ovalbumin, CpG-DNA, and poly(I:C). Vaccine-pulsed dendritic cells were co-cultured with total OT-1 splenocytes (left) or $CD8^+$ OT-1 splenocytes (right).

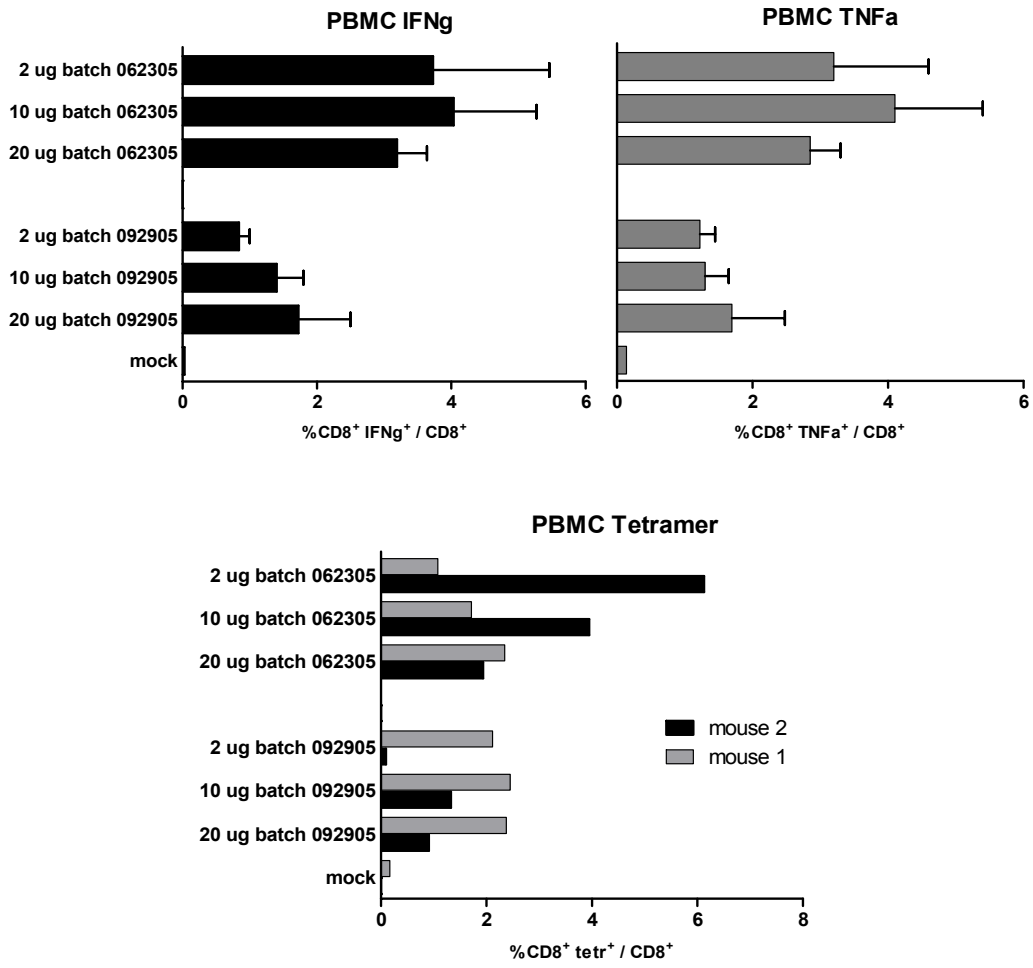


Figure 4.7. In vivo cross-priming of CD8+ T cells by micelles containing ovalbumin and CpG-DNA. PBMCs were stained for IFNγ, TNFα, or SIINFEKL tetramer.

Efficacy of Hybrid Polyketal/PLGA Vaccine Containing Ovalbumin and MPL

In Vitro OT-1 Cross-Priming of $CD8^+$ Splenocytes by Ovalbumin/MPL Vaccine

Microparticles containing ovalbumin were prepared by double emulsion method with a blend of polyketal and PLGA. MPL was co-encapsulated with Ova or was encapsulated alone using a single emulsion. As controls, Ova and MPL were administered in soluble form. Figure 4.8 shows the in vitro cross-priming of OT-1 splenocytes, in terms of $IFN\gamma^+$ cells as a subset of $CD8^+$ cells. The Ova dosages ranged from 0.005 to 0.5 μg/mL for the microparticle groups and from 0.005 to 50 μg/mL for the soluble Ova group. This experiment demonstrates that encapsulation of ovalbumin in polyketal-based microparticles dramatically enhances the efficacy as compared to soluble ovalbumin. The addition of MPL in the same particle, or in a separate particle, slightly increases the polyketal(Ova) response.

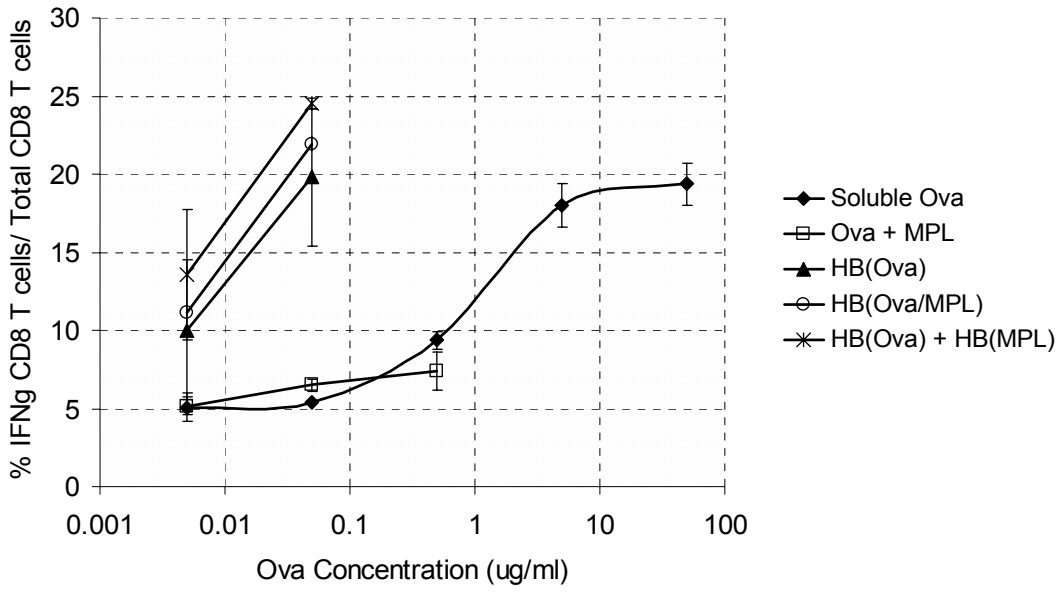


Figure 4.8. In vitro cross-priming of CD8+ T cells by hybrid polyketal/PLGA microparticles containing ovalbumin and MPL.

In Vivo Cross-Priming of $CD8^+$ T cells by Ovalbumin/MPL Vaccine

A 6-day vaccine efficacy study in mice was conducted with vaccine formulations containing ovalbumin and MPL. Hybrid polyketal/PLGA microparticles were prepared with Ova or MPL in separate particles, or Ova + MPL combined in the same particle batch. Figure 4.9 shows the cross-priming of PBMCs, presented in terms of $IFN\gamma^+$ cells as a subset of $CD8^+$ cells. The results show that delivery of ovalbumin and MPL in separate microparticles enhances the $CD8^+$ T cell response over the controls. Interestingly, the combined Ova/MPL particle did not have a strong response.

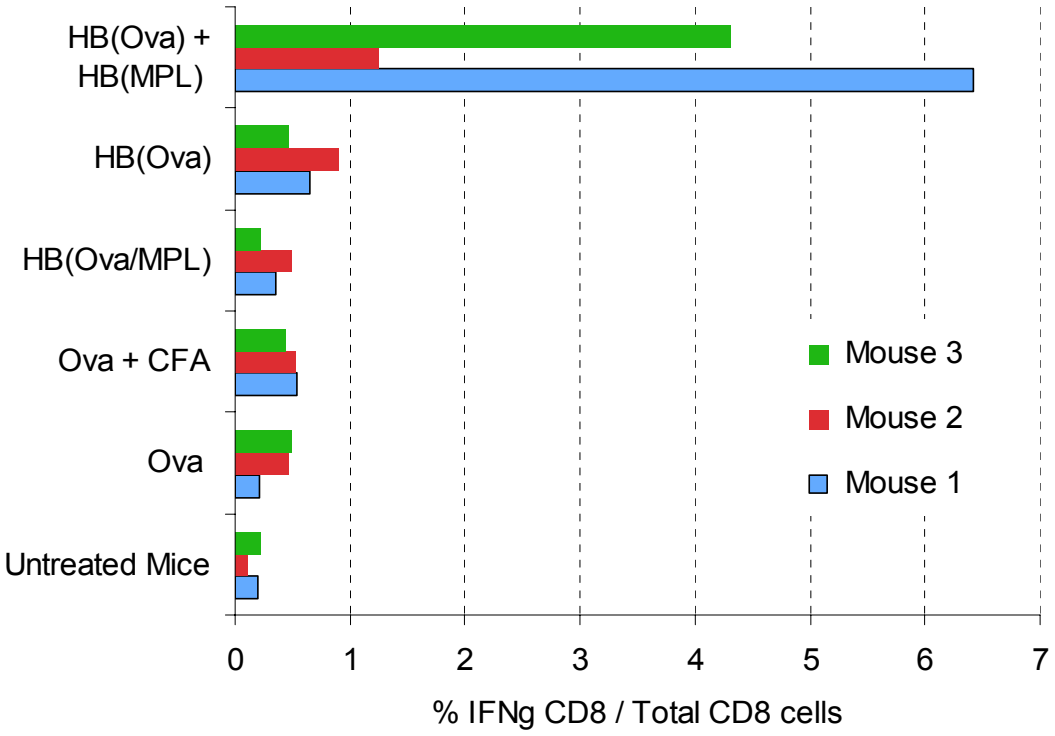


Figure 4.9. In vivo cross-priming of CD8+ T cells by hybrid polyketal/PLGA microparticles containing ovalbumin and MPL.

Efficacy of Polyketal-based Vaccine Containing Ovalbumin and Poly(I:C)

In Vitro OT-1 Cross-Priming of $CD8^+$ Splenocytes by Ovalbumin/poly(I:C) Vaccine

In this experiment, polyketal microparticles containing ion-paired ovalbumin and poly(I:C) were tested in vitro at dosage levels of 0.01, 0.1, and 1 μg/mL ovalbumin and 0.005, 0.05, and 0.5 μg/mL poly(I:C), respectively. An example of the flow cytometry gating is shown in Figure 4.10, for the lowest dosage groups. Figures 4.11, 4.12, and 4.13 show the levels of IFNγ, TNFα, and IL-2, respectively, for all three vaccine dosage levels. All results are presented as the percentage of $CD8^+$ cells that are positive for the particular cytokine. The IFNγ results in Figure 4.11 indicate that at dosage levels of 0.01 and 0.1 μg/mL ovalbumin, the polyketal microparticles containing ovalbumin and poly(I:C) induced significantly higher levels of $IFN\gamma^+$ T cells than ovalbumin-containing microparticles or soluble ovalbumin/poly(I:C). Notably, the 0.01 μg/mL dosage of ovalbumin/poly(I:C)-containing microparticles induced a stronger $IFN\gamma^+$ T cell response than a 10-fold higher dose (0.1 μg/mL) of ovalbumin microparticles. At the lowest dose (0.01 μg/mL), the co-delivery of antigen and TLR3 agonist also significantly enhanced the generation of $TNF\alpha^+$ and IL-2^+ cells when compared the control formulations (Figures 4.12 and 4.13). The $TNF\alpha^+$ and IL-2^+ cell levels induced by the ovalbumin/poly(I:C) microparticles at the 0.01 μg/mL dosage were slightly greater than that of the ovalbumin microparticles at a 10-fold higher dosage (0.1 μg/mL) (Figures 4.12 and 4.13). This was the second of two identical experiments with independent batches of polyketal, ion-pairing, and microparticles. The second batch performed slightly better

than the first batch, mostly likely due to better particle dispersion, but both had similar trends.

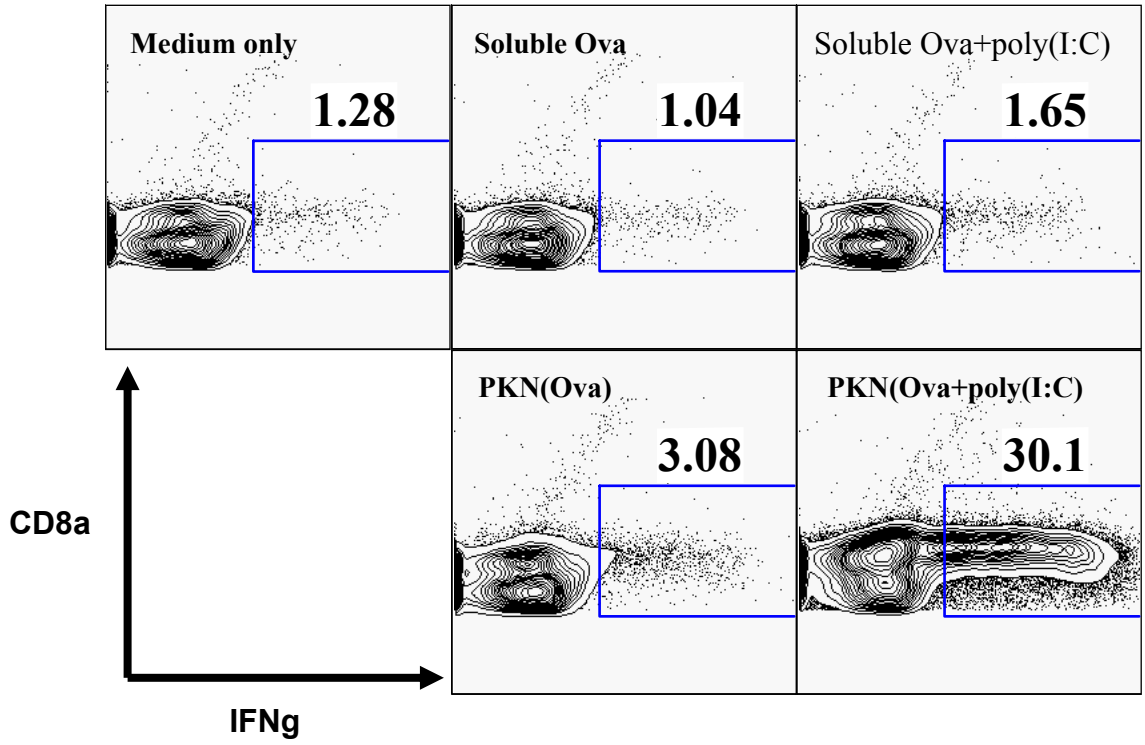


Figure 4.10. In vitro DC-OT1 cross-priming by polyketal(Ova-poly(I:C)) vaccine at 0.01 μg/mL Ova; flow cytometry scatter plots for $CD8^{+}IFN\gamma^{+}$ cells.

In Vivo Cross-Priming of $CD8^{+}$ T cells by Ovalbumin/poly(I:C) Vaccine

In this single experiment, polyketal microparticles containing ion-paired ovalbumin and poly(I:C) were tested in vivo at dosage levels of 20 μg/mouse ovalbumin and 10 μg/mouse poly(I:C). Figure 4.14 shows the percentage of cytokine-producing $CD8^{+}$ T cells from PBMCs, spleen cells, and lymph node cells. Both vaccine groups (soluble and microparticle formulations) showed an increase in levels of CD8+ T cell activation compared to untreated mice; however, there was no significant difference between the soluble control group and the microparticle group.

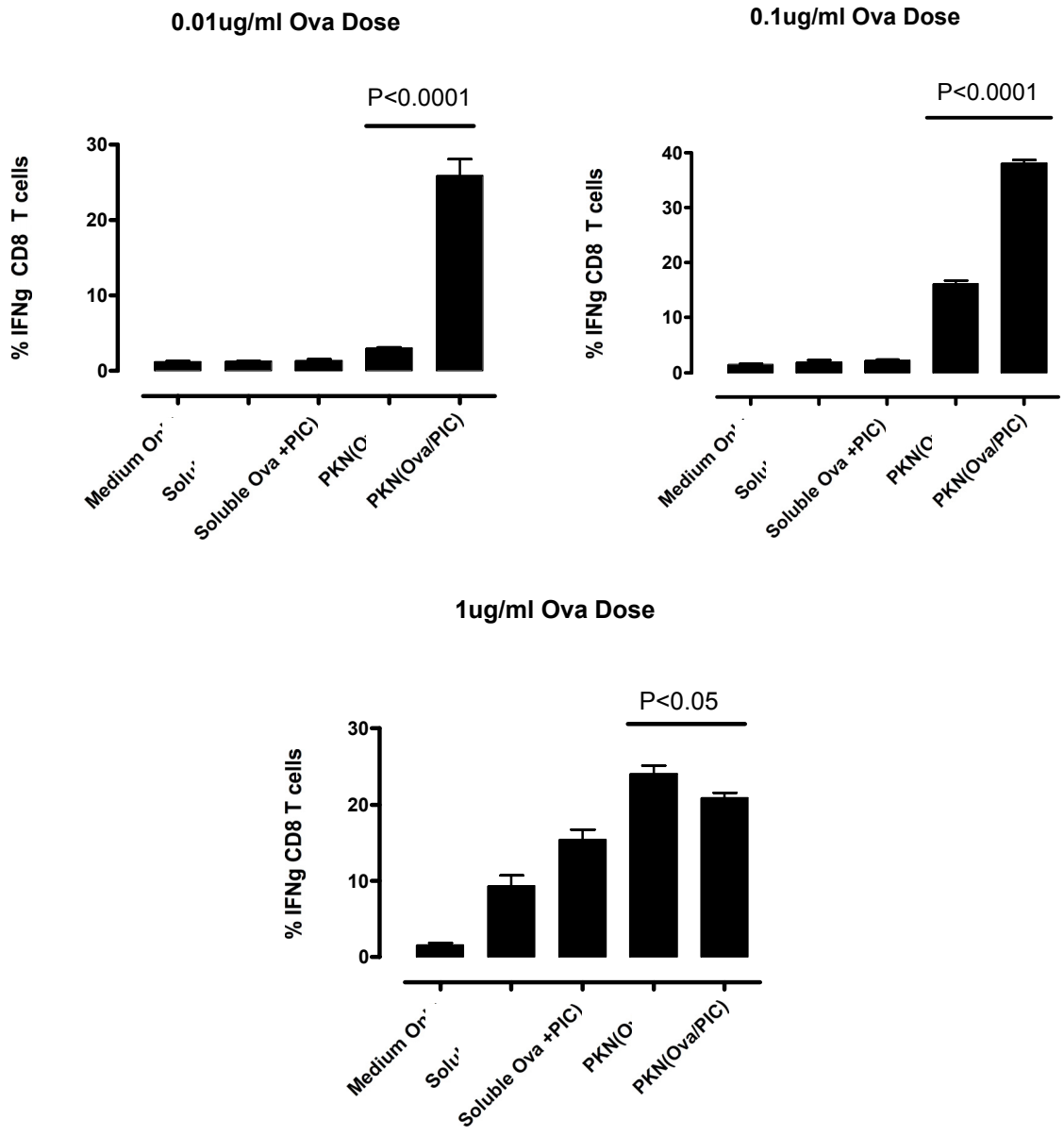


Figure 4.11. In vitro DC-OT1 cross-priming by polyketal(Ova-poly(I:C)) vaccine; percentage of IFNγ-producing $CD8^+$ cells, n = 4 wells per group.

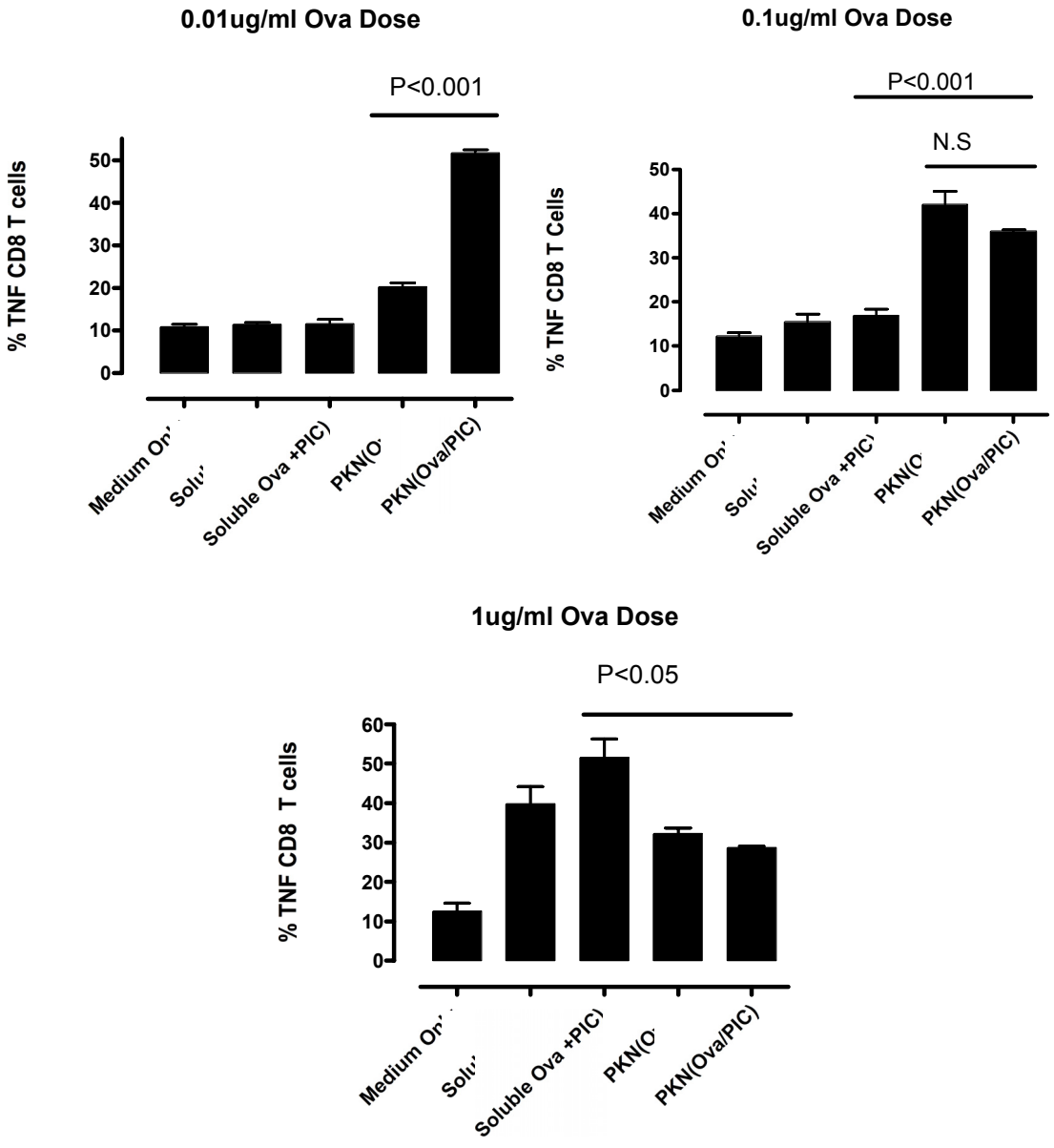


Figure 4.12. In vitro DC-OT1 cross-priming by polyketal(Ova-poly(I:C)) vaccine; percentage of TNFα-producing $CD8^+$ cells, n = 4 wells per group.

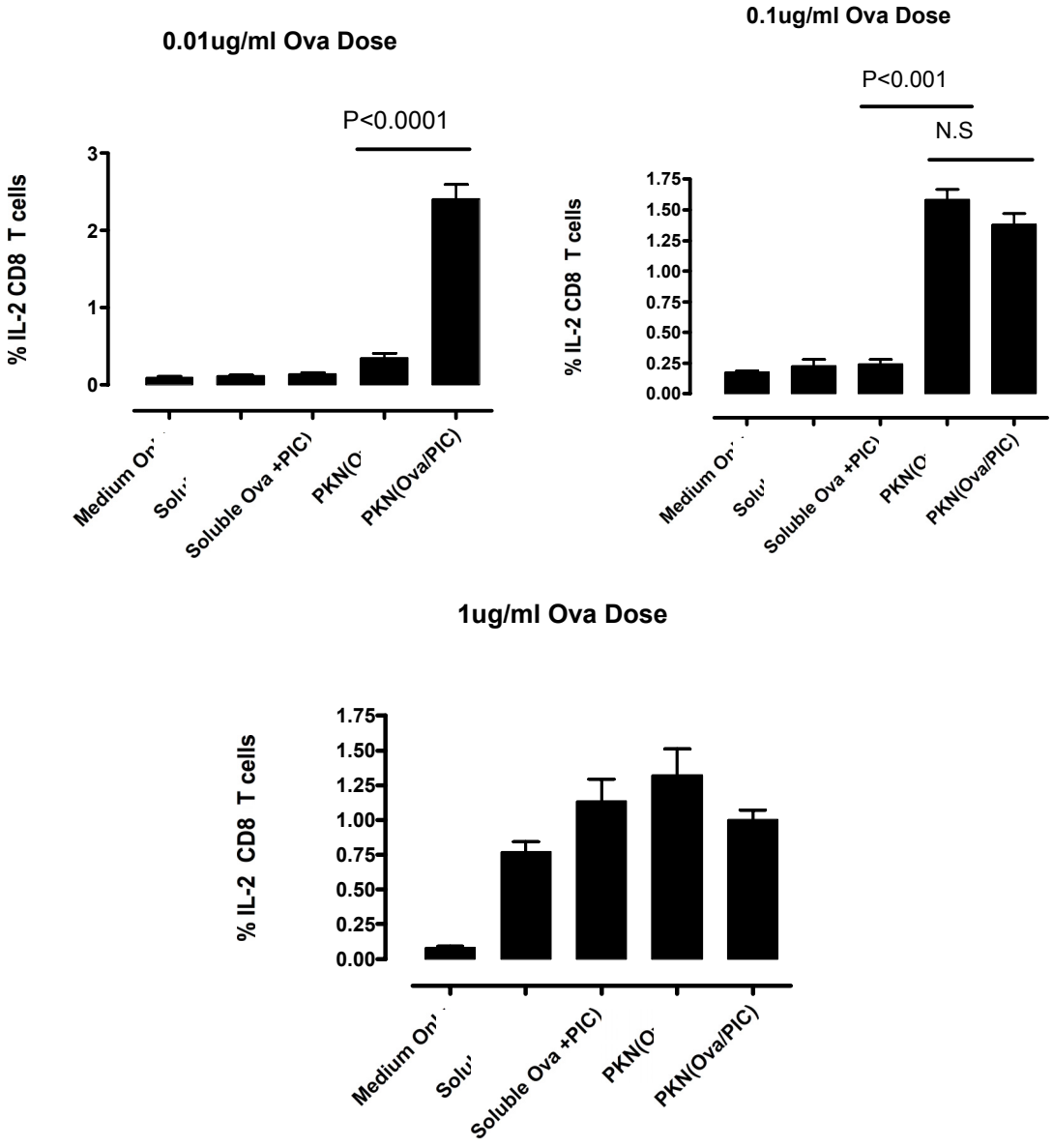


Figure 4.13. In vitro DC-OT1 cross-priming by polyketal(Ova-poly(I:C)) vaccine; percentage of IL-2-producing $CD8^+$ cells, n = 4 wells per group.

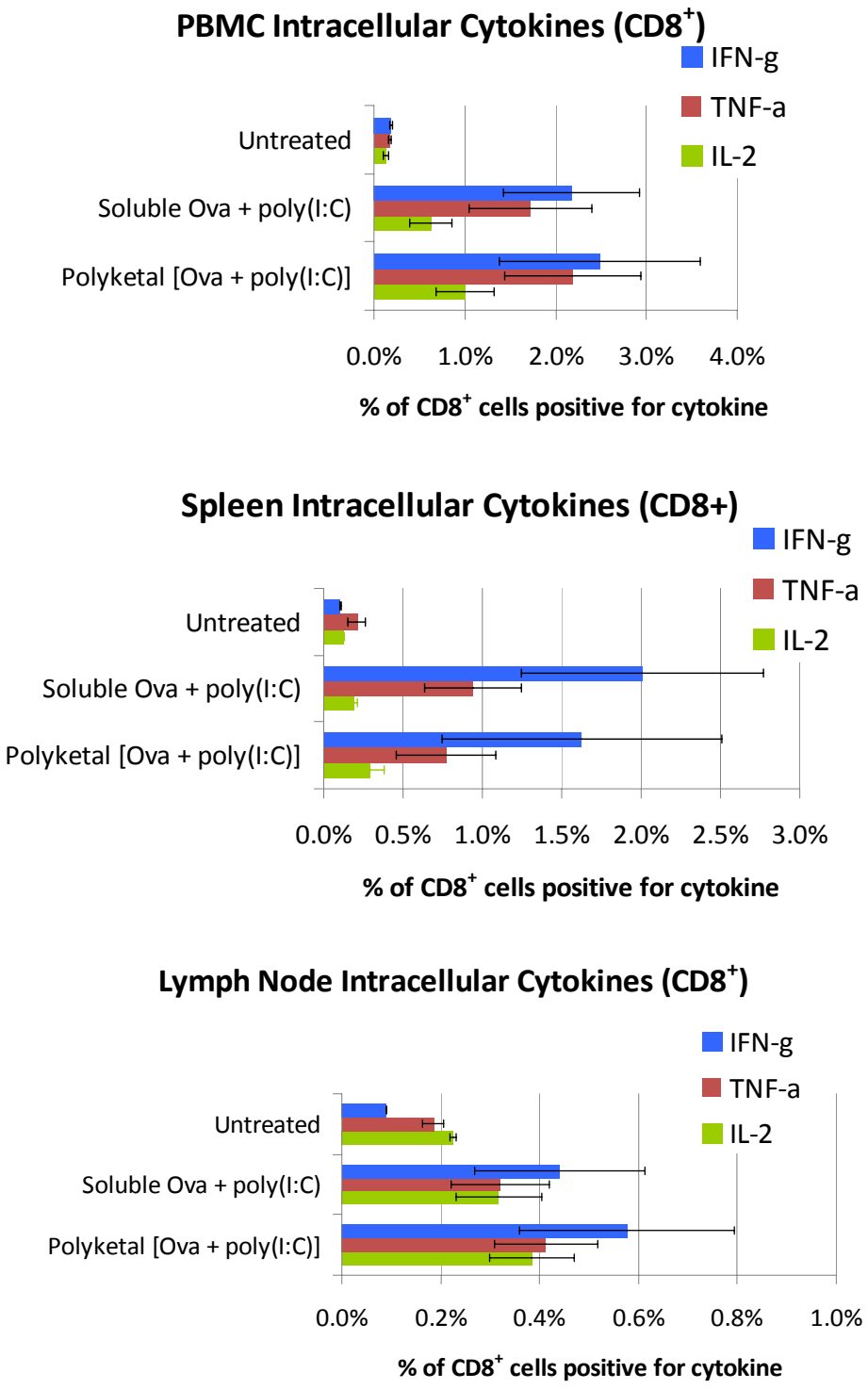


Figure 4.14. In vivo cross-priming of $CD8^+$ T cells by polyketal(Ova-poly(I:C)) vaccine. Data is shown as mean plus/minus standard deviation for n = 2 (untreated) or n = 4 (vaccine groups).

4.4 Discussion

Our objective in this study was to demonstrate that a vaccine delivery system could be developed which would enhance the cytotoxic T lymphocyte response of model vaccines containing protein antigen and immunostimulatory adjuvants. The initial experiments were conducted using the PEG-PLTP crosslinked micelle with ovalbumin antigen and CpG-DNA (TLR9 agonist). One particular batch of this micelle formulation showed a strong level of CTL cross-priming *in vitro* and *in vivo*; however, later batches could not reproduce the same level of dendritic cell uptake or CTL cross-priming. Possible causes were identified, such as differences in the PEG-PLL degree of polymerization or micelle degree of cross-linking, however efforts to pinpoint the root cause(s) of the batch-to-batch variation were unsuccessful. Through the process of improving the robustness of the micelle formulation, the SPDP-ovalbumin modification and step-wise crosslinking method were introduced, and characterization of these micelles demonstrated a high level of encapsulation and retention of ovalbumin and CpG-DNA, as presented in Chapter 2. Despite these improvement to the formulation, the micelles showed variable enhancement of uptake by DCs, and therefore we pursued solid microparticle formulations as an alternative.

Microparticle-based vaccine formulations were initially prepared by Sudhir Kasturi of the Pulendran laboratory using the polyketal with a double emulsion (water-oil-water) to encapsulated ovalbumin protein and TLR agonists. The double emulsion process yielded low encapsulation efficiency for ovalbumin, which was attributed to the material properties of the polyketals developed at the time. It was believed that the low

molecular weight of the polyketals resulted in less polymer entanglement which allowed protein to escape from the inner water phase to the external water phase in the double emulsion. Therefore, polyketal/PLGA blends were pursued, which would provide the pH-sensitivity of the polyketal along with the higher molecular weight of the PLGA. Hybrid polyketal/PLGA microparticles were prepared at polymer blend ratios of 50/50 and 25/75, encapsulating ovalbumin and MPL.

In subsequent work with double-stranded RNA as the adjuvant, it was deemed necessary to use a pure polyketal microparticle, which did not have acidic degradation products that might be deleterious to the encapsulated RNA. In order to encapsulate dsRNA and ovalbumin in a polyketal microparticle, we utilized the technique of ion-pairing to create hydrophobic complexes that could be encapsulated via a single emulsion method. Through this process, polyketal microparticles were formulated with ovalbumin and poly(I:C), and the efficacy of this formulation was tested using the *in vitro* DC-OT1 assay and *in vivo* testing in mice.

Our results demonstrate that the polyketal microparticles and PK/PLGA hybrid microparticles containing ovalbumin and TLR agonists are highly effective at cross-priming $CD8^+$ splenocytes in a DC/OT-1 co-culture system, and *in vivo* testing of the PK/PLGA hybrids showed that a single injection was capable of generating high levels of $IFN\gamma^+$ CTLs. The *in vitro* experiments with pure polyketal microparticles containing ion-paired ovalbumin and poly(I:C) showed that at the lower dosages, the microparticles significantly enhanced $CD8^+$ T cell cross-priming as compared to control groups with soluble antigen and adjuvant, as shown by high levels of IFNγ-, TNFα-, and IL-2-producing cells. The preliminary *in vivo* results with the ovalbumin/poly(I:C)

microparticles did not show any enhancement over the soluble control. A similar result was seen at the highest dosage tested *in vitro*, which suggests that a lower dosage would be more effective *in vivo*.

The *in vitro* tests for the polyketal-formulated Ova/poly(I:C) vaccine showed that the microparticles were highly effective at the lower dosages. For example, the polyketal-formulated Ova/poly(I:C) vaccine achieved a higher $IFN\gamma^+$ CTL response at 0.1 μg/mL antigen than that seen with a 10-fold higher dose of soluble antigen/adjuvant (Figure 4.11). This dose-sparing effect is seen as an advantage of microparticle-based protein vaccines because it enables higher production volumes of the vaccine in the case of an epidemic. Further studies are warranted to examine the effects of dosage and combinations of different TLR agonists.

The strong response even at low doses of antigen can be attributed to the rapid simultaneous delivery of antigen and TLR ligand to the phagosomes of dendritic cells. The pH-dependent hydrolysis exhibited by the polyketal family enables tuning of the hydrolysis kinetics to achieve rapid polymer degradation at lysosomal pH. The copolyketal (PK3) used in this study has a hydrolysis half-life on the order of 1 to 2 days at pH 4.5. This is within the timeframe that antigen presentation and cross-priming of CTLs occurs in vivo. The results are consistent with reported studies that show a strong T_H1-biased adaptive immune response through activation of TLRs 3, 4, and 9. For example, DCs treated with TLR agonists have been shown to express type I interferons (IFN-α and IFN-β), which promote a local antiviral response in infected cells, and pro-inflammatory cytokines such as interleukin (IL)-12p70 and IL-6 (Verdijk 1999, Alexopoulou 2001). The cytokines induced by TLR 3, 4, and 9 stimulation promote a

T_H1 bias and antigen-specific cross-priming of CTLs (Lore 2003, Schulz 2005, Zaks 2006).

An important feature of the polyketal(ovalbumin/poly(I:C)) formulation is that rapid degradation at lysosomal pH allows for delivery of the poly(I:C) at the site of TLR3 engagement. TLR3 is an endosomal receptor with its dsRNA binding site facing the interior of the endosome. After the polyketal particles are phagocytosed and the phagosome is acidified, the pH-sensitive polyketal should undergo rapid degradation and release poly(I:C) into the phagolysosome, where it can be recognized by TLR3. Furthermore, it has been shown that TLR3 interacts with poly(I:C) in a narrow pH window (pH 5.7 to 6.7) (de Bouteiller 2005); thus, as the pH of the phagosome is lowered from its initial level of pH 7.4, the accelerated degradation of the polyketal should allow for poly(I:C) to be released and engage TLR3 in the phagolysosome. We believe that the property of tunable, pH-sensitive degradation of polyketals makes this family of polymers well suited for delivery of ligands for endosomal TLRs, in particular TLR3.

The polyketal-based ovalbumin/TLR agonist vaccine formulations induce strong $CD8^+$ T cell responses, which indicates that antigenic peptides are presented by MHC-I molecules. This is consistent with reports in the literature that dendritic cells present phagocytosed exogenous protein antigen on MHC-I molecules. For example, reports have shown that ovalbumin conjugated to iron oxide beads is presented by bone marrow-derived DCs on MHC-I molecules (Shen 1997), and conjugates of ovalbumin and CpG-DNA induce T-helper cell-independent activation of CTLs (Cho 2000). Because MHC-I molecules originate in the endoplasmic reticulum, the processing of antigens trafficked through the endosomal-phagosomal system relies on a mechanism which brings the

antigen in contact with MHC-I processing machinery. While the exact processes have not been determined, various cellular mechanisms have been proposed in which protein antigens or MHC-I molecules are transported between intracellular compartments (Groothuis 2005).

An alternate pathway by which phagosomal antigen may access the MHC-I processing machinery is through antigen escape from the lysosome into the cytosol. This would enable the antigen to be processed and loaded onto MHC-I platforms in the same manner as endogenous proteins. In the case of microparticles prepared with CTAB as a hydrophobic ion-pairing agent, phagolysosome destabilization may occur as a result of interaction of CTAB, a cationic surfactant molecule, with the anionic endosomal membrane. Phagolysosome rupture may also occur due to the osmotic pressure imbalance resulting from rapid degradation of the polyketal microparticles into numerous small molecules within the phagolysosome. This mechanism has been proposed with other biodegradable delivery vehicles as a means of antigen escape from the endosome to the cytosol to achieve MHC-I presentation (Shen 2006, Standley 2007). Thus, while various controlled mechanisms are thought to be involved in endosome-derived antigen being presented on MHC-I platforms, alternate pathways involving phagolysosome rupture and antigen escape may contribute to MHC-I processing of ion-paired ovalbumin released from polyketal microparticles.

In conclusion, we have demonstrated the development of novel vaccine delivery systems for protein antigen and TLR-inducing adjuvants. The cross-linked micelle has been shown to be capable of high efficiency encapsulation of protein, DNA, and RNA; however, significant batch-to-batch variation was observed with regard to DC uptake and

CTL cross-priming. The issue of low uptake would need to be addressed, perhaps through the addition of a targeting ligand, to realize the potential of the cross-linked micelle as a vaccine delivery vehicle. Solid microparticles based on pH-sensitive polyketals were shown to have enhanced uptake by DCs, and various microparticle formulations were prepared using a polyketal/PLGA hybrid with a double emulsion, or a polyketal with ion-pairing and a single emulsion method. We have demonstrated that the polyketal-based vaccine formulations enhance the ability of dendritic cells to cross-prime cytotoxic T lymphocytes in vitro, as evidenced by production of IFN-γ, TNF-α, and IL-2. Very low doses of antigen and adjuvant are required, which can be attributed to the rapid intracellular release from the polyketal-based microparticles. Additionally, polyketal/PLGA hybrid formulations containing ovalbumin and MPLA have shown significant enhancement of CTL priming in mice.

CHAPTER 5

CONCLUSIONS AND FUTURE DIRECTIONS

5.1 Conclusions

In completing these specific aims, we have demonstrated the development of two versatile delivery systems for protein and nucleic acid biotherapeutics. The first system is a self-assembling block copolymer micelle that encapsulates proteins and nucleic acids via electrostatic interactions and disulfide cross-linking. The micelle has the advantages of high encapsulation efficiency and flexible batch scaling, and has a size range of 50-150 nm. The second system is a solid nanoparticle composed of a pH-sensitive biodegradable polyketal which is capable of encapsulating hydrophobic compounds as well as proteins and nucleic acids. The polyketal particles (300 nm to 3 μm) have high uptake by phagocytic cells, and the polyketal degrades into non-acidic molecules that are not detrimental to encapsulated biotherapeutics. The micelles and microparticles were evaluated for their efficacy as protein vaccine delivery vehicles, using ovalbumin as a model antigen and various TLR agonists as immunostimulatory adjuvants. These experiments focused on the cross-priming of $CD8^+$ T cells by DCs treated with vaccine formulations. The cross-linked micelles served as a proof-of-concept for the enhancement of $CD8^+$ T cell priming by encapsulation of ovalbumin (protein antigen) along with CpG-DNA (TLR9 agonist) and/or poly(I:C) (TLR3 agonist) in a delivery vehicle; however, variability in DC uptake and activation was observed among micelle batches. Solid microparticles based on pH-sensitive polyketals were developed, which improved the uptake by phagocytic cells. We demonstrated that polyketal-based

microparticle formulations containing ovalbumin and the TLR4 agonist MPL were capable of enhancing CD8+ T cell responses in vitro and in vivo, in comparison to soluble formulations. We also showed that polyketal microparticles could be prepared with hydrophobic ion-paired ovalbumin and poly(I:C) (TLR3 agonist), and that this vaccine formulation elicited strong CD8+ T cell responses in vitro at low antigen doses. From these results it can be concluded that the polyketal-based microparticles, in conjunction with the hydrophobic ion pairing technique, have significant potential as a delivery system for protein/TLR-agonist vaccines.

5.2 Future Directions

Several areas of future research have been considered to improve the functionality of the cross-linked micelles and polyketal microparticles, and to explore more effective protein-based vaccine formulations.

1. Further Development of Cross-linked Micelle Delivery System

a) Add a targeting ligand to improve uptake by DCs or other target cells. A simple method would be to conjugate a targeting peptide to the PEG-PLTP polymer by including a single cysteine residue at one end of the peptide and adding the peptide prior to the cross-linking step. Another method would be to conjugate the targeting ligand to the distal end of the PEG chain. This would require a heterobifunctional PEG with conjugation chemistry that is orthogonal to that used in cross-linking the interior of the micelle. For example, the click reaction between an alkyne and a azide could be used to attach the targeting ligand to free end of the PEG chain.

b) Synthesize a PEG-poly(Lys) dendrimer. A PEG-poly(Lys) dendrimer could be synthesized in a controlled manner, as opposed to the ring-opening polymerization currently used for the PEG-poly(Lys). This would produce a polymer with narrow distribution, and thus would improve the uniformity of the micelles. The dendrimer could be synthesized using Fmoc protecting groups on the Lysine, similar to the methods used in solid-state peptide synthesis. Preliminary experiments would be needed to demonstrate that the dendrimer block could complex proteins and nucleic acids as effectively as a linear polymer chain.

c) Develop non-reversible cross-linking chemistry. An alternative cross-linking scheme could be used for extracellular applications where it might be desired to keep the micelle intact for an extended time. One possible application is enzyme-cleavable prodrug therapy for cancer. Micelles loaded with enzyme would be delivered to the tumor site due to the enhanced permeability and retention effect (EPR), and the enzyme, which is active inside the core of the micelle, would convert the prodrug to the active drug at the tumor site.

d) Develop alternative method of conjugating proteins in the micelle core. The SPDP conjugation has been demonstrated for ovalbumin and catalase, both of which are relatively inexpensive proteins. If this process were to be scaled down, there would be some loss in material in the conjugation and purification steps. Thus, it would be preferred to use a method such as glutathione-GST affinity to bind a GST fusion protein in the core of the micelle.

2. Improvements to Polyketal Microparticles

a) Improve microparticle fabrication process. Optimization of the homogenization, solvent evaporation, and particle washing steps are needed to increase the particle yield.

b) Prevent aggregation of precipitated microparticles. Aggregation has been an issue with microparticles following the washing and lyophilization steps. This may be improved by analyzing the effects of the size and material properties of the polyketal microparticles, and modifying the chemistry or process parameters to reduce aggregation.

3. Improvements to Vaccine Formulations

a) Investigate synergy between TLR agonists. Evidence of synergy between TLR agonists has been reported in the literature (Napolitani 2005, Gautier 2005, Theiner 2008). The microparticle delivery system provides an excellent platform to explore various combinations of TLR agonist delivered simultaneously with protein antigen. A systematic evaluation of combinations of agonists for TLRs 3, 4, 7, and 9 could be performed using in vitro DC-splenocyte cross-priming and in vivo T cell proliferation models.

b) Optimize dosage of antigen and adjuvant. In vitro and in vivo results have suggested that high doses of microparticle-formulated antigen/adjuvant are not optimal. Various dosages should be explored to achieve the highest CTL response, and also to determine if the benefits of dose-sparing can be realized. Furthermore, experiments should be conducted with different ratios of antigen and adjuvant to optimize the dosages.

c) Delivery of siRNA for IL-10. The hydrophobic ion paring technique has been used to encapsulate siRNA in polyketal microparticles, and TNFα siRNA particles enhance the

suppression of TNF-α in RAW264.7 cells (Sungmun Lee, manuscript in preparation). We have also been interested in suppressing IL-10 for vaccine applications (Igietseme 2000, Liu 2004). Preliminary experiments, however, suggest that siRNA-mediated IL-10 suppression may be difficult to ascertain due to the non-specific stimulation induced by dsRNA through TLR3 or MDA-5. As an alternative, IL-12 expression can be measured, to determine if IL-10 siRNA is more effective at increasing IL-12 production than scrambled siRNA or other forms of dsRNA, such as poly(I:C).

APPENDIX A

MATERIALS AND SUPPLIES

Poly(riboinosinic acid)-poly(ribocytidylic acid) (Poly(I:C))	Amersham Biosciences (GE Healthcare)	Piscataway, NJ
1,2-dioleoyl-3-trimethylammonium-propane (DOTAP)	Avanti Polar Lipids, Inc.	Alabaster, AL
H-Lysine(Z)-OH	Bachem	Torrance, CA
Antibodies (BD Pharmingen) Fc block (Anti CD16/32, 2.4G2)	BD Biosciences	San Jose, CA
BD Cytofix/Cytoperm™ fixation/permeabilization solution	BD Biosciences	San Jose, CA
BD Cytofix™ fixation buffer	BD Biosciences	San Jose, CA
BD-Falcon™ round-bottom tube	BD Biosciences	San Jose, CA
Agarose Gel	Bio-Rad Laboratories	Hercules, CA
Ready Gel, 4-15% polyacrylamide gel, Tris-HCl, 50 μL, 10 wells	Bio-Rad Laboratories	Hercules, CA
Ethidium bromide	Bio-Rad Laboratories	Hercules, CA
C57B16 mice	Charles River Laboratories, Inc.	Wilmington, MA
Costar® 96-well, round bottom cell culture plates	Corning, Inc.	Corning, NY
Ovalbumin Class I SIINFEKL peptide	Emory University, Microchemical & Proteomics Core Facility	Atlanta, GA
CpG-DNA Oligonucleotide	Integrated DNA Technologies, Inc.	Coralville, IA
IDTE pH 7.5 buffer (10 mM Tris, 0.1 mM EDTA)	Integrated DNA Technologies, Inc.	Coralville, IA

Fluorescein-5-isothiocyanate (FITC 'Isomer I')	Invitrogen	Carlsbad, CA
OliGreen dye	Invitrogen	Carlsbad, CA
SYBR Green I dye	Invitrogen	Carlsbad, CA
Anti-CD11c magnetic beads	Miltenyi Biotech	Auburn, CA
MACS cell sorter	Miltenyi Biotech	Auburn, CA
α-methoxy-ω-amino-poly(ethylene glycol), MW 5000	Nektar Therapeutics	Huntsville, AL
N-Succinimidyl 3-(2-pyridyldithio)-propionate (SPDP)	Pierce Protein Reseach Products (Thermo Fisher Scientific)	Rockford, IL
Succinimidyl 6-(3-[2-pyridyldithio]-propionamido)hexanoate (LC-SPDP)	Pierce Protein Reseach Products (Thermo Fisher Scientific)	Rockford, IL
Polystyrene standards	Polymer Laboratories, Inc.	Amherst, MA
CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS reagent)	Promega	Madison, WI
1,4-Benzenedimethanol	Sigma-Aldrich	St. Louis, MO
1,4-Cyclohexanedimethanol	Sigma-Aldrich	St. Louis, MO
1,5-Pentanediol	Sigma-Aldrich	St. Louis, MO
2,2-Dimethoxypropane	Sigma-Aldrich	St. Louis, MO
2,2-Dithiopyridine (Aldrithiol)	Sigma-Aldrich	St. Louis, MO
3,6-Dioxa-1,8-octanedithiol	Sigma-Aldrich	St. Louis, MO

Acryloyl chloride	Sigma-Aldrich	St. Louis, MO
Albumin from chicken egg white (A5503)	Sigma-Aldrich	St. Louis, MO
Dithiothreitol	Sigma-Aldrich	St. Louis, MO
Docusate Sodium	Sigma-Aldrich	St. Louis, MO
Fluorescamine	Sigma-Aldrich	St. Louis, MO
Mercaptoethanol	Sigma-Aldrich	St. Louis, MO
Poly(vinyl alcohol)	Sigma-Aldrich	St. Louis, MO
p-Toluenesulfonic acid	Sigma-Aldrich	St. Louis, MO
Triphosgene	Sigma-Aldrich	St. Louis, MO
(5-carboxyfluorescein)-CGSIINFEKLGCG	SynPep	Dublin, CA
C57BL/6-Tg (OT-1) RAG<tm1Mom> mouse	Taconic Farms, Inc.	Hudson, NY

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VITA

Michael John Heffernan was born in Livonia, Michigan, on October 16, 1968 to James and Linda Heffernan. He and his older brothers Paul and Mark enjoyed hockey, skiing, sailing, and traveling with the family during the childhood years. The family moved to North Carolina in 1980, and Mike began playing the piano and running track and cross-country. Mike graduated from Chapel Hill High School in 1986 and attended Duke University on a mathematics scholarship from the state of North Carolina. He continued running varsity track and cross-country at Duke, and obtained his B.S. degree in mechanical engineering in 1990. Mike joined an engineering firm in Washington, DC, where he met his future wife, Kasey. Mike and Kasey were married in 1994. Kasey and Mike completed their M.S. degrees in mechanical engineering at Virginia Tech in 1994/1995, and then moved to Columbus, Indiana to work for Cummins Engine Co., and later relocated to Rocky Mount, NC. During this time they were blessed with three boys: Luke, Nathan, and Sean. Watching the miracle of their children grow and develop inspired an interest in biology and medicine, which led Mike to the field of biomedical engineering. With the support of his dear wife Kasey, he began the Ph.D. program at Georgia Tech in 2003. In Atlanta, Mike and Kasey joined St. Andrew Catholic Church in Roswell and became involved in several church ministries. They have watched their children thrive in school, scouting, music, and in the parish environment, and will celebrate fourteen wonderful years of marriage this summer. After graduation, Mike will work as a post-doctoral fellow at the National Cancer Institute in Bethesda, Maryland.