Development of Immunosuppressant and Peptide Loaded Microparticles as Tolerogenic Vaccines for Treatment of Autoimmune Diseases

DISSertation

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By

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Abstract

Multiple Sclerosis (MS) is an immune-mediated demyelinating disorder of the central nervous system (CNS) and most current treatments for the disease dampen the overall immune system. An antigen-specific T-regulatory (T-reg) population that would suppress the autoimmune signaling could treat MS. To this end a microparticle loaded with dexamethasone and autoimmune antigen that can passively target dendritic cells (DCs) was developed. To formulate the microparticle, a recently described polymer acetalated dextran (Ac-DEX) was used. Ac-DEX is a biocompatible biopolymer which undergoes tunable burst degradation at the acidic conditions that can be present in the phagosome after phagocytosis (pH 5) but slower degradation at extracellular conditions (pH 7.4), thereby making it an ideal candidate for immune applications. Dexamethasone-encapsulated Ac-DEX microparticles exhibited a dose sparing response when cultured with macrophages and bone marrow derived dendritic cells in vitro. Furthermore, a significant reduction in overall experimental autoimmune encephalomyelitis (EAE) score was observed in vivo with prophylactic administration of dexamethasone and peptide loaded Ac-DEX particles. Overall, this system displays good progress towards development of a tolerogenic vaccine for MS.

To optimize microparticle preparation for better drug encapsulation, particles size and degradation rates, studies were done with rapamycin (immunosuppressant) loaded
Ac-DEX microparticles using various molecular weights of dextran. Optimized microparticles were determined by varying the chemical and physical parameters during particle synthesis. Results showed that rapamycin-loaded microparticles prepared with 71k molecular weight Ac-DEX had higher encapsulation efficiency and slower overall degradation than microparticles synthesized from 10k Ac-DEX. These particles will be advantageous for future applications in immune suppression therapies.

Finally studies were carried out to investigate the stability of enzyme-loaded Ac-DEX particles over prolonged periods under various storage conditions. Horseradish peroxidase (HRP) was selected as a model enzyme for this study. Particles were prepared by sonication or homogenization techniques and were stored at different temperatures (-20°C, 4 °C, 25 °C, and 45 °C) over a period of 3 months. Irrespective of the preparation method, temperature, and storage time, all the particles were spherically shaped with no signs of degradation. However, in terms of retaining the enzyme activity, homogenized particles showed better shielding of HRP than probe sonicated particles at 25 °C or 45 °C. Overall Ac-DEX particles provided better stabilization of model enzyme as compared to the free enzyme, and can be advantageous in loading drugs or enzymes that need to be stored or protected at temperatures outside the cold chain.
Dedication

This document is dedicated to Almighty, my family, and to the committee members
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Publications


**Fields of Study**

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CHAPTER 1

INTRODUCTION

According to American Heritage Medical Dictionary, immunological tolerance is described as “acquired specific failure of the immunological mechanism to respond to a given antigen, induced by exposure to the antigen” (Anonymous 2007a). This tolerance can either be directed towards natural antigens, so as to prevent the attack of the immune system on the body’s own proteins or to external antigens such as during organ transplantation. Any disregulation of tolerogenic processes such as failure to identify “self” from “non-self” could result in autoimmune diseases such as multiple sclerosis, type 1 diabetes, rheumatoid arthritis, leukemia, or allergies (Bluestone and Tang 2004). According to the National Institute of Health (NIH), more than 80 types of autoimmune diseases have been identified and around 23.5 million Americans suffer from these diseases (Anonymous 2011b).
1.1 Multiple Sclerosis and the Role of Immune Cells

Multiple sclerosis (MS) is the most common immune mediated debilitating central nervous system (CNS) disorder. According to the National Multiple Sclerosis Society, more than 2.5 million people worldwide and approximately 400,000 Americans suffer from MS (Anonymous 2011a). Its occurrence is more prominent among women and Caucasians when compared to men or other ethnicities, respectively (Brodkey, et al. 2011). The common symptoms of MS are muscle weakness in the face, impairment of gait, eye disorders, improper function of bladder and bowel, depression, and having numbness or a tingling sensation (Brodkey, et al. 2011). MS occurs between the age groups of 10 to 60 with a higher incidence between ages 20 and 40. Probable reasons for the variation in occurrence of the disease might be associated with genetic, environmental factors (virus or bacteria), or due to autoimmunity (Rejdak, et al. 2010). There are no specific tests to confirm MS diagnosis. However, it can be identified through symptoms, MRI scans of brain and spinal cord, and through other neurological findings.

Although the etiology of MS has not been clearly established, histopathological studies have shown an aggressive immune attack on the myelin sheath and other components of the CNS by immune cells resulting in inflammation and demyelination of the CNS (Frohman, et al. 2006). Lipoproteinaceous myelin is a dielectric material that surrounds the axon of neurons. It helps in the transmission of signal from one neuron to another and is essential for proper functioning of the nervous system. Demyelination abruptly severs the signal transmission between the brain and other parts of the CNS, causing loss of vision, loss of hearing, and weakness of arms and legs. Major immune...
cells that play a role in demyelination are antigen-presenting cells (dendritic cells, macrophages) and lymphocytes (T and B cells). Apart from demyelination, MS can be hallmarked with the breakdown of the blood brain barrier (BBB), perivascular inflammation, and loss of axons (Lucchinetti, et al. 2000).

1.2 Dendritic and T Cells

Steinman and his colleagues first identified dendritic cells (DCs) (Steinman and Cohn 1973). DCs are a heterogenous collection of cells derived from the bone marrow and are extensively studied as targets for allo- and autoreactive T-cell responses (Steinman, et al. 2003). Precursors of DCs that have originated in the bone marrow, circulate through blood into non-lymphoid tissues and differentiate into immature DCs (Hart 1997)(Cella, et al. 1997). Immature DCs are characterized by high levels of major histocompatibility complex (MHC) products and a higher capacity to engulf antigens (Satthaporn and Eremin 2001). Immature DCs respond when encountering an antigen, and transform into mature DCs. Immature DCs can uptake antigen either by phagocytosis, macropinocytosis, or receptor mediated endocytosis (Sallusto, et al. 1995)(Seferian and Martinez 2000)(Caux, et al. 1992). After internalization, the antigen can be processed to peptides by the endosomal pathway for exogenous antigens or by the proteosmal pathway for endogenous antigens (Hackstein and Thomson 2004). These peptides can then be presented on the surface of antigen presenting cells (APC) on MHC I or II. For MHC I presentation which can result in stimulation of CD8+ T cells, the antigens can enter into the cytosol and become degraded by proteasomes before entering
the endoplasmic reticulum (ER). Inside the ER, the peptides can bind to MHC I and move to the surface of the cell for presentation (Lennon-Dumenil, et al. 2002). For MHC II presentation, which can stimulate helper CD4+ T cells, proteolysis of an antigen or protein can occur in the endosome, binds to MHC II in vesicles, and transfer to the surface of the cell (Hackstein and Thomson 2004). Mature DCs are rich in major histocompatibility complex (MHC), proinflammatory cytokines such as tumor-necrosis factor alpha (TNF-α), and costimulatory molecules (eg., CD40 ligand). Mature DCs migrate to secondary lymphoid organs and activate naïve CD4+ and CD8+ T cells. These T cells play a major role in both humoral and cell-mediated immunity (Anonymous 2011b). Graphical depiction of lifecycle of DC is shown in Figure 1.1 (Hackstein and Thomson 2004).

The migration of DCs from bone marrow to non-lymphoid tissues and subsequently to the lymphoid tissues can occur due to chemokines specific for DCs. For example, CC chemokine receptors (CCR) such as CCR1, CCR2, and CCR5 are present on immature DCs and respond to corresponding chemokines (CCL1, CCL2 and CCL5) present in non-lymphoid tissue. Upon maturation, DCs can downregulate the expression of these receptors and upregulate CCR7. This will bind to CCL19 and CCL 21 which can guide the DCs towards lymphoid tissues (Dieu, et al. 1998; Hackstein and Thomson 2004).

Activation of CD4+ and CD8+ T cells occurs with the interaction between the MHC complex on the DC and the T cell receptor, followed by co-stimulation between CD28 on the T cells with CD80 and CD86 expressed by DCs. Both CD4+ and CD8+ T
cells play a critical role in function of the immune system. Upon activation the presence of a specific cytokine signals, helper CD4+ T cells further differentiate into memory helper T cells (Th1, Th17, Th2, iTreg). The different classifications of helper T cells are based on their characteristic cytokine production and their function as shown in Figure 1.2 (Zhu, et al. 2010). Mosmann and Coffman were the first to identify different populations of CD4+ T cells, followed by Bottomly and her colleagues (Mosmann, et al. 2005) (Killar, et al. 1987). Naïve CD4+ T cells differentiate into Th1 in the presence of interleukin-12 (IL-12), and secrete interferon-gamma (IFN-γ) and TNF-α as their signature cytokine milieu. On stimulation Th2 cells secrete inflammatory cytokines IL-4, IL-5 and IL-13. It was assumed for more than a decade that only Th1 and Th2 exist, but in 2003 another classification of CD4+ T cell, Th17, was identified (Zhu, et al. 2010). In the presence of transforming growth factor β1 (TGF-β1) and proinflammatory cytokines IL-6 and IL-21, CD4+ T cells differentiate into Th17 cells and secrete IL-17 as their signature cytokine. Th1 and Th17 participate in cell-mediated immunity whereas Th2 cells are involved in humoral immunity. Contrary to Th1, Th2, and Th17 generation, in the presence of IL-10 and TGF-β1, CD4+ T cells can differentiate into inducible Tregs (iTregs). Immature tolerogenic DCs present self antigen to T cells, but fail to provide the required costimulatory signals for T cell activation and proliferation, resulting in T-cell anergy, T-cell death, or regulatory T-cell generation (Abbas, et al. 2004). Tolerogenic DCs are immature in nature and are characterized by their inability to activate inflammatory T cells, express low levels of co-stimulation to inhibitory signals, and induce formation of iT-reg (Adorini and Penna 2009).
Autoimmune diseases such as MS can occur when T cells act on self-antigens presented by DCs or other APCs, resulting in an attack on other cells of the body, partially due to the failure of regulatory T cells. MS can be caused by infiltration of T cells such as those showing Th1 and Th17 phenotype into the CNS and secretion of inflammatory cytokines and chemokines such as IL-12, IFN-γ, and IL-17 (Langrish, et al. 2005). Th1 and Th17 T cell phenotypes are the most prominent in initiating the disease when compared to other T cells (Langrish, et al. 2005). In addition, secretion of inflammatory cytokines and chemokines further attract other inflammatory cells into the CNS, exacerbating the disease (Brown 2006). The action of cytokines and direct cellular damage can cause myelin sheath degradation and disruption of nerve impulse conduction which can result in axonal loss, neuro-degeneration, and irreversible disability.

1.3 Treatment of Multiple Sclerosis

Present treatments available in market treat MS by decreasing the Th1 and Th17 cellular response. These include treatments with first line therapies consisting of Interferon-β (IFN-β) and glatiramer acetate (GA). Ninety seven percent of current MS treatments involve treatment with IFN-β (Avonex®, Rebif® and Betaseron®) and GA (Pan, D). IFN-β has been shown to lower the production of Th1 differentiation cytokine, IL-12, and increased production of anti-inflammatory cytokine, IL-10, in bone marrow derived dendritic cells (BMDCs) (Nakatsuji, et al. 2007). A greater than 80% reduction in inflammatory lesions has been shown in patients treated with IFN- β (Goodin, et al. 2006).
In spite of clinical benefits shown by IFN-β, long-term administration is associated with severe side effects such as hepatotoxicity, anemia, thrombocytopenia, and flu-like symptoms (Graber, et al. 2010). Also, patients can eventually develop antibodies against IFN-β. GA is one non-interferon drug that entered the market for MS treatment. It acts by mimicking myelin basic protein (component of myelin) and by promoting production of anti-inflammatory cytokines. However, administration of GA can cause a severe post-injection systemic reaction characterized by chest tightness (Vosoughi and Freedman 2010). The remaining three percent of the market is covered by treatment with a second line of therapies such as Mitoxatrone® (immunosuppressant), Natalizumab® (monoclonal antibodies) and glucocorticoids. Mitoxantrone® and Natalizumab® suppress the proliferation of T and B cells by decreasing proinflammatory cytokine production (Niino and Sasaki 2010). Usually, these are prescribed for more aggressive forms of the disease. Long term treatment with Mitoxantrone® causes severe adverse effects such as cardiac toxicity, acute leukemia, and infertility (Ellis and Boggild 2009). Adverse effects associated with use of Natalizumab® are multifocal leukoencephalopathy, a fatal viral infection of the CNS (Anonymous 2011a). Glucocorticoids are most frequently used and are given to treat acute exacerbations and first acute events of MS. They act by decreasing Th17 cells (Hackstein and Thomson 2004; Liu, et al. 2009). Long term treatment with glucocorticoids results in osteoporosis, high blood pressure, cataract formation, decreased wound healing and opportunistic infections (Habermann, et al. 2008). All of these aforementioned treatments can decrease the symptoms of MS, but do not provide a cure and can dampen the overall immune
system, resulting in the potential for severe side effects. Hence, new treatments are necessary to treat MS.

An ideal therapy for MS would be either to prevent the initiation of the inflammation process or to halt its progress. One possible way to treat MS is to induce an antigen specific T-regulatory (T-reg) population. A subset population of T cells, T-reg, expressing CD25+ and FOXP3+, play a major role in maintaining the equilibrium between immunity and tolerance (Bluestone and Tang 2004). T-reg act by maintaining immunological tolerance to self-antigens and can decrease the pathogenesis of MS by suppressing the inflammatory response of effector T cells (Costantino, et al. 2008). T-reg act by producing immunosuppressive agents such as transforming growth factor beta (TGF-β) and interleukin-10 (IL-10) (Thomson 2010). Stern et al. has shown suppression of experimental autoimmune encephalomyelitis (EAE, animal model of MS) in mice with IL-10 secreting regulatory T cell lines were administered (non antigen specific) (Stern, et al. 2008). T-reg act by secreting high amounts of IL-10, which also inhibit Th1 and Th17 responses (McKinstry, et al. 2009). To help generate T regs, development of a tolerogenic vaccine containing a glucocorticoid or immunosuppressant and an autoimmune antigen was hypothesized. Dendritic cells, upon exposure to anti-inflammatory agents or immunosuppressive agents, can become tolerogenic. These agents act by affecting DC differentiation and function by different mechanisms (Morelli and Thomson 2007). For example, Vitamin D3 and corticosteroids act by suppressing DC differentiation and maturation. Rapamycin, an immunosuppressant, acts by suppressing antigen uptake and maturation, whereas sanglifehrin, a new cyclophilin-binding
immunosuppressant, acts by blocking the production of bioactive IL-12. A graphical representation of various drug-related effects on DCs is shown in Figure 1.3 (Morelli and Thomson 2007).

A tolerogenic DC when triggered with an antigenic protein or peptide interacts with naïve T cell to induce formation of antigen specific inducible T-reg (iT-reg). Formation of iT-reg can hinder the inflammatory action of Th1 or Th17 cells (O’Garra and Murphy 2009)(McKinstry, et al. 2009) and also has been shown to suppress EAE in rats and mice (Schif-Zuck, et al. 2006)(Stern, et al. 2008). Researchers are currently investigating the use of ex vivo techniques to generate tolerogenic DCs. In ex vivo methods, DCs are removed from patients, made tolerogenic by exposing them to tolerogenic agents, and reinjected into the body. Although promising results were obtained, carrying out this process is laborious, expensive, and time consuming (Mende and Engleman 2007) (Van der Walt, et al. 2010). Targeting of DC’s in vivo could help to avoid ex vivo manipulation which could shift the current methods for MS treatment.

1.4 Particulate Carriers

A novel approach to treat MS is to target DC’s with tolerogenic vaccines in vivo. However, one of the major problems associated with current vaccines and immunotherapy is the ability to deliver the drug/antigen to the target organ/cell and trigger a specific immune response (Kwon, et al. 2005). It would be important if a vaccine was able to deliver the tolerizing drug and antigen to DCs specifically and induce the required T cell response. By exploiting the fact that DCs can phagocytize particulate matter above 200 nm (Reddy, et al. 2006a), it is possible to passively target DCs.
Particles less than 100 nm are ingested by most cells through pinocytosis or receptor-mediated endocytosis. However, antigen presenting cells such as DCs and macrophages can uptake particles that are up to several microns in diameter (Manolova, et al. 2008). Research showed that when particles ranging from 1000 to 2000 nm were injected in vivo; DCs (CD11c+CD11b− or CD11c+CD11b+) phagocytized the particles 50 times more effectively than the macrophages (CD11c−CD11b+ cells). Additionally, results indicated that large particles are predominately transported by DCs from the injection site to the lymph node; whereas smaller particles are cleared by resident macrophages (Manolova, et al. 2008). Immature DCs internalize exogenous particulate matter such as nano- and microparticles through receptor-mediated endocytosis or phagocytosis. Particulate matter loaded in this manner is internalized and released into DC in a controlled manner.

A potential way to passively target APCs is by encapsulating the active ingredient or antigens into particulate matter. Although the study of particulate materials (either natural or synthetic) for vaccines started over 20 years ago, it has been only recently that these carriers were studied to release tolerizing agents in a controlled manner (O'Hagan and Valiante 2003). Naturally obtained materials are safe and can be metabolized by biological systems through established pathways. However the major disadvantage of using materials of natural origin is their non-reproducibility and batch-to-batch variation in production (Angelova and Hunkeler 1999; Langer, et al. 1997). With synthetic materials, physio-chemical properties such as degradation, molecular weight and solubility in water can be altered to meet specific applications. Nevertheless, they might
not possess biological recognition and may have compatibility issues (Angelova and Hunkeler 1999; Langer, et al. 1997). Passive targeting does not require addition of targeting moieties to the particle surface for APCs uptake of the particles when administered intravenously or by other routes. Whereas active targeting can be achieved by modifying the surface of the particle with targeting entities such as antibodies, adhesion molecules, and antigens (Steeber and Tedder 2000)(Simone, et al. 2009). Hence active or passive targeted drug delivery can be achieved using using particulate matter. Additionally, use of particulate materials to deliver the drug not only increases specific targeting to DCs but may also decrease the side effects associated with the systemic toxicity of the drug.

Commonly used biomaterials for drug delivery are liposomes, degradable polymers such as polyesters [poly(lactic acid), poly(glycolic acid), and their copolymers], polyorthoesters, polyanhydrides and polycarbonates.

1.4.1 Liposomes

Liposomes are artificial vesicles made up of phospholipids with mixed lipid chains (Figure 1.4A). They were first described by Dr. Alec Bangham in 1961 and can encapsulate both hydrophilic and hydrophobic antigens and molecules. Loading of antigens or drugs into liposomes can help in decreasing systemic toxicity and can prevent the drug degradation in the body. Another advantage of using liposomes is that they can pass through the blood brain barrier (BBB) and can target dendritic cells. The work of Copland et al. has shown that DCs internalize liposomes through receptor-mediated
endocytosis and present them to T cells more effectively than free antigens (Copland, et al. 2003). DCs can also uptake liposomes by phagocytosis (White, et al. 2006). Metal-chelating linkages with tagged antibodies, incorporating protein A with attached receptor specific antibodies have been employed to specifically target DCs using liposomes (Reddy, et al. 2006b). Despite the success obtained with liposomal delivery to DCs, liposomes have their own share of drawbacks. Preparations of liposomes are tedious to prepare, expensive to develop, difficult to scale up; and have sterility issues (Hamdy, et al. 2011)(Lasic and Papahadjopoulos 1998). Furthermore, small sized liposomes experience high shear forces during formation which might deteriorate the protein of interest (Lasic and Papahadjopoulos 1998).

1.4.2 Polyester particles

Polymers linked by ester groups constitute polyesters. Among different varieties of polyesters, poly(lactic-co-glycolic acid) (PLGA) is a widely used drug delivery vehicle with US Food and Drug Administration (FDA) approval. PLGA is a copolymer obtained from lactic acid and glycolic acid linked together by an ester bond (Figure 1.4B). It is biodegradable, biocompatible, and is used to produce a variety of biomedical devices such as sutures, grafts, and implants. Similar to liposomes, PLGA can encapsulate both hydrophilic and hydrophobic molecules or antigens, protect them from proteolytic degradation, and facilitate delivery to DCs in a targeted and controlled manner. However, unlike liposomes, PLGA can alter degradation rates by changing the relative concentrations of lactic acid to glycolic acid (Hamdy, et al. 2011). Lactic acid is more
hydrophobic than glycolic acid and increased hydrophobicity can be achieved with higher lactic acid content (Hamdy, et al. 2011).

Several research groups have shown the uptake of PLGA particles by human or mouse DCs (Kempf, et al. 2003)(Lutsiak, et al. 2002)(Elamanchili, et al. 2004)(Waeckerle-Men and Groettrup 2005). Within 24h of incubation more than 90% of PLGA particles were taken up by mouse bone marrow-derived DCs (Elamanchili, et al. 2004) and human peripheral blood-monocytes-derived DCs (Lutsiak, et al. 2002). Increased targeting of PLGA particles to DCs can be achieved by conjugating PLGA particles with anti DEC-205, anti-CD11c antibodies, or by decorating the surface of the particle with mannose (Hamdy, et al. 2011). Also, by tailoring the degradation rates of PLGA, less frequent doses can be used in the treatment regimen (Kazzaz, et al. 2006).

For targeting DCs using PLGA vaccines, nanoparticles given by intradermal injection are preferred over microparticles given intraperitoneally. Newman et al. has shown higher uptake of nanoparticles by dendritic cells with intradermal injection compared to macrophages which show higher uptake with intraperitoneal injection (Newman, et al. 2002). Similarly Cruz et al., show comparison between micro- and nano-particles of PLGA with regards to uptake by DCs. Nanoparticulate formulations of PLGA were shown to specifically target DCs when compared to microparticles. Other immune cells such as macrophages engulfed most of the microparticles (Cruz, et al. 2010).

Overall, profound research has been done to test whether PLGA is a good vehicle to deliver molecules or antigens to DCs. However, one of the major drawbacks associated with these polymers is the time taken for degradation. The degradation rates of PLGA
vary from weeks to a month depending upon the lactic acid content (Hamdy, et al. 2011)(Lu, et al. 2000)(Miller, et al. 1977). Hydrolysis of the ester bond in PLGA results in creation of an acidic microenvironment, which may alter stability of recombinant protein drugs (Wang, et al. 2004). Moreover, upregulation of CD80, CD86, and CD40 were observed when targeted with blank PLGA particles resulting in maturation of DCs (Cruz, et al. 2010). Hence PLGA might not be a good vehicle to deliver tolerogenic drugs/molecules to DCs.

1.4.3 Acid degradable polymers

An ideal polymer particle for targeting DCs should be easy to make, degradable by hydrolysis, nontoxic to cells and upon degradation should not create an acidic environment. Additionally, the polymer should have high drug/antigen/protein loading capacity (Wang, et al. 2004). Widely used drug delivery vehicles such as liposomes and polyesters are not able to satisfy these required parameters. Additionally it would be ideal to have a polymer particle for targeting APCs that can degrade rapidly in acidic conditions inside DCs phagosome. Unlike other cells, APCs can engulf polymer microspheres of 1-10 μm in diameter (Wang, et al. 2004). These engulfed particles move into the phagosome where the pH changes from 7.4 to 5. For this purpose, acid degradable particles have been synthesized. These particles are stable at pH 7.4 but degrade more readily in slightly acidic conditions, releasing the drug into the phagosome or in some instances, the cytoplasm. To satisfy all the above properties and to target DCs, new acid degradable polymers, such as poly(ortho ester), encrypted polymers,
polyurethanes, acetalated dextran, and benzylidene acetal crosslinker have been synthesized and studied for these applications.

A. Poly(ortho esters) (POEs)

POEs were synthesized by condensation copolymerization of dikete acetals and diols (Wang, et al. 2004). At high concentration of 1 mg/ml, POEs were shown to be nontoxic and have rapid release of DNA at pH 5 when compared to 7.4. Gel electrophoresis results showed stable DNA conformation within POEs microspheres, when exposed to acidic conditions. Also, stimulation of T cells was also significantly higher with POE microspheres when compared to PLGA particles (Wang, et al. 2004). However, a major drawback with this polymer is that upon hydrolysis, it produces monomeric diols and small amounts of acid, which might be toxic to the encapsulated material.

B. Encrypted polymer

Murthy et al. developed an encrypted polymer consisting of a membrane disruptive backbone with acid degradable acetal linkages. The polymer is masked with polyethylene glycol (PEG), which protects the disruptive backbone at physiological pH (Murthy, et al. 2003). At lower pH, acetal groups degrade exposing the membrane’s disruptive backbones, releasing the encapsulated drug. Hydrolysis experiments in phosphate buffer at pH 5.4 and 7.4 have shown that more than 50% of the polymer degraded in acidic conditions compared to neutral (<3%). However, there is currently no data showing the physical and chemical characterization of the encrypted polymer.
C. Acrylamide/polyurethanes/acetalated dextran

The Frechet group at the University of California, Berkeley carried out intense research in the development of acid degradable polymers and resulting particles. By co-polymerizing acid degradable crosslinker with acrylamide, acid-sensitive loaded particles were synthesized. These particles showed pH-sensitive release and increased T cell activation when compared to other non-degradable polymers. To specifically target the DCs, acid-degradable particles were conjugated with anti-DEC-205 monoclonal antibodies (mAbs). Unlike macrophages, which express mannose and Fc receptors, DCs and thymic endothelial cells express the endocytosis receptor, DEC-205 (Kim, et al. 2002). Both mature and immature DCs were targeted with anti-DEC-205 mAbs coated particles. Results showed higher uptake of these particles by draining inguinal lymph nodes, indicating efficient uptake of particles by DCs. However, upon degradation, these particles release non-biodegradable linear polyacrylamide. Moreover, the use of acrylamide to synthesize particles may cause toxicity and biocompatibility issues (Cohen, et al. 2009). Similar toxicity problems might exist for polymers such as poly(β-amino) ester, poly(ketal), and poly(acetal) (Cohen, et al. 2009). In order to overcome these drawbacks, the same lab developed polyurethanes. Libraries of polymers were prepared from polyureas and polyurethanes with varying degrees of hydrophobicity (Paramonov, et al. 2008). These polymers contain dimethyl acetyl moieties, which upon hydrolysis yield small molecule diols and acetone (Cohen, et al. 2009). Unlike acrylamide particles, polyurethane particles degrade to produce small molecule diols and acetone.
Another kind of polymer generated was acetalated dextran (Ac-DEX, Figure 1.4C). Dextran is a homopolysaccharide of glucose (Naessens, et al. 2005). Under mild acidic conditions, addition of 2-methoxypropene to dextran results in formation of acetalated dextran, containing both cyclic and acyclic acetals (Bachelder, et al. 2008). During this process, the hydroxyl groups of dextran are converted to acetal groups, rendering it insoluble in water. Due to the acetal groups, Ac-DEX degrades much faster in acidic environment compared to the physiological (Beaudette, et al. 2009)(Bachelder, et al. 2008). Accelerated degradation of particles at lower pH causes the triggered release of encapsulated agents in the phagosomal compartments of phagocytes upon uptake. This pH-sensitivity allows for a significant increase to both CD8+ and CD4+ T cells (Broaders, et al. 2009; Cohen, et al. 2009). 

Broaders et al., 2009 investigated ovalbumin loaded particles made from Ac-DEX polymers with different degradation half-lives (at pH 5.0). Polymer degradation rates has significantly affected the MHC I presentation efficiency and faster degrading polymers generally lead to better MHC I presentation. Additionally, comparision of Ac-DEX (t1/2=1.7 h) with other materials such as cross-linked acrylamide (PA), PLGA, and iron oxide for their ability to elicit MHC I presentation was performed. Degradation of Ac-DEX (t1/2=1.7 h) and PA (t1/2~2 h) particles performed an order of magnitude better when compared to PLGA and iron oxide. In contrast to the above-mentioned polymers, Ac-DEX has tunable degradation rates due to the formation of both cyclic and acyclic acetal groups with significantly different rates of hydrolysis (Broaders, et al. 2009). The different degradation rates of the materials can aid in precise and sustained release of tolerizing agents into DCs.
Ac-DEX is more biocompatible than the previous discussed materials because upon degradation, Ac-DEX forms dextran, acetone and very small amounts of alcohol. Dextran is a FDA approved plasma expander, acetone is a nontoxic metabolic intermediate, and methanol is non-toxic in small quantities (Paine and Davan 2001). Unlike PLGA, degradation of Ac-DEX does not lead to the formation of acidic byproducts which may degrade the encapsulated protein. Furthermore, it may encapsulate both hydrophobic and hydrophilic molecules.

Using Ac-DEX, nano/microparticles can be fabricated for immune applications. These particles can be phagocytized by DCs to induce formation of tolerogenic DC to induce iT-reg formation. This system has the potential of dampening the immune response in an antigen specific manner potentially overcoming the barrier of systemic immune suppression.
1.5 OBJECTIVES OF DISSERTATION

The first aim of this dissertation was to induce an antigen specific T reg population by preparing microparticles containing glucocorticoid and autoimmune antigen encapsulated within Ac-DEX. The formed particles were targeted to immature dendritic cells which in turn activate naïve CD4+ T cells to promote the formation of antigen specific iT regs (chapter 2).

Second aim was to optimize physical and chemical parameters of emulsion process for better encapsulation efficiency, degradation and yield with rapamycin loaded Ac-DEX particles (Chapter 3). Finally studies were carried out to determine the physiochemical behavior of both polymer and encapsulated ingredient over time at different storage conditions (Chapter 4).
Figure 1.1: Various check points of DCs immunology. Immature dendritic cells develop from hematopoietic stem cells (HSC). Upon processing of antigen, it matures. Mature DCs, rich in major histocompatibility complex (MHC), proinflammatory cytokines, and co-stimulatory molecules, migrate to secondary lymphoid organs and activate naïve CD4+ and CD8+ T cells (Hackstein and Thomson 2004).
Figure 1.2: Different subsets of CD4+ T cells. Descents of helper T cells are classified based on the cytokine secretion and function. Th1 and Th17 cells are responsible for cell mediated immunity and iTregs play a major role in tolerance. (Zou and Restifo 2010)
Figure 1.3: A graphical representation of the drug-related effects on DCs. Vitamin D3 and corticosteroids act by suppressing DC differentiation and maturation. Rapamycin, an immunosuppressant, acts by suppressing antigen uptake and maturation, whereas sanglifehrin, a new cyclophilin-binding immunosuppressant, acts by blocking the production of bioactive interleukin-12 (Hackstein and Thomson 2004).
Figure 1.4:
(A) A graphical representation of liposome. Liposomes can encapsulate hydrophobic molecules inbetween the lipid layers and hydrophilic molecules such as DNA in the core. (Torchilin 2005)

(B) Structure of Acetalated Dextran. (Kauffman, et al. 2012)

CHAPTER 2

NOVEL TREATMENT FOR MULTIPLE SCLEROSIS USING
DEXAMETHASONE AND PEPTIDE LOADED ACETALATED DEXTRAN
MICROPARTICLES

2.1 INTRODUCTION

To generate tolerogenic DCs and to promote formation of antigen specific T-reg population, dexamethasone and myelin oligodendrocyte glycoprotein (MOG) peptide were loaded into acetalated dextran (Ac-DEX) microparticles.

Dexamethasone (DXM) is a glucocorticosteroid that is available in the market for treatment of acute exacerbations of MS. The work of Kang et al. showed induction of antigen-specific iT-reg population when non-obese diabetic mice were treated with DXM and antigen (Kang, et al. 2008). Prediabetic mice when treated with DXM and insulin showed increased survivability when compared to controls. Tolerogenic DCs that stimulated an antigen specific inducible T regulatory (iT-reg) population, and suggests the possibility of DXM acting as a “tolerogenic adjuvant”. Similarly work by Barrat et al. showed production of IL-10 by regulatory cells when treated with DXM (Barrat, et al.
Based on extensive literature and its ability to create tolerance in DC, DXM was considered as a potential drug.

The main aim was to create antigen specific T regs by trigerring the immature DC’s and generating naïve CD4+ T with Ac-DEX loaded dexamethasone and autoimmune antigen microparticles. Ac-DEX microparticles loaded with MOG35-55 and DXM were characterized for their physical and chemical properties. These characterized particles were then used to evaluate the therapeutic efficacy of the particle system as well as the mechanism of action, both in vitro and in vivo. For in vitro assessment, studies were done in RAW 264.7 macrophages and BMDCs and for in vivo evaluation; experimental autoimmune encephalomyelitis (EAE), a model for MS was used.

2.2 MATERIALS AND METHODS

2.2.1 Materials

The following materials were obtained from Sigma Aldrich (St. Louis, MO): dextran from *Leuconostoc mesenteroides* (71,400 and 9,000-11,000 MW), pyridinium p-toluenesulfonate (PPTS, 98%), 2-methoxypropene (2-MOP, 97%, stabilized), 3,3′,5,5′-Tetramethylbenzidine (TMB) Liquid Substrate System, tetraethylamine (TEA, ≥ 99%), poly(vinyl alcohol) (PVA, 87-89% hydrolyzed, 13-23k MW), poly(DL-lactide-co-glycolide) (PLGA, 85:15 lactide:glycolide, 50,000–75,000 MW), sodium acetate (SA), Dimethyl sulfoxide (DMSO; anhydrous, 99.9%), RBC lysing buffer (Sigma-Aldrich), deuterium oxide (D₂O, 99.9 atom% D, 0.75 wt% TSP), deuterium chloride (DCl, 35 wt%
solution in D$_2$O, 99 atom% D), and thiazoly blue tetrazolium bromide (MTT). Dexamethasone was obtained from Alfa Aesar and peptides myelin oligodendrocyte glycoprotein and myelin basic protein were obtained from Invitrogen. Glacial acetic acid (ACS grade), anhydrous ethanol, and phosphate buffer (PBS, pH 7.4) were obtained from Thermo Fisher Scientific (USA). Dichloromethane (DCM) was purchased from Honeywell Burdick & Jackson (Muskegon, MI) and Alexis Biochemicals (Enzo Life Sciences, Plymouth Meeting, PA), respectively. All the biomaterials, reagents, solvents purchased from commercial sources and used without further purification. Pertussis toxin was obtained from List Biological Laboratories) and M. tuberculosis H37Ra, CFA from Difco. Myelin oligodendrocyte glycoprotein (MOG35-55) peptide and myelin basic protein (MBP Ac1-11) peptide were obtained from Invitrogen (Green Island, NY). All the antibodies used, Ionomycin, GolgiPlug, Fc-block, Cytofix/Cytoperm, FACSCanto II were bought from BD Biosciences. Sodium Pyruvate, Non Essential Amino Acids, HEPES and beta-mercaptoethanol are obtained from cellgro (Manassas, VA). APC-conjugated anti-FOXP3 was purchased from ebioscience (Sandiego, CA). Basic water was prepared by adding 0.01% of triethylamine to nanopure water (Branstead water filter) to attain a pH of 9.0.

### 2.2.2 Synthesis of Acetalated dextran (Ac-DEX)

Ac-DEX was prepared as described by Broaders et al. but with some modifications (Broaders, et al. 2009). Lyophilized dextran ($M_w = 10,000$ Da) and pyridinium p-toluenesulfonate (0.0617 mmol) were dissolved in anhydrous DMSO for
one hour before reacting with 2-methoxypropene (37 mmol) under nitrogen gas. After six hours, the reaction was quenched with triethylamine (7 mmol) and the modified dextran was precipitated in basic water (pH 9). The modified dextran was collected by vacuum filtration and was repeatedly washed with basic water (100 ml). The resultant product was lyophilized for two days to yield a fluffy white solid. To remove any impurities present in the obtained product, the resultant white solid (≈1.07 g) was again dissolved in 100% ethanol (6 ml), centrifuged at 10000 rpm for 5 minutes (Beckman RA-21, Los Angeles, CA, USA). The supernatant was collected and was reprecipitated in basic water (200 ml, pH 9), vacuum filtered and lyophilized for two days to yield final acetalated dextran (Ac-DEX).

2.2.3 Ac-DEX Nuclear Magnetic Resonance (NMR) Analysis

NMR analysis was performed to determine the relative proportion of cyclic:acyclic acetal substrate of Ac-DEX. It was carried out similarly, as described by Broaders et al. (Broaders, et al. 2009). Ac-DEX was placed in an NMR tube and deuterium oxide (D$_2$O) was added to the tube. The NMR tubes were tilted roughly to 30° from horizontal and deuterium chloride (DCl) was placed inside the rim of the tube. Immediately, the tube was capped and sealed with parafilm before straightening it and allowing the DCl to react with the suspension. Next the tube was vortexed for 1-5 minutes until the solution became clear and the NMR analysis was carried out after two hours using a 300 MHz $^1$H-NMR (Bruker 300 Ultrashield). The hydrolysis of one cyclic acetal produces one acetone molecule and the hydrolysis of one acyclic acetal produces...
one acetone and one methanol molecule. Therefore, from the relative ratio of the peaks from acetone, methanol, and the anomeric carbon on dextan, the cyclic:acyclic ratio of acetal substitution could be determined (Broaders, et al. 2009).

2.2.4 Particle Formation

A. Preparation of Ac-DEX microparticles encapsulating DXM using single emulsion techniques

DXM loaded Ac-DEX microparticles (DXM-Ac-DEX-MPs) were prepared using oil in water emulsion (o/w) techniques similar to the procedure described by Bachelder et al. (Bachelder, et al. 2008). Briefly, DXM at three different concentrations (1, 0.1, 0.01 mg) was dissolved in chloroform: ethanol (95:5) mixture along with Ac-DEX and added to higher volumes of 3% polyvinyl alcohol (PVA), (MW-13,000-23,000 g/mol, 87-89% hydrolyzed) in phosphate buffer solution (PBS) (water phase). The two immiscible solvents were probe sonicated using a Misonix CL5 Sonicator (Branson, Los Angeles, CA, USA), with a 0.5 in. flat tip and with an output setting of 4 on ice for 30 seconds to form a primary emulsion. The formed primary emulsion was then immediately added to a second PVA solution (0.3% w/w in PBS) and stirred for 3 h to evaporate the organic solvent. To recover the microparticles, each formulation was subjected to centrifugation at 10°C for 14 min at a speed of 21000 rpm (Beckman RA-25.5, Los Angeles, CA, USA). The supernatant was discarded, and was washed twice with basic water to remove surface drug from the microparticles. To remove any residual water, the microparticles were
freeze-dried (Labconco, Kansas City, MO, USA) and stored at 4°C until further use. Blank particles were prepared using similar techniques as described above but without the addition of active ingredients or proteins/peptides.

B. Preparation of Ac-DEX microparticles encapsulating Dexamethasone and MOG35-55 antigen

DXM in combinations with autoimmune antigen were encapsulated into Ac-DEX microparticles (DXM-MOG-Ac-DEX-MPs) using a double-emulsion water/oil/water (w/o/w) method. Briefly, 100 μl of 4 mg/ml concentrated MOG35-55 solution in PBS was added to 1 ml (CHCl₃: EtOH; 95:5) of organic solvent containing DXM and Ac-DEX. The resulting mixture was probe sonicated on an ice bath for 30 sec (Misonix CL5 Sonicator 450, with a 0.5 in. flat tip with an output setting of 4 and a duty cycle of 10%) to form a w/o emulsion. To this primary emulsion, 3% PVA solution was added and was probe sonicated again for 30 seconds with the same settings to form a w/o/w emulsion. This results in encapsulation of both peptide and DXM within the Ac-DEX. The formed double emulsion was added to a second PVA solution (40 mL, 0.3% w/w in PBS) and the remaining process carried out was similar to the one described for preparation of single emulsion. Blank particles were prepared using the similar techniques as described above but without the addition of active ingredients or proteins/peptides.
C. Preparation of PLGA loaded DXM particles

Instead of Ac-DEX, poly(lactic-co-glycolic acid) (PLGA, lactide:glycolide 85:15, MW – 50,000-75,000 g/mol) was used to prepare the PLGA loaded DXM microparticles using a single emulsion technique (DXM-PLGA-MPs) using the procedure outlined above. Blank particles were prepared using the similar techniques/method as described above but without the addition of active ingredients or proteins/peptides.

2.2.5 Physical and Chemical Characterization of Particles

A. Scanning Electron Microscopy (SEM)

To study the surface morphology and to understand the shape of the lyophilized particles, samples were imaged using SEM. Particles were suspended at 20 mg/ml concentrations in basic water (pH 9.0) and 20 μl was added to a silicon wafer attached to a pin stub. The samples were allowed to air dry, and then sputter coated with a layer of palladium/gold alloy for 120 seconds before imaging with a FEI NOVA NanoSEM 400.

B. Particle Size Analysis and Zeta Potential

The size distribution of the prepared microparticles were measured using a light scattering size analyzer by suspending the particles in basic water (Brookhaven, 25°C, 90° angle). Lyophilized particles were suspended at 0.05 mg/ml concentration in basic water and were analyzed. Zeta potential distribution was measured using Zeta PALS instrument (Brookhaven Instruments Corp., Worcestershire, NY). Samples were prepared
by diluting 100 μl of 1 mg/ml suspended particles (PBS) in 1.4 ml PBS solution and measurements were reported as the mean of 5 readings.

C. Differential Scanning Calorimetry (DSC)

DSC studies were carried out as described by Rouse et al. (Rouse, et al. 2007). Briefly, lyophilized particles weighing 0.5-1.5 mg were placed in an aluminum pan, which was hermetically sealed with a pinhole in the lid. An empty aluminum pan containing a pinhole acted as a reference. Both the reference and samples pan were heated from 0°C to 450°C at a rate of 10°C/min under dynamic argon atmosphere (HP DSC827, Mettler Toledo, USA). The results were analyzed using Mettler STAR software. DSC scans of blank Ac-DEX particles; lyophilized Ac-DEX, physical mixture of DXM, Ac-DEX and DXM alone were used as controls.

2.2.6 Determination of DXM and MOG35-55 encapsulation

The amount of DXM loaded into polymer was determined using Liquid Chromatography Mass Spectroscopy (LC-MS). The particles (with or without DXM) were dissolved in acetonitrile at 1 mg/ml concentration. To remove any impurities or undissolved polymer the dissolved solution was centrifuged for 5 min at 15 g, followed by filtering through 0.2 μm filters. Samples were diluted with acetonitrile as needed to avoid an excessive concentration. Desoxymethasone was used as an internal standard. The experiment was carried out using a C18 column (C18*2.5um*100*100) coupled with a Beta basic C8 guard column. The LC-MS/MS analysis was carried out on a Thermo
TSQ Quantum triple quadrupole mass spectrometer operated in positive electrospray ionization (ESI) mode. The mobile phase was 50% acetonitrile containing 0.1% formic acid and was used in an isocratic mode at a flow rate of 0.2 mL/min. The total run time of the analysis for the method was 7 min. Dexamethasone and desoxymethasone were quantified using selected ion reaction monitoring. The ion transitions m/z 393→237, m/z 377→339, at the collision energy 25% were used for the determination of DXM and desoxymethasone respectively.

A fluorescamine assay was used to determine peptide content in the microparticles. Particles were suspended at a 1 mg/ml concentration in sodium acetate buffer (0.0188M, pH 5.0) and were incubated at 37°C on a shaker plate at 150 rpm for 72 h. Standards were prepared by adding the MOG35-55 peptide to sodium acetate buffer. After incubating for 72 h, 3 mg/ml of a concentrated fluorescamine solution in acetone was added to the samples or standards in a 96 well plate and was read at 400 nm excitation, and 460 nm emissions via a plate reader (FlexStation 3 Benchtop Multi-Mode Microplate Reader). The fluorescence values obtained from blank particles was subtracted for background correction and served as controls. The final concentration of MOG35-55 loaded into the particles was determined using as standard curve.

The encapsulation efficiency (EE) was then determined by the formula

\[
Encapsulation\ Efficiency = \frac{Actual\ DXM/peptide\ Loading}{Theoretical\ DXM/peptide\ Loading}
\]
2.2.7 In vitro release profile studies

DXM loaded Ac-DEX microparticles at 1 mg/ml concentration were suspended in triplicate in sodium acetate buffer (0.0188M, pH 5.0) and in PBS (pH 7.4). The samples were kept at 37°C on a shaker plate at 150 rpm and at defined time intervals (0-72h); aliquots were withdrawn and centrifuged (15000 x g, 4°C, 5min). The supernatant was collected, freeze-dried and reconstituted in the mobile phase. Using LC-MS, the amount of DXM released was determined. A plot of time versus percent of DXM released at both pH 5.0 and pH 7.4 was generated with blank microparticles serving as a control.

2.2.8 In vitro Cell Culture studies

A. Cell Study Preparation

Macrophages:

Macrophages (RAW 264.7; ATCC, Manassas, VA) were grown and maintained as per the manufacturer’s guidelines. To make the cell media, 50 mL of fetal bovine serum (Hyclone, Pittsburgh, PA) and 5 mL of penicillin-streptomycin (Fischer, Pittsburgh, PA) were added to 500 mL of Dulbecco’s Modified Eagle’s Medium (Fischer, Pittsburgh, PA). Cells were grown in an incubator at 100% relative humidity, 37°C, and 5% CO₂ for the duration of the experiments.
**Dendritic Cells:**

BMDCs were obtained from femur bone of 6 week old BALB/C mice. The cells inside the bone were collected and centrifuged for 5 min at 1500 rpm. After centrifugation, the remaining cells were resuspended in J558L media. BMDCs were plated at concentration of $2 \times 10^6$ cells/mL in each petri dish containing 10 ml of media and incubated at $37^\circ$C containing 5% CO$_2$. On day 6 and 8, the media was removed; the cells were spun down and resuspended in 10 ml of fresh J558L supplemented media. The J558L media was supplemented with FBS (10% v/v), penicillin (100 units/mL), streptomycin (100 μg/mL), L-glutamine (2 mM, GIBCO/BRL), IL-4 (both 5 ng/mL, Peprotech) and granulocyte macrophage colony-stimulating factor (GM-CSF, 20 ng/ml).

**B. Nitrite Analysis**

Greiss reagent was used to assess nitric oxide (NO) production in RAW 264.7 macrophages. Macrophages were plated at concentration of $1 \times 10^5$ cells/mL in a 96 well plate and incubated for 24 hours. After 24 hours, the media in each well was replaced with media containing optimized DXM encapsulated Ac-DEX or PLGA microparticles, blank Ac-DEX/PLGA microparticles, or free dexamethasone at the same concentrations. LPS (10 ng/mL) was added to the media in one plate to promote nitric oxide (NO) production. A plate with media containing no LPS served as a control. After 24 h, supernatants were removed, centrifuged at 15,000 x g, 4 °C, and 10 min before pipetting 50 μL of the resultant supernatant into a 96 well plate. The Greiss reagent (Promega, Fitchburg, WI) was added as per the manufacturer’s instructions, and standard nitrite
concentrations were prepared. The absorbance was quickly measured at 535 nm via a plate reader (FlexStation 3 Benchtop Multi-Mode Microplate Reader) and compared with the standard curve to determine nitrite concentration and subsequent nitric oxide production.

C. Tumor necrosis factor – alpha (TNF – α) production from BMDCs

After 10 days of growing BMDCs in a petri dish with J558L supplemented media, the cells were harvested at 50000-cells/well into a 96 well plate with media containing optimized DXM encapsulated microparticles (ranging from 0.001 to 0.1 μM), blank Ac-DEX microparticles and free dexamethasone at the same concentrations. All the formulations were added in triplicate. The plates were incubated for 24 h at 37°C. One hundred fifty microliters of media was transferred from each well into centrifuge tubes. To remove suspended DCs and microparticles, samples were centrifuged for 5 minutes at 1400 rpm at 4°C. The TNF alpha level in the supernatant was then determined by ELISA. Before the addition of cell supernatants to the ELISA plate, the plate was preincubated with anti-tumor necrosis factor – alpha (anti TNF α) (200 μg/ml concentration) for 24 hrs at 4°C and also washed twice with PBS-tween. TNF-α standards prepared with EAE buffer was added to the ELISA plate. The EAE media was prepared by supplementing 500 mL of RPMI 1640 media with FBS (50 ml), penicillin (5 ml), Sodium Pyruvate (5 ml), Non Essential Amino Acids (5 ml), HEPES (6.5 ml) and beta-mercaptoethanol (500 μl). Seventy microlitres of both standards and supernatants were added to the plate. The plate was further incubated for 24 hrs at 4°C and washed twice with PBS-tween. Hundred
microlitres of 0.2 ng/ml concentrated biotin anti TNF α and 1% bovine serum albumin (BSA) were then added to each well. The plates were incubated for one hour at room temperature before washing with PBS-tween. Twelve millilitres of 1% BSA containing 30 µl of avidin peroxidase was added, followed by incubation for 30 minutes. The plates were washed with TBS-Tween prior to adding hydrogen peroxidase and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) substrate. Following storage in the dark for 10 minutes, the absorbance was measured at 405 nm via a plate reader (FlexStation 3 Benchtop Multi-Mode Microplate Reader).

**D. Cell Viability**

RAW macrophage viability was determined using a 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay. Twenty microlitres of a 5 mg/ml solution of MTT in cell culture media was added to macrophages and incubated for 1 hr. Formazhen crystals were formed and dissolved by adding 200 µl of isopropanol; the resultant colored product was read on a microplate reader at 560 nm and background absorbance at 670 nm was subtracted to measure the cell viability.

BMDC viability assays were carried out using MTT reagent as well. After incubating the BMDC for 24 hrs with media containing formulations, the media was removed and 150 µl fresh EAE buffer was added to each well containing 0.1 mg/ml concentrated MTT. The plates were incubated for 1-3 hr. Cell viability was determined as described above.
2.3.6 *In vivo* experiments

A. Mice experiments

C57Bl/6 and B10.PL mice were purchased from Jackson Laboratory. B10.PL Vα2.3 and Vβ8.2 TCR transgenic mice were obtained from J. Goverman (Goverman, et al. 1993). All mice were bred and kept in a pathogen-free facility at The Ohio State University (OSU). All animal protocols were approved by The Institutional Animal Care and Use Committee at The Ohio State University.

B. Induction of EAE

Induction of EAE in C57Bl/6 mice was modified from methods described by Smith *et al.* (Smith 2011). Briefly, the mice were immunized subcutaneously over four sites at the flanks of mice with complete Freund’s adjuvant (CFA) emulsion containing *Mycobacterium tuberculosis* at 2 mg/ml concentration and MOG35-55 (200 µg). 200 µl of pertussis toxin (1ng/µl) was injected intraperitoneally (i.p.) on day 0 along with immunization and 48h later. The mice were monitored from day 1 to 90 for clinical signs of EAE. After immunization, the mice are expected to develop EAE within 7–15 days. The mice were monitored 3 times a week initially, then daily after showing signs of disease. EAE scoring was performed as described elsewhere (Huss, et al. 2010; Smith 2011). Briefly EAE was scored on a scale of 0-6; 0, no abnormality; 1, a limp tail; 2, moderate hind limb weakness; 3, severe hind limb weakness; 4, complete hind limb
paralysis; 5, quadriplegia or premoribund state; and 6, death. Mice were euthanized if they have lost more than 20% of their weight or reached a grade 5 clinical EAE score.

Another mode of induction of EAE in mice is adoptive transfer of myelin reactive T cells (Stromnes and Goverman 2006). Myelin basic protein (MBP), a component of the nervous system is responsible for myelination of nerves. The capacity of T cells to identify MBP Ac1-11 peptide in Vβ8.2 TCR transgenic mice is very high, making them highly susceptible for EAE onset (Smith 2011). These highly specific T cells for MBP Ac1-11 peptide were isolated from immunized mice or TCR transgenic mice and can be passively transferred to other mice to induce EAE (Bettelli, et al. 2003)(Goverman, et al. 1993)(Lafaille, et al. 1994)(Pollinger, et al. 2009)(Waldner, et al. 2000). Induction of EAE by adoptive transfer of myelin specific T cells modified from previously described methods (Huss, et al. 2010; Smith, et al. 2011). Briefly single cell splenocyte suspensions were prepared by removing spleens from TCR transgenic mice and cultured in a 24 well plate at 1.5 X 10^6 cells/well concentration. These splenocytes were irradiated with B10.PL splenocytes and later activated in vitro in 24-well plates at 2 ml/well with the addition of 2 μg/ml MBP Ac1-11. After 3 days of culture, cells were washed with sterile PBS and 2-10 x 10^6 total cells were injected i.p. into B10.PL mice. The mice receiving the reactive cells were expected to develop EAE within 4 – 10 days.
C. Prophylactic treatment of CB57Bl/6 mice

Study 1:

CB57Bl/6 mice were treated separately with Ac-DEX particles containing dexamethasone and MOG35-55 peptide (DXM-MOG-Ac-DEX-MPs), blank Ac-DEX MPs, PBS for two weeks successively once every week (-7, -14 days) before induction of EAE and once after induction of EAE (day 4). For induction of EAE, on day 0 the mice were immunized subcutaneously with MOG35-55 in an emulsion with CFA (2 mg/ml). 200 μl of pertussis toxin (1 ng/μl) was given. A diagram of the study design is shown in Figure 2.12A.

Study 2:

CB57Bl/6 mice were injected with PBS over four sites at the flanks of mice on day 0. Mice were prophylactically treated separately with Ac-DEX particles containing dexamethasone and MOG peptide (DXM-MOG-Ac-DEX-MPs), blank Ac-DEX particles (Blk MPs), free dexamethasone and MOG35-55 (Free DXM & MOG) or PBS on days 7, 10, and 14. The mice were sedated with isoflurane administered via an inhalation machine before injecting them subcutaneously with 200 μl (100 μl on either side of hind limbs) of respective formulations. Each mouse received 8 μg of dexamethasone per injection and the MOG concentration given was calculated based on the amount of MOG35-55 encapsulated in Ac-DEX particles containing 8 μg of DXM. On day 17, the mice were challenged with EAE (1 mg/ml of CFA), followed by pertussis vaccine on day 20. Clinical EAE scoring was started from day 1 and continued till day 35. Graphical presentation of injections given to mice was shown in Figure 2.12B.
D. Cytokine evaluation using ELISA, intracellular staining and flow cytometry

To evaluate the cytokine production and FOXP3 expression, flow cytometry analysis was performed on CNS infiltrating cells and an ELISA was performed on the supernatant from splenocytes cultured with MOG 35-55 for 48 hrs. Single cell splenocyte suspensions were isolated as described previously (Huss, et al. 2010; Smith, et al. 2011) and stimulated with MOG35-55 for 48 hrs to assess antigen specific cytokine production. The supernatants were collected and analyzed for cytokines IL-10, IL-12, and interferon-gamma (IFN-γ) using ELISA techniques as described by Yang (Yang, et al. 2009). Briefly, the ELISA plates were incubated overnight at 4°C with 50 μl of primary antibody. After 24 hrs of incubation, wells were washed twice with PBS/0.05% Tween 20 and blocked with 200 μl/well of 1% BSA in PBS for 2 h. The plates were then twice washed with PBS/Tween 20 before adding 200 μl of splenocytes supernatant into each well, followed by incubation for 24 hrs at 4 °C. After 24 hrs, the plates were washed with PBS/Tween 20 twice and 100 μl of biotinylated rat antimouse secondary antibodies diluted in 1% BSA were added and incubated for 1 hr. The plates were washed again with PBS/Tween 20 before adding 100 μl avidin-peroxidase (2.5 μg/ml) and incubated for 30 minutes. After washing the plates eight times with PBS/Tween, 100 μl ABTS substrate containing 0.03% H₂O₂ was added to each well. The plate was stored in the dark for 10-20 min and absorbance was read at 405 nm. A standard curve was generated from the GM-CSF, IFN-γ, IL-10, and IL-12 standards, and used to calculate the respective cytokine concentration in the unknown samples.
CNS infiltrating cells were prepared as previously described (Gocke, et al. 2007; Huss, et al. 2010; Yang, et al. 2009). After eighteen days post induction of EAE, the mice were sacrificed, and the brain and spinal cords were harvested. Tissues from the same treatment group were combined and passed through a 70-µm-nylon mesh cell. Mononuclear cells were separated and collected from the grouped cells by washing twice in 37% percoll followed by centrifugation at 2118 x g for 15 min at 22 ºC over a 30%/70% percoll gradient. The separated mononuclear cells were resuspended in RPMI 1640 media and cultured for 48 h, with or without MOG (2 µg/ml). After 44 h of incubation, phorbol myristic acetate (PMA) (50 ng/ml) and ionomycin (750 ng/ml) were added. Four hours before staining, the cytokine secretin was blocked by adding 1 µl/ml GolgiPlug. Before the addition of staining buffer, CD 16/32 was added to block the Fc receptors and incubated for 15 minutes. The cells were resuspended in staining buffer (4% Fetal calf serum (FCS) and 0.1% sodium azide in PBS) and incubated with monoclonal antibodies (mAbs) for 30 min. Using Cytofix/Cytoperm solution, the cells were permeabilized for 20 min at 4°C and then stained for intracellular cytokines with mAb diluted in PermWash solution for 30 min at 4°C. The cells were then washed, resuspended in staining buffer, and fixed in 1% paraformaldehyde. For each cell samples 80,000 to 100,000 events were acquired on a FACSCanto (BD Biosciences, MD) and the data was analyzed using FlowJo software (Flow Jo, OR). The following mAbs were used: PerCP-conjugated anti-CD45, allophtocyanin-conjugated anti-Vα2 and fluorescein isothiocyanate (FITC)-conjugated anti-V8, Phycoerythrin (PE)-conjugated anti-IL-17 and PE-conjugated anti-IFN-γ (BD Pharmingen, MD).
For FOXP3 intracellular staining, surface-stained (CD4+ and CD25+) cells were fixed in E-bioscience fixation and permeabilization buffer (Ebioscience, CA) for 30 minutes and washed three times in E-bioscience Perm/Wash solution (Ebioscience, CA). Cells were incubated with anti-CD16/32 in Perm/Wash buffer to block the Fc receptors for 15 minutes before resuspending in FOXP3-APC antibodies diluted in Perm/Wash for 45 minutes. After three washes in Perm/Wash, cells were resuspended in FACS buffer (BD Biosciences, MD) and run on a FACSCanto flow cytometer.

E. Treatment of CB57Bl/6 mice and B10.PL mice

Study 1:

Male CB57Bl/6 mice were induced with EAE by immunizing subcutaneously over four sites at the flanks with CFA emulsion containing 1 mg/ml *M.tuberculosis* and 200 µg of MOG35-55. Pertussis toxin was injected i.p. on Day 0 along with immunization and 48 h later. EAE scoring was performed beginning on day 1. In this study the treatment was delayed until clinical signs of EAE were observed in the mice. On day 14 after induction of EAE, the mice were separated into four different groups, with each group having a mean clinical EAE score of 2. Each group was treated separately either with Ac-DEX particles containing dexamethasone and MOG peptide suspended in PBS (DXM-MOG-Ac-DEX-MPs), free dexamethasone in PBS (free DXM), dexamethasone and MOG dissolved in PBS (Free DXM & MOG) or PBS on days 14, 17, 19 and 21. Each mouse received 8 µg of dexamethasone per injection and the MOG concentration given was calculated based on the amount of MOG35-55
encapsulated in Ac-DEX particles containing 8 µg of DXM. Graphical presentation of injections given to mice was shown in Figure 2.12C.

Study 2:
In this study, the mice were induced with EAE by adoptive transfer of $10 \times 10^6$ MBP-specific T cells Ac1-11 i.p. (maximum volume 200 µl) into B10.PL mice. EAE scoring was done from day 7 onwards. On day 7, the mice were separated into four different groups with the same mean clinical EAE score (2.1). Each group was treated separately either with Ac-DEX particles containing dexamethasone (DXM-Ac-DEX-MPs), dexamethasone (free DXM), dexamethasone and MBP Ac1-11 dissolved in PBS (Free DXM & MBP), or PBS alone on days 7 and 11. Mice treated with dexamethasone received 8 µg of DXM per a injection and 4.4 µg of MBP/injection. A diagram of the study was shown in Figure 2.12D.

2.3 RESULTS AND DISCUSSION

2.3.1 Ac-DEX synthesis and NMR analysis
The work of Broaders et al., has shown that the synthesis of a library of Ac-DEX’s with tunable degradation can be done by changing the reaction times (Broaders, et al. 2009). With an increase of the reaction time, a higher number of thermodynamically stable cyclic acetals are formed when compared to kinetically favored acyclic acetals. Hence, the degradation half-life of Ac-DEX corresponds to the thermodynamically more
favored cyclic acetal content and the relative content of cyclic to acyclic acetals formed can be determined using NMR.

A representative NMR spectrum showing methanol and acetone signals is shown in Figure 2.1B. Hydrolysis of acyclic acetals produces one mole each of methanol (3.34 ppm, 3H) and acetone (2.08 ppm, 6H), whereas degradation of cyclic acetals produces one mole acetone. By comparing the integration of the acetone peak to the methanol peak, cyclic acetal coverage of Ac-DEX can be determined. All integrations were normalized to the number of protons on each molecule. Six-hour reaction time 10k Ac-DEX was shown to have a relative cyclic acetal coverage of 68%.

2.3.2 Particle Formation and Characterization

Table 2.1 provides the yield and drug loading for all the formulations containing DXM and with or without peptide (MOG35-55). High loaded DXM with Ac-DEX (High LD-DXM-Ac-DEX-MPs) or PLGA (High LD-DXM-PLGA-MPs) formulations have a DXM loading of 1 mg per 100 mg of Ac-DEX or PLGA in comparison to low loaded DXM (low LD-DXM-Ac-DEX-MPs, low LD-DXM-PLGA-MPs) and medium loaded DXM (Med LD-DXM-Ac-DEX) at 0.01 and 0.1 mg respectively. For in vivo experiments, both DXM (2% w/w) and MOG35-55 (0.4% w/w) were loaded into Ac-DEX particles (DXM-MOG-Ac-DEX-MPs) and were characterized. Particles prepared using a single emulsion technique showed a higher yield when compared to particles prepared by the double emulsion technique. In part, this is because a higher input power
is needed to generate double emulsion, which results in a break down of the microspheres resulting in a reduced yield (Yang, et al. 2001).

2.3.3 Particle Size and Zeta Potential

Ac-DEX or PLGA microparticles encapsulating DXM ± MOG35-55 were characterized to evaluate the effect of polymer and drug loading on particle size, as shown in Table 2.1. All the formulations showed a mean diameter in the range of 800 – 2000 nm, suitable for passively targeting to dendritic cells. Immature DCs internalize exogenous particulate matter such as micro- and nanoparticles (which mimic pathogens but are not infectious) through phagocytosis (Reddy, et al. 2006b), making them excellent targets for drug delivery. Particles less than 200 nm are ingested by most cells through pinocytosis or receptor-mediated endocytosis. However, antigen presenting cells such as dendritic cells and macrophages can uptake particles, which are up to several microns in diameter (Manolova, et al. 2008). Work by Manolova showed that when particles ranging from 1000 to 2000 nm were injected in vivo; DCs (CD11c+CD11b– or CD11c+CD11b+) phagocytized the particles 50 times more than the macrophages (CD11c–CD11b+ cells) (Manolova, et al. 2008). Furthermore, research done by Manolova and group, showed predominant transport of large particles by DCs from the injection site to the lymph node; whereas smaller particles are cleared by resident macrophages (Manolova, et al. 2008). Hence the particle size obtained from probe sonication can be ideal for passive targeting of DCs. Also, variations in loading of drug have not shown any significant difference in particle size. Additionally with the same
drug loading, PLGA particles attained higher particle size when compared to Ac-DEX particles. This might be due to the higher viscosity of organic phase formed with PLGA polymer when compared to Ac-DEX. Furthermore, the addition of peptide to the formulation also increased the particle size (DXM-MOG-Ac-DEX-MPs).

The zeta potentials of all the particles prepared using Ac-DEX or PLGA were negatively charged with zeta potential varying from -15 to -16 mV. Similar to the results obtained by Panyam et al., PLGA particles were negatively charged with values from -40 to -50 mV. The reason behind the lower zeta potential for PLGA particles may be the presence of free carboxylic groups on the surface of polymer (Panyam, et al. 2004). According to Muller (1991) formulations with a surface charge of -10 to -30 mV show moderate stability, hence all particles prepared using Ac-DEX fall in the category of moderate stability (Muller, et al. 2000; Ravichandran 2009).

2.3.4 Scanning Electron Microscopy (SEM)

SEM images of the particles prepared are shown in Figure 2.2. Both the Ac-DEX and PLGA microparticles formed were spherical in structure and without aggregation in solution. Also, addition of drug (DXM) and peptide (MOG35-55) had no effect on the surface morphology of the particles.

2.3.5 Encapsulation Efficiency (EE)

Encapsulation efficiencies of DXM with Ac-DEX and PLGA loaded microparticles are shown in Table 2.1. An increase in encapsulation efficiency of DXM
was observed with decreased drug loading. With decreased drug loading, the amount of drug that needs to be encapsulated per mg of polymer is modest; due to the escape of the drug into aqueous continuous phase. The lower encapsulation efficiency of DXM into Ac-DEX particles can be explained with the sparingly soluble nature of DXM in water and also because of use of ethanol. Ethanol is a versatile solvent miscible with water which results in consequent partitioning of DXM to the aqueous environment. Interestingly, Ac-DEX particles showed higher encapsulation of DXM when compared to PLGA particles. The amount of DXM entrapped within PLGA matrix obtained was similar to that obtained by Panyam et al., showing fairly low encapsulation when compared to Ac-DEX particles (Panyam, et al. 2004).

Fluorescamine assay was used to determine the encapsulation of MOG35-55 peptides. Fluorescamine is a protein dye used to measure minute amounts of proteins and/or peptide in solution (Baker 1983). Ac-DEX has a higher encapsulation of MOG35-55 peptide (80%) as shown in Table 2.1.

2.3.6 Differential Scanning Calorimetry (DSC)

*In vivo* and *in vitro* release of DXM is influenced by its physical nature in the polymer. DSC studies were performed to understand the physical nature of DXM in microparticles and also to assess for any possible interactions between DXM and Ac-DEX polymer. DSC thermograms of pure DXM, lyophilized Ac-DEX, DXM loaded Ac-DEX particles and other controls are shown in Figure 2.3. Pure DXM (Curve D) showed a sharp endothermic melting peak at 257 °C similar to that reported by Fialho *et al.* and
Rodrigues et al. (Fialho, et al. 2008)(Rodrigues, et al. 2009). The sharp endothermic peak obtained at 257 °C represents the crystalline nature of dexamethasone. Pure Ac-DEX (Curve B), exhibits a broad endothermic peak at 160 °C and an exothermic peak at 340 °C. The broad endothermic peak of Ac-DEX might correspond to the glass transition temperature of the polymer. The DSC thermogram for dexamethasone encapsulated Ac-DEX particles is represented as curve E in Figure 2.3. DXM loaded Ac-DEX particles show a relaxation peak similar to pure Ac-DEX and blank Ac-DEX particles (curve A) indicating that the micro encapsulation process did not affect the polymer or that the amount encapsulated was too low to be detected. Since no sharp endothermic peak was observed at 257 °C for DXM in loaded Ac-DEX particles, the drug might exist either in an amorphous or a disordered-crystalline phase. Because of lack of significant controls, results interpreted with DSC thermograms were not completely conclusive and further studies such as X-ray diffraction and Tg analysis are necessary to elucidate the underlying phenomenon.

2.3.7 Release rate

Ac-DEX microparticles encapsulating DXM showed pH sensitive drug release, with 80% of drug released within 24 h at pH 5.0, as shown in Figure 2.4. At pH 7.4, Ac-DEX microparticles encapsulating DXM showed a biphasic DXM release with an initial burst effect around 20% within the first 6 h, followed by a steady release for the next 48h. Previous experiments showed significant increase in MHCI and MHCII presentation with Ac-DEX when compared to PLGA, because of the acid sensitivity of the polymer in the
phagolyososomal compartments of DCs (Broaders, et al. 2009). The pH sensitivity nature of Ac-DEX can be used to maximize the drug release inside the DC.

2.3.8 Nitric Oxide Analysis and Cell Viability

Nitric oxide experiments showed no significant difference in nitrite reduction between high loaded or medium loaded DXM Ac-DEX particles and free DXM (Figure 2.5 and 2.6). As shown in Figure 2.7, the low loaded DXM Ac-DEX particles showed dose dependent decrease in nitrite production, similar to free DXM. However the nitrite concentration is significantly lower with low loaded Ac-DEX particles compared to free DXM, showing the dose sparing nature of DXM loaded Ac-DEX particles. This change in cellular response with the three different formulations occurs because the number of particles available for macrophages to phagocytize was reduced with high and medium loaded Ac-DEX particles when compared to low loaded DXM particles. Hence to increase the number of particles, drug loading was decreased. Due to their pH sensitive degradation, Ac-DEX dexamethasone microparticles showed a dose sparing response in comparison to PLGA dexamethasone microparticles in the reduction of nitrite production assessed in vitro in LPS stimulated macrophages (Figure 2.8). Cell viability showed no significant difference between the different formulations used (Figure 2.9). Based on the reduction of nitrite in macrophages, it indicates that encapsulation of DXM into Ac-DEX particles significantly increased drug activity, and facilitates dose sparing.
2.3.9 TNF-\(\alpha\) production and cell viability

Low loaded DXM Ac-DEX particles show dose sparing with RAW macrophages (Fig 2.7), a similar batch was used to determine TNF-\(\alpha\) production in BMDC. As shown in Figure 2.10, production of TNF-\(\alpha\) was compared between low loaded DXM Ac-DEX particles and free dexamethasone, both were stimulated with 100 ng/ml of LPS. Similar to the macrophage results, DXM loaded particles showed a significant decrease in TNF-\(\alpha\) production when compared to free DXM. This again shows a dose sparing effect of encapsulated DXM over free DXM. Cell viability results showed no significant difference in BMDC number between DXM encapsulated Ac-DEX particles and free dexamethasone treatments as shown in Figure 2.11.

2.3.10 In vivo experiments

In vivo experiments were performed to confirm the ability of DXM and antigen loaded microparticles in forming iT-regs. As a first step towards this aim, experiments were planned to study the effect of microparticles administered prophylactically in C57Bl/6 mice and to compare their clinical EAE score with PBS and blank microparticles treated mice. The mice were injected subcutaneously in the flank with the respective formulations on day -14, -7 and +4 relative to EAE induction protocol. Eighteen days after the EAE induction protocol, C57Bl/6 mice, DXM and peptide loaded particle (DXM-MOG-Ac-DEX-MPs) treated mice showed a significant decrease in median clinical EAE score (Mann-Whitney U test) when compared to controls (Figure 2.13A). The mice were weighed on respective days and the mean percentage change in
mice weight is shown in Figure 2.13B. With the progression of EAE symptoms mice lost weight as observed in blank particles and PBS group treated mice. However with administration of DXM and MOG35-55 loaded particles, weight was not significantly decreased, suggesting no development of EAE. The mean onset of EAE for DXM loaded particle treated mice was 16 days in comparison to 11 days for PBS treated group (Table 2.2). This shows delayed progression of disease when DXM and antigen loaded particles are given prophylactically. T cells isolated from the spleens of mice isolated on day 14 were stimulated with MOG35-55 peptide. Results of ELISA performed on cell supernatant suggest an increased production of IL-10 and reduced production of IL-12 cytokines (Figure 2.14A). Cells derived from the CNS of treated mice were cultured with MOG35-55 peptide and analyzed by flow cytometry for Treg populations (high expression of CD25$^+$ and FOXP3). Furthermore, DXM treated mice showed higher T-reg population (CD25$^+$ and FOXP3) in the central nervous system (CNS) when compared to controls (Figure 2.14B). Also, cells derived from the CNS displayed decreased intracellular concentrations of less production of inflammatory cytokines IL-17 and IFN–γ (Figure 2.14B). Inflammatory cytokines IL-17 and IFN–γ are the signature cytokines secreted by Th17 and Th1 cells. Overall, the data shows that the prophylactic injection of DXM-MOG-Ac-DEX-MPs a systemic iT-reg population was generated, which trafficked to the brain to suppress EAE.

A second prophylactic study was planned in female C57Bl/6 mice by injecting PBS on day 0 and injecting the mice with respective formulations on day 7, 10 and 14. On day 17, the mice were challenged with EAE (1 mg/ml of CFA), followed by pertussis
vaccine on day 20. The study was carried out till day 35 and no further treatments were given. Mice treated with PBS, free DXM and MOG peptide formulation showed higher scores of EAE when compared to mice treated with either blank particles or DXM and MOG35-55 loaded particles. By day 35, the median EAE score was highest for free DXM and MOG (2.5) followed by PBS (1.0) as shown in Table 2.3. The median scores for both DXM and MOG encapsulated Ac-DEX particles and blank particles were zero and the mean score was 0.5 and 0.7 respectively. After day 35 the scoring of mice was stopped. The higher clinical EAE score for free DXM & MOG treated group can be explained by the non specific targeting action of free DXM, thus resulting in generation of less tolerance. Also, the free MOG35-55 peptide given along with DXM further initiated additional inflammatory immune response against the peptide augmenting the clinical EAE symptoms in mice. Evaluation of weight showed lowering of mice weight with free DXM and MOG treated group, reflecting EAE symptoms (Figure 2.15B). No significant difference in mouse weights was observed between the other treatment conditions.

An initial treatment study was planned by starting treatment after initial clinical signs of EAE. The EAE induction protocol (2 mg/ml) was performed on day zero along with pertussis vaccine on day 0 and day 2. EAE scoring was started from day 1 and the treatment was started on day 14 when the mean EAE score per group was 2.2. The mice were injected with particles on day 14, 17, 19 and 21 relative to the EAE induction protocol. Five mice were included in each group: PBS, free DXM group and free DXM & MOG. Seven mice were selected for DXM and MOG35-55 loaded particles group. The
median EAE scores of mice are shown in Figure 2.16A. Fourteen days after induction of EAE all groups showed reduction in clinical symptoms of EAE. The scoring of mice was stopped 31 days after the EAE induction protocol and the median EAE score showed no significant difference between the treatments. Mouse weights increased after day 14 (first day of treatment), corresponding to a reduction in clinical EAE symptoms (Figure 2.16B). Before the mice were treated, all mice showed decreased weight indicating the progression of EAE in mice. However after sedating with isoflurane, the mouse weight started increasing. By day 31 the median EAE score for all the treatments is 0 as shown in Table 2.4.

Hence to avoid the inconsistency of EAE induction in CB57Bl/6 mice, adoptive transfer of myelin specific T cells was carried in B10.PL mice. The mice had the EAE induction protocol on day 0 and the treatment was started on day 7 when the mean EAE score per group was 2.2. Mice were injected with formulations on day 7 and 11 and the median EAE score is shown in Figure 2.17. Studies were planned to inject the mice without the use of isoflurane. However because of small weight of the mice, it was hard to inject particles without sedation and hence isoflurane was used. The mice were scored till day 20 and the results showed higher median EAE score with free DXM and MBP group than with particles containing DXM. 100% of mice had EAE progression by day 20 and there was no significant difference in median EAE scores between the treatments as shown in Table 2.5. One possible reason for this might be high EAE induction and late treatment of mice with formulation.
In summary, promising results were obtained with *in vitro* experiments showing dose sparing and the preliminary *in vivo* studies were inconclusive. In future more intense and detailed *in vivo* studies are needed to confirm the affirmative treatment action of dexamethasone and antigen loaded Ac-DEX microparticles in generation of antigen specific T cells.
Figure 2.1:

(A) The reaction scheme to synthesize acetalated dextran (Ac-DEX) from dextran. (Kauffman, et al. 2012).

(B) A representative NMR for 10k Ac-DEX showing the acetone and methanol peaks.
Figure 2.1

A

Dextran

\[ \text{PPTS (cat.)} \]

\[ \text{DMSO} \]

\[ 6 \text{ hr.} \]

\[ \text{OMe} \]

\[ \text{H}_3\text{O}^+ \]

Acetalated Dextran (Ac-DEX)

B

Methanol

Acetone

56
Figure 2.2: Representative scanning electron micrographs of (A) high loaded dexamethasone encapsulated acetalated dextran particles, (B) medium loaded dexamethasone encapsulated acetalated dextran particles, (C) low loaded dexamethasone encapsulated acetalated dextran particles and (D) low loaded dexamethasone encapsulated PLGA particles.
Figure 2.3: Differential scanning calorimetry thermograms for (A) blank Ac-DEX particles, (B) Ac-DEX, (C) physical mixture of Ac-DEX and dexamethasone, (D) Dexamethasone, and (E) Dexamethasone encapsulated Ac-DEX particles.
Figure 2.4: Release profiles of dexamethasone encapsulated in acetalated dextran particles under acidic (pH 5.0) and physiologic conditions (pH 7.4). Each data point is presented as the mean ± standard deviation (n=3).
Figure 2.5: Nitrite production of macrophages cultured with dexamethasone encapsulated in Ac-DEX microparticles (High LD-DXM-AcDEX-MPs) at a drug loading of 1:100 or unencapsulated dexamethasone (free DXM) at equivalent drug concentrations. Half of these cultures were stimulated with lipopolysaccharide (LPS) to induce a pro-inflammatory response. Values are presented as average ± standard deviation (n=3). *p < 0.01 with respect to free drug.
Figure 2.6: Nitrite production of macrophages cultured with dexamethasone encapsulated in Ac-DEX microparticles (Med LD-DXM-Ac-DEX-MPs) at a drug loading of 1:1000 or unencapsulated dexamethasone (free DXM) at equivalent drug concentrations. Half of these cultures were stimulated with lipopolysaccharide (LPS) to induce a pro-inflammatory response. Values are presented as average ± standard deviation (n=3). *p < 0.01 with respect to free drug.
Figure 2.7: Nitric Oxide production of macrophages cultured with dexamethasone encapsulated in Ac-DEX microparticles at a drug loading of 1: 10000 (Low LD-DXM-AcDEX-MPs) or unencapsulated dexamethasone (free DXM) at equivalent drug concentrations. Half of these cultures were stimulated with lipopolysaccharide (LPS) to induce a pro inflammatory response. Values are presented as average ± standard deviation (n=3). *$p < 0.01$, **$p < 0.001$, with respect to free drug.
Figure 2.8: Nitrite production of macrophages cultured with dexamethasone encapsulated in Ac-DEX microparticles (Low LD-DXM-AcDEX-MPs) or PLGA microparticles (Low LD-DXM-PLGA-MPs) at a drug loading of 1: 10000 or unencapsulated dexamethasone at equivalent drug concentrations. Half of these cultures were stimulated with lipopolysaccharide (LPS) to induce a pro inflammatory response. Values are presented as average ± standard deviation (n=3). *p < 0.01, **p < 0.001, with respect to free drug
Figure 2.9: Cell viability of RAW macrophages for dexamethasone encapsulated Ac-DEX particles (Low LD-DXM-AcDEX-MPs, Med LD-DXM-AcDEX-MPs) and free dexamethasone (Free DXM) with and without lipopolysaccharide (LPS) stimulation. Each bar is presented as the mean ± standard deviation (n=3).
Figure 2.10: Tumor necrosis factor (TNF-α) production of bone marrow derived dendritic cells (BMDCs) cultured with dexamethasone encapsulated in Ac-DEX microparticles (Low LD-DXM-Ac-DEX-MPs) at a drug loading of 1:10000 or unencapsulated dexamethasone (Free DXM) at equivalent drug concentrations. These cultures were stimulated with lipopolysaccharide (LPS) to induce a pro-inflammatory response. Values are presented as average ± standard deviation (n=3). *p < 0.05, **p < 0.001, with respect to free drug.
Figure 2.11: Cell viability of bone marrow derived dendritic cells (BMDC’s) for dexamethasone encapsulated Ac-DEX particles (Low LD-DXM-Ac-DEX-MPs) and free dexamethasone (Free DXM) with lipopolysaccharide (LPS) stimulation. Each bar is presented as the mean ± standard deviation (n=3).
A. Prophylactic treatment study 1: Mice were injected subcutaneously in the flank with the formulations on day -14, -7 and +4 relative to EAE induction on day zero.
B. Prophylactic treatment study 2: Mice were injected subcutaneously in the flank with the formulations on day 7, 10 and 14 relative to PBS injection. EAE was induced on day 17.
C. Treatment study 1: Mice were injected subcutaneously in the flank with the formulations on day 14, 17, 19 and 21 relative to EAE induction on day zero.
D. Treatment study 2: Mice were injected subcutaneously in the flank with the formulations on day 7 and 11 relative to EAE induction with adoptive transfer on day zero.
Figure 2.12

A

B

C

D
Figure 2.13: Median EAE score of mice injected with phosphate buffer solution (PBS), empty Ac-DEX microparticles (Blank MPs), or Ac-DEX microparticles encapsulating MOG and dexamethasone (DXM-MOG-Ac-DEX-MPs). Mice were injected subcutaneously in the flank with the formulations on day -14, -7 and +4 relative to EAE induction. Five mice were included in each group.
Figure 2.13

A

Median EAE score

Time (Days)

- PBS Group
- Blank MPs
- DXM-MOG-AcDEX-MPs

B

% Change in weight

Time (days)

- PBS
- Blank MPs
- DXM-MOG-AcDEX-MPs
Figure 2.14: The cells were extracted from mice 14 days after EAE was induced (A) ELISA results of supernatant isolated from spleen cells extracted from mice on day 14 after induction with EAE and stimulation in vitro with MOG peptide. (B) Surface markers and intracellular cytokines of CNS infiltrating cells extracted from mice 18 days after EAE was induced.
Figure 2.15: (A) Median EAE score and (B) % change in weight of mice injected with phosphate buffer solution (PBS), empty Ac-DEX microparticles (Blank MPs), free dexamethasone in combination with MOG (Free DXM & MOG) and Ac-DEX microparticles encapsulating MOG (DXM-MOG-Ac-DEX-MPs). Mice were injected subcutaneously in the flank with the formulations on day 7, 10 and 14 relative to PBS injection. EAE was induced on day 17 with pertussis boast on day 17 and 20. Values are presented as median ± standard error of mean. Seven mice were included each for PBS and Blank MPs and eight mice for free DXM & MOG and DXM-MOG-Ac-DEX-MPs.
Figure 2.16: (A) Median EAE score and (B) % change in weight of mice injected with phosphate buffer solution (PBS), dexamethasone alone (Free DXM), dexamethasone in combinations with MOG (Free DXM & MOG), Ac-DEX microparticles encapsulating MOG (DXM-MOG-Ac-DEX-MPs). Mice were injected subcutaneously in the flank with the formulations on day 14, 17, 19 and 21 relative to EAE induction. EAE was induced on day 0 with pertussis booster on day 0 and 2. Values are presented as median ± standard error of mean. Five mice were included each for PBS, free DXM and free DXM & MOG and seven mice for dexamethasone and MOG encapsulated particles.
Figure 2.16

A

Median EAE score

Time (Days)

- PBS
- Free DXM
- Free DXM & MOG
- DXM-MOG-AcDEX-MPs

B

% Change in Weight

Time (Days)

- PBS
- Free DXM
- Free DXM & MOG
- DXM-MOG-AcDEX-MPs
Figure 2.17: Median EAE score of mice injected with phosphate buffer solution (PBS), dexamethasone alone (Free DXM), dexamethasone in combination with MBP (Free DXM & MBP), or Ac-DEX microparticles (DXM-Ac-DEX particles). Mice were injected subcutaneously in the flank with the formulations on day 7 and 11 relative to EAE induction with adoptive transfer of 2 * 10^6 myelin specific T cells. Values are presented as median ± standard error of mean.
Table 2.1: Drug loading (LD), yield, encapsulation efficiency (EE), particle size, zeta potential for dexamethasone (DXM) ± MOG encapsulated acetalated dextran (Ac-DEX) or poly lactic-co-glycolic acid (PLGA) particles prepared by single or double emulsion techniques.
<table>
<thead>
<tr>
<th></th>
<th>DXM conc (mg/mg of polymer)</th>
<th>Yield (%)</th>
<th>EE (DXM, MOG %)</th>
<th>Diameter (nm)</th>
<th>Zeta Potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>High LD-DXM-Ac-DEX-MPs</td>
<td>$10^{-2}$</td>
<td>53</td>
<td>10.2 ± 0.8</td>
<td>1310</td>
<td>-14.8 ± 0.73</td>
</tr>
<tr>
<td>Med LD-DXM-Ac-DEX-MPs</td>
<td>$10^{-3}$</td>
<td>63</td>
<td>16.5 ± 2.1</td>
<td>896</td>
<td>-16 ± 0.79</td>
</tr>
<tr>
<td>Low LD-DXM-Ac-DEX-MPs</td>
<td>$10^{-4}$</td>
<td>66</td>
<td>33.6 ± 0.4</td>
<td>1083</td>
<td>-15 ± 0.47</td>
</tr>
<tr>
<td>DXM-MOG-Ac-DEX-MPs</td>
<td>$2 \times 10^{-2}$</td>
<td>40</td>
<td>11.5 ± 4.6</td>
<td>2184</td>
<td>-16 ± 0.62</td>
</tr>
<tr>
<td>Blank Ac-DEX-MPs</td>
<td>--</td>
<td>70</td>
<td>--</td>
<td>--</td>
<td>-20.14±1.47</td>
</tr>
<tr>
<td>High LD-DXM-PLGA-MPs</td>
<td>$10^{-2}$</td>
<td>69</td>
<td>4.4 ± 0.14</td>
<td>1732</td>
<td>-41.72 ± 2.54</td>
</tr>
<tr>
<td>Low LD-DXM-PLGA-MPs</td>
<td>$10^{-4}$</td>
<td>62</td>
<td>28.1 ± 4.8</td>
<td>1561</td>
<td>-51.4 ± 0.64</td>
</tr>
<tr>
<td>Blank PLGA-MPs</td>
<td>--</td>
<td>84</td>
<td>--</td>
<td>697</td>
<td>-47.62 ±0.82</td>
</tr>
</tbody>
</table>

Table 2.1
Table 2.2: Table showing the mean onset time and median EAE score of mice injected with phosphate buffer solution (PBS), empty Ac-DEX microparticles (Blank MPs), or Ac-DEX microparticles encapsulating MOG (DXM-MOG-Ac-DEX-MPs). Mice were injected subcutaneously in the flank with the formulations on day -14, -7 and +4 relative to EAE induction.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Median EAE Score on Day 18</th>
<th>Incidence (%)</th>
<th>Mean onset of EAE (Days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS</td>
<td>3.0 ± 0.6</td>
<td>5/5 (100)</td>
<td>11 ± 1.4</td>
</tr>
<tr>
<td>Blank MPs</td>
<td>3.0 ± 0.4</td>
<td>5/5 (100)</td>
<td>12 ± 2</td>
</tr>
<tr>
<td>DXM-MOG-Ac-DEX-MPs</td>
<td>2.0 ± 0.4</td>
<td>5/5 (100)</td>
<td>16 ± 3.4</td>
</tr>
</tbody>
</table>
Table 2.3: Table showing the median EAE score and % incidence of EAE in mice injected with phosphate buffer solution (PBS), empty Ac-DEX microparticles (Blank MPs), dexamethasone in combinations with MOG (Free DXM & MOG), Ac-DEX microparticles encapsulating MOG (DXM-MOG-Ac-DEX-MPs). Mice were injected subcutaneously in the flank with the formulations on day 7, 10 and 14 relative to PBS injection. EAE was induced on day 17 with pertussis booster. Values are presented as median ± standard error of mean.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Median EAE Score on Day 17</th>
<th>Median EAE Score on Day 35</th>
<th>Incidence (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS</td>
<td>0.0 ± 0.0</td>
<td>1.0 ± 0.0</td>
<td>5/7 (71)</td>
</tr>
<tr>
<td>Blank MPs</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.5</td>
<td>2/7 (29)</td>
</tr>
<tr>
<td>Free DXM &amp; MOG</td>
<td>0.0 ± 0.0</td>
<td>2.5 ± 0.6</td>
<td>5/7 (71)</td>
</tr>
<tr>
<td>DXM-MOG-Ac-DEX-MPs</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.3</td>
<td>2/7 (29)</td>
</tr>
</tbody>
</table>
Table 2.4: Table showing the median EAE score and % incidence of EAE in mice treated with phosphate buffer solution (PBS), dexamethasone alone (Free DXM), dexamethasone in combinations with MOG (Free DXM & MOG) or Ac-DEX microparticles encapsulating MOG (DXM-MOG-Ac-DEX-MPs) injected subcutaneously in the flank with the formulations on day 14, 17, 19 and 21 relative to EAE induction. EAE was induced on day 0 with pertussis booster on day 0 and 2. Values are presented as median ± standard error of mean.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Median EAE Score on Day 14</th>
<th>Median EAE Score on Day 31</th>
<th>Incidence (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS</td>
<td>2.0 ± 0.4</td>
<td>0.0 ± 0.6</td>
<td>5/5 (100)</td>
</tr>
<tr>
<td>Free DXM</td>
<td>2.0 ± 0.2</td>
<td>0.0 ± 0.2</td>
<td>5/5 (100)</td>
</tr>
<tr>
<td>Free DXM &amp; MOG</td>
<td>2.0 ± 0.2</td>
<td>0.0 ± 0.5</td>
<td>5/5 (100)</td>
</tr>
<tr>
<td>DXM-MOG-Ac-DEX-MPs</td>
<td>2.0 ± 0.4</td>
<td>0.5 ± 0.3</td>
<td>7/7 (100)</td>
</tr>
</tbody>
</table>
Table 2.5: Table showing the median EAE score and % incidence of EAE for mice injected with phosphate buffer solution (PBS), dexamethasone alone (Free DXM), dexamethasone in combination with MBP (Free DXM & MBP), or Ac-DEX microparticles encapsulating dexamethasone (DXM-Ac-DEX-MPs). Mice were injected subcutaneously in the flank with the formulations on day 7 and 11 relative to EAE induction with adoptive transfer of $2 \times 10^6$ myelin specific T cells. Values are presented as median ± standard error of mean.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Median EAE Score on Day 20</th>
<th>Incidence (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS</td>
<td>$3.0 \pm 0.4$</td>
<td>9/9 (100)</td>
</tr>
<tr>
<td>Free DXM</td>
<td>$3.0 \pm 0.4$</td>
<td>7/7 (100)</td>
</tr>
<tr>
<td>Free DXM &amp; MBP</td>
<td>$5.0 \pm 0.6$</td>
<td>8/8 (100)</td>
</tr>
<tr>
<td>DXM-Ac-DEX-MPs</td>
<td>$4.0 \pm 0.4$</td>
<td>9/9 (100)</td>
</tr>
</tbody>
</table>
CHAPTER 3

OPTIMIZATION OF RAPAMYCIN-LOADED ACETALATED DEXTRAN MICROPARTICLES FOR IMMUNOSUPPRESSION

3.1 INTRODUCTION

Immunosuppression can be useful in the treatment of autoimmune diseases and for patients undergoing organ transplants. Various immunosuppressive drugs have been developed to treat autoimmune diseases ranging from rheumatoid arthritis (Drosos 2002) to multiple sclerosis (Hartung, et al. 2002). Also, immunosuppressive drugs such as rapamycin or cyclosporine can be administered to patients receiving organ transplants to discourage organ rejection, greatly increasing the survival rates of these patients (Khan 2008). As a point of therapeutic intervention, phagocytes like dendritic cells (DCs) and macrophages can be targeted because they are considered as gateways to immune suppression responses. Several small molecules have been shown to skew phagocytes’ immune responses \textit{ex vivo}, such as rapamycin (Fischer, et al. 2009) (Turnquist, et al. 2007), dexamethasone (Barrat, et al. 2002; Bosma, et al. 2008), Vitamin D3 (Griffin, et al. 2000), dimethylfumaric acid ester (Litjens, et al. 2004; Zhu and Mrowietz 2001),

Rapamycin, also known as sirolimus, is an inexpensive immunosuppressive drug that has been well-studied in renal transplantation (Kahan 2003) and in drug-eluting stents as an anti-inflammatory/anti-proliferative agent (Venkatraman and Boey 2007). Rapamycin is typically administered as an oral solution under the commercial name Rapamune; however, regular administration of rapamycin even in low doses (1 to 2 mg/day) can produce side-effects in humans including diarrhea and headaches, but more seriously, myelosuppression, hyperlipidemia, and over-immunosuppression (Saunders, et al. 2001).

These side-effects could potentially be reduced by using a polymeric micro-particle system to deliver rapamycin by passively targeting phagocytes. The phagocytosis of rapamycin-loaded microparticles would allow for localized drug release to phagocytes, thereby reducing side-effects and perhaps requiring less drug (Bachelder, et al. 2010). The polymer most commonly used to create microparticles for drug delivery applications is poly(lactic-co-glycolic acid) (PLGA). Previous research has shown that rapamycin-loaded PLGA microparticles significantly increased drug efficacy and reduced T-cell activation when the particles were incubated with phagocytic dendritic cells (DCs) for four days (Jhunjhunwala, et al. 2009). Similarly, rapamycin-loaded PLGA microparticles have been shown to inhibit DC maturation far better than the free drug form (Haddadi, et al. 2008). These results indicate that rapamycin has immunosuppressive effects on phagocytes when passively targeted to the cells.
Although PLGA and other polyesters (e.g., poly(lactic acid), polycaprolactone) are commonly used in drug delivery applications, they have several disadvantages. Most notably, they have fixed degradation rates on the order of months, degrade into acidic byproducts which can significantly lower the local pH (Lu, et al. 2000), and release nearly half of their drug payloads in a burst fashion (Allison 2008). Acetalated dextran (Ac-DEX) is a new biodegradable polymer that is formed by replacing the hydroxyl groups on dextran, a water-soluble homopolysaccharide of glucose, with cyclic and acyclic acetal groups to render the polymer water-insoluble (Bachelder, et al. 2008). Ac-DEX is particularly promising because it can overcome many of the inherent disadvantages of polyesters for application in the body. In contrast to fixed degradation rates, Ac-DEX exhibits tunable degradation rates which can range from minutes to months, due to the differing rates of hydrolysis for cyclic and acyclic acetal groups. Specifically, Ac-DEX microparticles undergo tunable burst release in acidic phagosomal conditions (pH 5) but have a slower, tunable release profile in extracellular conditions (pH 7.4). The pH-sensitivity of Ac-DEX microparticles allow them to release their drug payloads in the phagolysosome once they are passively targeted to the phagocyte. Importantly, this pH-sensitivity may minimize systemic toxic side-effects of the encapsulated drug by ensuring that the maximum amount of drug is released intracellularly in the phagolysosome instead of systemically, which could also lead to the administration of less drug for equivalent drug effect (i.e. dose sparing). Furthermore, materials sensitive to the acidic environment present in the phagolysosomal compartments of macrophages and DCs (pH 5) (i.e. Ac-DEX and polyacrylamide) have a
significantly increased efficacy of the immunological synapse compared to degradable (PLGA) and non-degradable (iron oxide) materials (Broaders, et al. 2009). These characteristics make Ac-DEX microparticles a more desirable vehicle to deliver rapamycin to phagocytes than microparticles made from other available biopolymers. 

Previously, Ac-DEX had been synthesized from dextran with a MW of 10,500 (10k) (Bachelder, et al. 2008). However, production of Ac-DEX with an increased MW of 71,400 (71k) was hypothesized to increase drug encapsulation efficiency, particle size, and degradation rates primarily due to its higher viscosity and longer chain lengths; it has been previously shown that increased molecular weight of polymer correlates to increased encapsulation efficiency with chitosan (Xu and Du 2003) and lengthened degradation rates with PLGA and poly(lactic acid) (Alexis (2005)) Because 71k Ac-DEX has not yet been investigated in the literature, optimized parameters were first established to create the best rapamycin-loaded Ac-DEX microparticles in terms of yield and encapsulation efficiency, similar to work completed by Mao et al. with PLGA (Mao, et al. 2007). Additionally, the release of rapamycin from Ac-DEX microparticles was characterized in acidic (pH 5) and neutral conditions (pH 7.4). Lastly, cell studies were performed with RAW macrophages stimulated with pro-inflammatory lipopolysaccharide (LPS) to determine the efficacy of the drug in its free and encapsulated form. In summary, the objective of this study was to synthesize 71k Ac-DEX rapamycin-loaded immunosuppressive microparticles, which could potentially reduce the toxic side-effects of the encapsulated immunosuppressant in vivo due to the controlled and localized release of drug.
3.2 MATERIALS AND METHODS

All materials were purchased from Sigma-Aldrich (St. Louis, MO) and used as received unless otherwise noted.

3.2.1 Synthesis and NMR analysis of Ac-DEX

Ac-DEX was prepared and analyzed with NMR as described in chapter 2.

3.2.2 Particle Preparation

A. Ac-DEX Particle Formation via Homogenization

Homogenized microparticles were prepared via a single-emulsion technique (water/oil) (Bachelder, et al. 2010). To fabricate the homogenized microparticles, Ac-DEX (100 mg) and rapamycin (1 mg, LC Laboratories, Woburn, MA) were dissolved in dichloromethane (DCM, 1 mL) and added to 3% poly(vinyl alcohol) (PVA) in PBS (17 mL). The resultant mixture was homogenized for 30 seconds (Polytron PT 10-35 Homogenizer, 20,500 RPM) and the emulsion was immediately poured into a spinning solution of 0.3% PVA (40 mL). The reaction mixture was allowed to spin for two hours to evaporate the solvent and allow for particle hardening. To recover the microparticles, each formulation was subjected to centrifugation (5 min, 20,000x g, 4 °C). The supernatant was discarded, and the resulting microparticle sediment was washed with basic water to remove excess drug and PVA (2 x 5 min, 20,000 x g, 4 °C). The microparticles were then suspended in basic water and lyophilized for two days to yield
rapamycin-loaded Ac-DEX microparticles. To produce blank microparticles, the same procedure was followed, except no rapamycin was added.

B. Ac-DEX Particle Formation via Sonication

To synthesize sonicated microparticles, Ac-DEX (100 mg) and rapamycin (1 mg) were dissolved in DCM and added to 3% PVA. The mixture was sonicated for 30 seconds (Misonix Ultrasonic Liquid Processor, amplitude 2, 4, or 8%, duty cycle 50%) and the formed emulsion was immediately pipetted into a spinning solution of 0.3% PVA. The same washing procedure was performed as described for the homogenized microparticles.

3.2.3 Physical and Chemical Characterization of Particles

A. Scanning Electron Microscopy (SEM) and Particle Size Analysis

To study the surface morphology of the prepared formulation, samples were imaged using FEI NOVA Nano SEM 400. The size and size distribution of the prepared microparticles were measured by using light scattering size analyzer and zeta potential distribution was measured using ZetaPALS Zeta Potential Analyzer (Brookhaven). The samples were prepared and analyzed using the same technique as described in chapter 2.
B. Ac-DEX Particle Degradation Analysis

Blank Ac-DEX microparticles were suspended in sodium acetate buffer (pH 4.90) or in PBS (pH 7.4). The samples were kept at 37°C on a shaker plate at 150 RPM. At various timepoints (0 to 240 h), aliquots were withdrawn and centrifuged (15,000 x g, 4 °C, 5 min). Microparticles collect on the sides of the tubes, so when aliquots were withdrawn, the mass of microparticles in the medium was unchanged. The supernatants were then stored at -20°C in a 96-well polystyrene plate. The supernatants were analyzed with a microplate reductometric bicinchoninic acid based assay in triplicates according to the manufacturer’s protocol (Protein Assay Kit; Pierce, Rockford, IN). The resultant absorbances were measured at 562 nm with a platereader (FlexStation 3 Benchtop Multi-Mode Microplate Reader). The assay measured the amount of the degradation product dextran in the supernatant with time. A curve was fit to this data, and the polymer degradation t_{1/2} was defined to be the time on the curve at which 50% of the Ac-DEX had degraded.

C. Determination of Rapamycin Loading

Rapamycin-loaded Ac-DEX microparticles were fully dissolved in acetonitrile (5 mg/mL). The solution was vortexed, bath sonicated for 10 min, centrifuged (5 min, 15,000 x g, 4 °C) and the supernatant was withdrawn and analyzed with HPLC (Agilent 1100 series, column: C18*5μm*150mm*4.6mm, 1 mL/min, 20 min, mobile phase: 65% acetonitrile/35% water) at 278 nm. The experimental rapamycin concentration in each particle was determined by comparison with a standard curve of rapamycin in
acetonitrile. The encapsulation efficiency was determined by the equation: 

\[ \text{EE} (\%) = 100 \times \frac{\text{experimental rapamycin concentration}}{\text{theoretical rapamycin concentration}}. \]

Also, the rapamycin percent weight loading (w/w) was determined by the equation: 

\[ \text{weight loading} (\%) = 100 \times \frac{\text{loaded rapamycin in mg}}{\text{amount of polymer in mg}}. \]

**D. Drug Release from Microparticles**

The release of rapamycin from Ac-DEX microparticles was performed by collecting aliquots in the same manner used for the degradation analysis. The aliquots were centrifuged (15,000 x g, 4 °C, 5 min) and the supernatants were analyzed via HPLC at 278 nm, using the same parameters as described in determining the encapsulation efficiency. After plotting the concentration of rapamycin versus time, a curve was fit to the data and the drug release \( t_{1/2} \) was defined to be the time on the curve at which 50% of the encapsulated rapamycin was released.

**3.2.4 Cell Studies**

**A. Cell Study Preparation**

Macrophages (RAW 264.7; ATCC, Manassas, VA) were grown and maintained as per the manufacturer’s guidelines and described in chapter 2.

Macrophages were plated at concentration of 1 x \( 10^4 \) cells/mL and incubated for 24 hours in two 96-well plates. After 24 hours, the media in each well was replaced with media containing optimized rapamycin-loaded Ac-DEX microparticles ranging from 0.001 to 0.1 μg rapamycin/mL, blank Ac-DEX microparticles, or free rapamycin at the
same concentrations, all in triplicate. The LPS (100 ng/mL) was added to the media in one plate to promote nitric oxide (NO) production, whereas the media used in the other plate contained no LPS to act as a control. The cells were incubated for another 24 hours at these conditions and nitric oxide production was assessed.

B. Nitrite Analysis

A Griess assay was performed as described in chapter 2. The NO concentrations for the microparticles and free drug were standardized with respect to 0 μM.

C. Cell Toxicity Analysis

The MTT assay was performed as described in chapter 2. The absorbance measurements were standardized with respect to 0 μM to obtain percent viability.

3.3 RESULTS AND DISCUSSION

3.3.1 71k Versus 10k Ac-DEX Particle Degradation

Figure 2.1 shows the reaction scheme to synthesize Ac-DEX. The average diameter of the microparticles was 829 nm with a polydispersity of 0.17, indicating that the particles were fairly uniform, and the shape of the particles was spherical with no observable porosity at higher magnification.

In Figure 3.1, the degradation of 71k and 10k Ac-DEX microparticles at two different pHs (4.9 – phagosomal, and 7.4 – physiological) is shown. Table 3.1 also
quantifies various properties of the Ac-DEX polymer and microparticles. The 71k Ac-DEX microparticles require about three times longer to fully degrade at pH 5 than 10k Ac-DEX particles at the same pH. Furthermore, 71k Ac-DEX microparticles exhibit an initially quick degradation period that results in approximately 50% degradation in 40 hours. This is followed by a slower degradation until about 210 hours when the particles become fully degraded. At pH 7.4, the 71k Ac-DEX particles degrade much slower and in a more linear fashion, with only approximately 20% degradation in 240 hours. The results from the 10k Ac-DEX particles mirror the degradation profiles of 10k Ac-DEX particles as reported by Broaders et al. (Broaders, et al. 2009). Qualitatively, the particle degradation in both pH 5 and pH 7.4 are in agreement with the time-lapse pictures showed by Bachelder et al. (2008); i.e. the suspension of particles and buffer change from cloudy-white to a transparent solution as the particles degrade.

The initial quick degradation of Ac-DEX microparticles is due to hydrolysis of acyclic acetal groups on the polymer, followed by the steady and rate-limiting hydrolysis of the cyclic acetal groups (Broaders, et al. 2009). In addition, Figure 3.1 indicates that increasing the molecular weight of the polymer also varies the degradation rate, since both the 10k and 71k Ac-DEX were synthesized with the same reaction time of 6 hours. The lengthened degradation of Ac-DEX with a higher molecular weight is consistent with other polymers such as PLGA and poly(lactic acid) (Alexis (2005)).

As shown in Table 3.1, NMR results indicated that the 71k Ac-DEX had a cyclic acetal coverage of 0.86 per glucose residue, with a relative cyclic acetal coverage of 61%, whereas the 10k Ac-DEX had a cyclic acetal coverage of 0.89 per glucose residue and a
relative cyclic acetal coverage of 64%. Because the cyclic acetal coverage of the 10k and 71k Ac-DEX were approximately the same, the lengthened degradation rate is possibly due to the higher viscosity and longer chain length of the 71k Ac-DEX and not differing cyclic acetal coverage. In addition to Broaders et al. research displaying the tunable degradation rates of 10k Ac-DEX polymer via simple variance of reaction time (2009); the range of degradation rates can also be altered by changing the MW of the dextran.

3.3.2 Encapsulation of Rapamycin in 71k and 10k Ac-DEX Microparticles

The default 71k Ac-DEX microparticles were determined to have an encapsulation efficiency (EE) of rapamycin of 64 ± 2%, whereas the 10k Ac-DEX microparticles had an EE of 19 ± 9%. The positive correlation between EE and molecular weight is in contrast to PLGA, for which the molecular weight and encapsulation efficiency are generally inversely related (Hans and Lowman 2002). One such example of this trend is the increasing EE of encapsulated albumin for decreasing PLGA molecular weight (Song, et al. 1998). This is, however, not necessarily indicative of all polymers and all types of encapsulated drugs; lowering the molecular weight of PLGA increases the number of its carboxylic end groups, possibly resulting in more covalent bonding with the drug and thus a better EE (Fernandez-Carballido, et al. 2004). The polymer chitosan exhibits a positive correlation between EE and molecular weight, perhaps because the longer chains of polymer make entrapment of drug easier (Xu and Du 2003). In addition to longer chain lengths, the greater viscosity of 71k Ac-DEX may facilitate encapsulation of the drug by preventing the diffusion of the drug into the
continuous phase after emulsification. 71k Ac-DEX is also less soluble in DCM solvent than 10k Ac-DEX, and lower solubility of polymer in organic solvent leads to faster solidification of microparticles resulting in a higher EE (Yeo and Park 2004). Although it can be concluded that higher molecular weight Ac-DEX results in a higher EE of rapamycin, this trend may be different with other types of drugs due to different drug molecular weights, functional groups, and hydrophilicities.

3.3 Optimization of 71k Ac-DEX Microparticles

Based on previous research by Mao et al. (2007), seven parameters in the single-emulsion particle formation procedure were chosen to be varied in order to optimize the rapamycin-loaded Ac-DEX microparticles in terms of size, yield, and encapsulation efficiency. The parameters included Ac-DEX molecular weight, Ac-DEX concentration, continuous phase PVA concentration, continuous phase PVA volume, spinning PVA concentration, spinning PVA volume, and either homogenization speed or sonication amplitude. Each particle was compared to the default particle, which was synthesized using the parameters given in the particle preparation section. Table 2 presents the yield, encapsulation efficiency, size, and polydispersity for each particle synthesized with the given parameters varied.

Of the seven parameters investigated, the most apparent trend was observed in varying the MWs of Ac-DEX, as seen in Figure 3.2, and no clear correlations were generally observed between the other six parameters and EE, yield, and particle size. As discussed previously, 71k Ac-DEX microparticles had a superior EE to 10k Ac-DEX
microparticles (64 ± 2% compared to 19 ± 9%). However, microparticles synthesized from even higher MWs of Ac-DEX of 150k and 2 million did not increase either EE or yield. This could be attributed to fact that the 150k and especially the 2m Ac-DEX exhibited high enough viscosity that the homogenizer was unable to impart sufficient energy to form a perfect emulsion of Ac-DEX, drug, PVA, and solvent. Regardless, the 150k and 2m Ac-DEX would likely have such lengthy degradation times that their microparticles would be impractical for the delivery of immunosuppressive agents as proposed.

Although the yields and encapsulation efficiencies were generally consistent, there were some deviations. For example, the particle system with 3% PVA spinning solution had a yield of over 100%, likely due to residual shards of PVA remaining in the microparticles since the PVA concentration was 10 times larger than the default value. Also, the particle system with 2% sonication amplitude exhibited a low yield of 21% and EE of 15 ± 2%, possibly because too little energy was imparted by the sonicator to effectively emulsify the solution.

The optimized particle was determined to be the particle system synthesized via sonication with 8% amplitude. This particle synthesis method had 63% yield, 91 ± 1% EE, 0.91% weight loading, and an average particle diameter of 473 nm. The weight loading of these particles were much higher than reported weight loadings of rapamycin-loaded PLGA microparticles and microparticles, while also having slightly higher EE. Haddadi et al. achieved 81 ± 8% EE for rapamycin-loaded PLGA microparticles 150 to 450 nm in diameter with a 0.05% weight loading (Haddadi, et al. 2008). Jhunjhunwala et
al. reported 74 ± 5% EE for rapamycin-loaded PLGA microparticles 3 to 4 μm in diameter with a 0.37% weight loading (Jhunjhunwala, et al. 2009).

3.4 Characterization of Drug Release for Optimized Particle System

The drug release of the optimized rapamycin-loaded Ac-DEX particle system was characterized in acidic and pH-neutral conditions, and the t1/2 values are shown in the last column of Table 1. Due to the acid sensitivity of Ac-DEX, rapamycin was released at a much faster rate at pH 5 conditions (50% in 45 hr), which simulates phagosomal conditions, compared to minimal release in the pH 7.4 conditions (5% in 240 hr), which simulates extracellular conditions. Since rapamycin has no ionizable functional groups, pH has no effect on its aqueous solubility (Simamora, et al. 2001). Therefore, it may be concluded that the pH-sensitivity of Ac-DEX microparticles is responsible for the differing drug release rates and not pH-dependent drug solubility.

Additionally, it should be noted that the rapamycin is very poorly soluble in the aqueous buffers (2.6 μg/mL) (Simamora, et al. 2001). Haddadi et al. circumvented this problem for PLGA microparticles by using a 9:1 v/v buffer: ethanol mixture in which rapamycin is more soluble (Haddadi, et al. 2008); however, Ac-DEX is soluble in ethanol, so aqueous pH 5 and pH 7.4 buffers were used in these studies. Moreover, it was important to most accurately model in vivo conditions, so using ethanol or any other organic solvent would not be appropriate.

The pH-sensitivity of the drug release implies that rapamycin-loaded Ac-DEX microparticles that passively target phagocytes would release their drug payload upon
phagocytosis, and drug would be released at a significantly reduced rate while residing in the extracellular environment. The pH-sensitive release of rapamycin from the Ac-DEX microparticles also suggests that the maximum amount of drug could be delivered intracellularly to the macrophage with a minimum amount of drug being released in other pH-neutral locations in the body, meaning drug-loaded Ac-DEX microparticles could both maximize the efficacy of the drug and minimize systemic and local toxicity. Therefore, a smaller amount of drug could be needed, which would reduce both harmful side-effects and cost. Bachelder et al. have demonstrated dose sparing by treating macrophages and DCs with imiquimod-encapsulated Ac-DEX microparticles, finding that less drug was required when encapsulated in Ac-DEX microparticles (Bachelder, et al. 2010).

3.5 NO Production in LPS-stimulated RAW Macrophages Exposed to Rapamycin-Loaded Ac-DEX Microparticles

Two commonly used methods to ensure phagocytosis of the microparticles are ligand-mediated targeting and passive targeting via particle sizing. Beaudette et al. have previously shown that it is possible to chemoselectively ligate alkoxyamine-bearing molecules such as cell-penetrating peptides to Ac-DEX particles (Beaudette, et al. 2009). Other researchers have proposed ligand-mediated targeting with polymeric particles to increase uptake in phagocytic cells. Hamdy et al. ligated mannan, a toll-like receptor 4 (TLR-4) agonist, to the surface of antigen-loaded PLGA microparticles and noted a non-statistically significant increase in particle uptake (< 5%) in DCs (Hamdy, et al. 2011).
For tolerance applications, TLR activation would be counter-productive and exacerbate the disease, but this study shows the impact of ligand-mediated particle uptake by phagocytic cells. Alternatively, the passive targeting of phagocytes has been well-established to be dependent on the size of the particle. Phagocytes like DCs and macrophages will phagocytose microparticles less than approximately 10 μm in diameter (Johansen, et al. 2000). Unlike most cell types in vivo, macrophages have phagocytic properties (Foged, et al. 2005) allowing them to internalize particles larger than 100 nm (Foged, et al. 2005; Hirota, et al. 2007). Studies have shown that particles in the range of 100-1,000 nm are more likely to be phagocytosed by CD11c- cells (macrophages) than CD11c+ cells (DCs); results indicated that smaller particles are cleared by resident macrophages (100-1,000 nm) and larger particles are predominately transported by DCs from the injection site to the lymph nodes (Manolova, et al. 2008). Therefore, it was determined that the diameter of optimized rapamycin-loaded Ac-DEX microparticles (473 nm) was ideal for passive targeting of macrophages. Although ligation to the particles is possible, phagocytosis could be best optimized through passive targeting and particle sizing; the small increase (< 5%) of particle uptake with ligation shown by Hamdy et al. is only a marginal improvement (Hamdy, et al. 2011). Additionally, preliminary in vivo work has shown that downstream signaling such as production of antigen specific antibodies and T-cells without ligation (data not shown). For these experiments, microparticles were injected into the sub-cutaneous area of the flank or nape of the neck. Ac-DEX microparticles can also be introduced through both needle and
needle-free methods, including intraperitoneal, intravascular, intratracheal, or internasal introduction.

Free rapamycin and Ac-DEX microparticles with and without encapsulated rapamycin were incubated with RAW macrophages, and LPS was added or not added to evaluate the formulations’ efficacy. Macrophages identify LPS through TLR-4 on their surface (Paul, 1994) and phagocytose the microparticles through non-opsonic receptors. The addition of LPS was used to stimulate an inflammatory response in the macrophages and thereby induce pro-inflammatory signaling such as nitric oxide production. The production of nitric oxide and related reactive oxygen species by macrophages has also been associated with diseases such as atherosclerosis and sepsis (Droge 2002). Rapamycin, an immunosuppressant, should reduce inflammatory responses, thereby decreasing nitric oxide levels. Thus, the efficacy of the drug is inversely related to the concentration of nitric oxide measured.

Figure 3.3 shows the nitric oxide production from RAW macrophages as determined with the Griess assay. For LPS-stimulated RAW macrophages, there was a dose-dependent response for both the rapamycin-loaded Ac-DEX microparticles and free rapamycin. Although dose sparing was not observed, there was no significant difference in nitric oxide production in LPS treated macrophages between the Ac-DEX microparticles and free drug, with the sole exception at the rapamycin concentration of 0.005 μM. There was constant and low nitric oxide production from RAW macrophages which were not stimulated with LPS. Additionally, Figure 3.4 shows that Ac-DEX microparticles and free rapamycin demonstrate favorable cell viability with and without
LPS, indicating that the rapamycin and microparticles had no cytotoxic effects on the cells and variance in nitric oxide production was not the result of disparity in cell viability.

The Griess assay results indicate that immunosuppression can be achieved with rapamycin-loaded Ac-DEX microparticles just as efficiently as with free rapamycin. The Ac-DEX microparticle advantage over free drug, however, is that the drug could be passively targeted to the macrophages in vivo rather than delivered systemically throughout the body. Furthermore, once the microparticles are phagocytosed, the drug payload would be released in a burst fashion intracellularly in the phagosomal acidic environment. Unlike PLGA microparticles, Ac-DEX microparticles have a relatively low release of rapamycin at pH 7.4, which could potentially reduce toxic systemic side-effects of the drug in vivo. The encapsulation of immunosuppressive drugs with harmful side-effects like rapamycin in microparticles made from acid-sensitive, biocompatible polymers like Ac-DEX could improve health in patients with autoimmune diseases or patients undergoing organ transplants.
Figure 3.1: Degradation rates of 10k and 71k Ac-DEX microparticles in acidic and pH-neutral buffers as determined by a reductometric bicinchoninic acid based assay, which measured the amount of degraded dextran in the sample. Each data point is presented as the mean ± standard error mean (n=3).
Figure 3.2: Effect of molecular weight of Ac-DEX on yield, encapsulation efficiency (EE), and diameter of the resultant particles. Yields and diameters are presented as means, and EEs are presented as the mean ± standard error mean (n=3). Increasing the molecular weight resulted in decreasing the yield and increasing the diameter of the particles; EE is maximum at 71k to 150k.
Figure 3.3: Nitric oxide production from RAW 264.7 macrophages incubated with optimized 71k Ac-DEX particles loaded with and without rapamycin, and free rapamycin. Each bar is presented as the mean ± standard error (n=3). The figure shows a dose-dependent response for LPS-stimulated RAW macrophages (w/ LPS), and also includes no LPS (w/o LPS) as a control.
Figure 3.4: Cell viability for Ac-DEX particles and free rapamycin with and without LPS stimulation. Each bar is presented as the mean ± standard error mean (n=3).
Table 3.1: Ac-DEX polymer and microparticle properties, including molecular weight (MW), reaction time, cyclic acetal coverage (per glucose residue and relative) as determined by NMR analysis, and polymer degradation $t_{1/2}$ (the time for 50% of the microparticle to degrade) and drug release $t_{1/2}$ (the time for 50% of encapsulated rapamycin to be released) for pH 5 and pH 7.4 buffers. For pH 7.4 buffers, 50% polymer degradation and drug release were not observed, so the percentage of polymer degraded or drug released is reported at its respective time.

<table>
<thead>
<tr>
<th>MW (kDa)</th>
<th>Reaction Time (hr)</th>
<th>Cyclic Acetal Coverage (Per Glucose Residue)</th>
<th>Cyclic Acetal Coverage (Relative)</th>
<th>Polymer Degradation $t_{1/2}$ (hr)</th>
<th>Optimized Drug Release $t_{1/2}$ (hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>6</td>
<td>0.89</td>
<td>64%</td>
<td>45</td>
<td>5% at 75</td>
</tr>
<tr>
<td>71</td>
<td>6</td>
<td>0.86</td>
<td>61%</td>
<td>40</td>
<td>20% at 240</td>
</tr>
</tbody>
</table>

For pH 7.4 buffers, 50% polymer degradation and drug release were not observed, so the percentage of polymer degraded or drug released is reported at its respective time.
Table 3.2: Yield, encapsulation efficiency (EE), diameter, and polydispersity of all twenty batches of Ac-DEX particles. EE is presented as the mean ± standard error mean (n=3). Each parameter changed is given with its respective default value in parenthesis. * Elevated yield is likely due to contamination from PVA solution.

<table>
<thead>
<tr>
<th>Parameter Changed (Default value)</th>
<th>Yield (%)</th>
<th>EE (%)</th>
<th>Diameter (nm)</th>
<th>Polydispersity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Default, unloaded (Blank)</td>
<td>61</td>
<td>0</td>
<td>1034</td>
<td>0.23</td>
</tr>
<tr>
<td>Default, loaded Rapamycin</td>
<td>72</td>
<td>64 ± 2</td>
<td>1027</td>
<td>0.19</td>
</tr>
<tr>
<td>Ac-DEX Concentration (100 mg/mL)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>50 mg/mL</td>
<td>78</td>
<td>31 ± 5</td>
<td>829</td>
<td>0.17</td>
</tr>
<tr>
<td>200 mg/mL</td>
<td>57</td>
<td>43 ± 5</td>
<td>1396</td>
<td>0.34</td>
</tr>
<tr>
<td>Continuous Phase Volume (17 mL)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 mL</td>
<td>49</td>
<td>79 ± 11</td>
<td>696</td>
<td>0.29</td>
</tr>
<tr>
<td>25 mL</td>
<td>57</td>
<td>67 ± 3</td>
<td>946</td>
<td>0.14</td>
</tr>
<tr>
<td>Spinning PVA volume (40 mL)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100 mL</td>
<td>71</td>
<td>79 ± 6</td>
<td>1093</td>
<td>0.19</td>
</tr>
<tr>
<td>250 mL</td>
<td>60</td>
<td>67 ± 6</td>
<td>1200</td>
<td>0.29</td>
</tr>
<tr>
<td>RPM Homogenization speed (20500 RPM)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8850 RPM</td>
<td>66</td>
<td>71 ± 7</td>
<td>1123</td>
<td>0.24</td>
</tr>
<tr>
<td>23750 RPM</td>
<td>64</td>
<td>44 ± 5</td>
<td>972</td>
<td>0.20</td>
</tr>
<tr>
<td>Continuous Phase PVA concentration (3%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.3% PVA</td>
<td>52</td>
<td>55 ± 8</td>
<td>338</td>
<td>0.24</td>
</tr>
<tr>
<td>1% PVA</td>
<td>54</td>
<td>81 ± 9</td>
<td>2187</td>
<td>0.28</td>
</tr>
<tr>
<td>Spinning Solution PVA Concentration (0.3%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1% PVA</td>
<td>60</td>
<td>72 ± 13</td>
<td>1039</td>
<td>0.16</td>
</tr>
<tr>
<td>3% PVA</td>
<td>102*</td>
<td>52 ± 4</td>
<td>1038</td>
<td>0.16</td>
</tr>
<tr>
<td>Molecular Weight Ac-DEX (71k)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10k Ac-DEX</td>
<td>76</td>
<td>19 ± 9</td>
<td>446</td>
<td>0.30</td>
</tr>
<tr>
<td>150k Ac-DEX</td>
<td>59</td>
<td>63 ± 19</td>
<td>1631</td>
<td>0.11</td>
</tr>
<tr>
<td>2m Ac-DEX</td>
<td>46</td>
<td>22 ± 4</td>
<td>2405</td>
<td>0.52</td>
</tr>
<tr>
<td>Sonication Amplitude (n/a)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2%</td>
<td>21</td>
<td>15 ± 2</td>
<td>370</td>
<td>1.05</td>
</tr>
<tr>
<td>4%</td>
<td>50</td>
<td>48 ± 14</td>
<td>132</td>
<td>0.09</td>
</tr>
<tr>
<td>8% (optimized)</td>
<td>63</td>
<td>91 ± 1</td>
<td>473</td>
<td>0.66</td>
</tr>
</tbody>
</table>
CHAPTER 4

PHYSIO-CHEMICAL CHARACTERIZATION OF ACETALATED DEXTRAN MICRO PARTICLES AFTER STORAGE

4.1 INTRODUCTION

Use of vaccines is considered to be one of the most successful interventions in the field of medicine (Heininger 2011). Current research has been focused on developing vaccines containing polymer which are capable of reducing the required number of doses via controlled release. One of the potential benefits associated with the use of polymer to encapsulate vaccine components is their thermal stability, especially for application in developing countries with a tropical climate (Wiggan, et al. 2011).

Due to its inherent biodegradability and low toxicity poly (lactic-co-glycolic acid) (PLGA) is one of the most widely used vaccine carriers. Based on the amount of lactic acid present, the degradation rate of PLGA varies from weeks to months, and it is not pH-sensitive (Hamdy, et al. 2011)(Lu, et al. 2000)(Miller, et al. 1977). Also, hydrolysis of the ester bonds in PLGA results in the creation of an acidic microenvironment (~pH 1.5), which is harmful for the stability of recombinant proteins used in immunotherapy (Mallapragada and Narasimhan 2008). Furthermore, it would be ideal to have a material
sensitive to the acidic environment, such as is present in the phagosomal pH of macrophages, and dendritic cells, resulting in accelerated release of protein (Haining, et al. 2004). Due to these setbacks with PLGA, new polymeric carriers were designed for vaccine applications and one such polymer developed was acetalated dextran (Ac-DEX).

Ac-DEX particles can be prepared using probe sonication or homogenization techniques as described previously (Kauffman, et al. 2012). The probe sonicator works on the principle of converting the electric energy into vibrational energy, which in turn is transferred to the solution being sonicated (Anonymous 2007b). On the other hand, a homogenizer consists of positive displacement pump, which forces the emulsion through a valve assembly resulting in formation of homogenous emulsion (Anonymous 2009). Although preparation of particles using various techniques has been extensively studied, there can exist problems regarding particle stability (Sameti, et al. 2003). Among them is to maintain the stability of physiochemical behavior of both polymer and encapsulated ingredient over time at different storage conditions with time.

The main objective of this work is to study the physical and chemical characterization of protein loaded Ac-DEX particles that were prepared by sonication or homogenization techniques and stored at different temperatures (-20°C, 4 °C, 25 °C, and 45 °C) over a period of 3 months. Horseradish peroxidase (HRP), an enzyme with four lysine residues and a molecular weight of 44-kDa was chosen as a model enzyme to be encapsulated into Ac-DEX. HRP is heme-containing oxidoreductase, isolated from horseradish roots capable of reducing hydrogen peroxide (Temocin and Yigitoglu 2009). On days 0, 15, 30, 60 and 90 the particles were taken out and various physical and
chemical characterization studies were carried out. To view the morphological appearance of the Ac-DEX loaded with HRP, scanning electron microscopy (SEM) was used. To determine the particle size and surface charge on the particles, dynamic light scattering and zeta potential instruments were used and to check the activity of encapsulated enzyme over time, tetramethyl benzidine (TMB) was used. Additionally, on day 0, 45 and 90 differential scanning caloriometry was carried out to investigate changes, if any, in polymer composition due to prolonged exposure to various storage conditions.

4.2 MATERIALS AND METHODS

4.2.1 Materials

The following materials were obtained from Sigma Aldrich (St. Louis, MO): Horseradish peroxidase type VI (44-kDa, EC 1.11.1.7), 3,3’,5,5’-Tetramethylbenzidine (TMB) liquid substrate system, tetraethylamine (TEA, ≥ 99%), were obtained from Sigma Aldrich (St. Louis, MO) and the remaining materials used were similar to those described in previous chapters. All the biomaterials, reagents, solvents purchased from commercial sources and used without further purification.

4.2.2 Synthesis and NMR Analysis of Ac-DEX

Synthesis of Ac-DEX and NMR analysis was carried out as described in previous chapters and elsewhere (Kauffman, et al. 2012). Lyophilized dextran (MW = 71,000 Da) was used for this project.
4.2.3 Preparation of PLGA/Ac-DEX particles encapsulating HRP by probe sonication method

Ac-DEX loaded HRP particles were prepared using a double-emulsion water/oil/water (w/o/w) method similar to that described by Broaders et al. Briefly, HRP solution in phosphate buffer saline (PBS) was added to an organic phase (dichloromethane) containing Ac-DEX or PLGA (1% weight loading). The resulting mixture was probe sonicated in a ice bath for 5 sec (Branson Sonifier 450, with a 0.5 in. flat tip with an output setting of 4 and a duty cycle of 10%). To this primary emulsion a 3% polyvinyl alcohol (PVA) solution was added and probe sonicated again for 30 seconds to form a w/o/w emulsion. The formed primary emulsion was then immediately added to second PVA solution (0.3% w/w in PBS) and stirred for 3 h to evaporate the organic solvent. To recover the nanoparticles, each formulation was subjected to centrifugation at 10 °C (10 min x 16500 rcf, Beckman RA-21, Los Angeles, CA, USA). The supernatant was discarded, and the resulting sediment was washed twice with basic water to remove excess PVA. Finally, the particles were freeze-dried at -70 °C and 25 mTorr for 24 hrs (Labconco, Kansas City, MO, USA) and stored under specified conditions until further use. Blank particles were prepared using the similar method as described above but without the addition of enzyme.
4.2.4 Preparation of Ac-DEX particles encapsulating HRP by homogenization method

For these particles, HRP dissolved in PBS was added to an organic phase (dichloromethane) containing Ac-DEX (1% weight loading). The resulting mixture was homogenized for 30s using Polytron PT 10-35 Homogenizer (Westbury, NY). To this primary emulsion 3% polyvinyl alcohol (PVA) solution was added and homogenized again for 30 seconds to form w/o/w emulsion. The formed primary emulsion was then immediately added to second PVA solution (0.3% w/w in PBS) and stirred for 3h to evaporate the organic solvent. To recover the particles, each formulation was subjected to centrifugation at 10°C (10 min x 9000 rcf, Beckman RA-21, Los Angeles, CA, USA). The supernatant was discarded; the particles were washed twice with basic water, freeze-dried at -70 °C and 25mTorr for 24hrs (Labconco, Kansas City, MO, USA) and stored under specified conditions until further use. Blank particles were prepared using the similar method as described above but without the addition of enzyme.

4.2.5 Baseline Enzyme Activity

The activity of HRP encapsulated into the particles was determined by using tetramethyl benzidine (TMB) reagent. TMB is an analog of benzidine and is used as a substrate for enzyme immunoassays (Le Goff, et al. 2011). Ac-DEX particles containing HRP were suspended in triplicate in 0.3M sodium acetate buffer (pH 5) for 24 hours. After this, aliquots were withdrawn and placed in a 96-well plate. Then, the TMB reagent was added to the samples, followed by the addition of 0.1N sulphuric acid
solution after nine minutes to stop the reaction. The activity of HRP was determined by measuring the absorbance at 450nm via a plate reader (FlexStation 3 Benchtop Multi-Mode Microplate Reader). Blank Ac-DEX particles were analyzed in a similar fashion to determine background absorbance. A standard curve of HRP in sodium acetate buffer was prepared to relate absorbance to drug concentration.

Additionally on a specific day of analysis, particles as well as free HRP stored at different temperatures were suspended at 1mg/ml concentration in sodium acetate buffer (pH 5.0) and incubated at 37°C on a shaker plate (150 rpm) for 24hrs, after which aliquots were withdrawn and analyzed for HRP content using TMB reagent as described previously. The activity of HRP at a particular time point was normalized to that on day ‘0’ and the results were calculated using the following equation. The activity obtained on day ‘0’ was considered to be 100% for free and HRP loaded particles separately.

\[
\text{Relative Activity (\%)} = \frac{\text{Enzyme activity on respective day}}{\text{Enzyme activity on Day "0"}}
\]

4.2.6 Degradation studies

Degradation studies were carried out similarly as described in previous chapters.

4.2.7 Activity of Released HRP

The activity of the released HRP from Ac-DEX particles was performed by collecting aliquots in the same manner as described for the baseline enzyme activity
analysis. The aliquots were centrifuged (15,000 x g, 4 °C, 5 min) and the supernatants were analyzed using TMB reagent as described in section 2.2.5.

4.2.8 Physical and Chemical Characterization studies of particles

A. Scanning Electron Microscopy (SEM), Particle Size Analysis and Zeta Potential

To study the surface morphology of the prepared formulation, SEM and Zeta potential was performed using the same technique as explained in previous chapters.

B. Differential Scanning Calorimetry (DSC)

To understand the nature of polymer at various storage temperatures glass transition temperature was determined using DSC (Mettler Toledo, UK) as described in Chapter 2. DSC scans of blank particles prepared by probe sonication and homogenization techniques immediately after lyophilization were used as controls.

4.3 RESULTS AND DISCUSSION

4.3.1 Preparation and Characterization of HRP encapsulated Acetalated Dextran Microparticles

The Ac-DEX polymer used in this study was prepared using 71k dextran and had relative cyclic acetal coverage of 43%. Table 1 shows the yield and baseline enzyme activity of HRP encapsulated Ac-DEX particles prepared by probe sonication and
homogenization techniques. Particles prepared using the homogenization technique showed a higher yield (89%) and increased baseline enzyme activity (86%) of HRP as compared to those prepared by probe sonication method, 55% and 54% respectively. The smaller size (100-200 nm) of probe sonicated particle is likely the main cause of the lower yield (due to incomplete settling down of particles at the time of centrifugation). Also proteins encapsulated into particulates using emulsion techniques, become susceptible to denaturation, aggregation, oxidation and cleavage, particularly at the aqueous phase-solvent interface. Hence it is anticipated that the high energy associated with probe sonication as compared to mechanical homogenization technique may have caused more denaturation of HRP in the present study.

Ac-DEX microparticles prepared by both probe sonication and homogenization, techniques showed faster degradation at phagosomal pH ($t_{1/2} < 3\text{hr}$) as compared to physiological pH condition ($t_{1/2}=72-75\text{hrs}$) (Table 4.1). The $t_{1/2}$ values obtained here suggest that Ac-DEX microparticles take about seventy times longer fully degrade at pH 7.4 as compared to that at pH 5.0. Degradation of polymer at a lower pH facilitates release of agents into the phagosomal compartments of dendritic cells and macrophages. Degradation profiles of Ac-DEX particles are shown in Figure 4.1. Drug loading had no effect on the degradation times of the polymer.

### 4.3.2. HRP Enzyme Activity

The activity of free or encapsulated HRP was determined by using TMB as a substrate similar to one described by Le Goff et al. (Le Goff, et al. 2011). Hydrogen
peroxide, in the presence of peroxidases oxidizes TMB yielding a characteristic blue color reaction product. This reaction is stopped with the addition of sulfuric acid, resulting in characteristic yellow color that can be determined spectrophotometrically.

Figure 4.2 shows the influence of the particle preparation method on the stability of HRP encapsulated acetalated dextran particles over time. Percent change in enzyme activity was measured over time (up to 90 days) for particles prepared by probe sonication, homogenization methods and HRP at predefined temperatures (-20°C, 4°C, 25°C, 45°C). HRP loaded Ac-DEX particles show higher thermal stability with time when compared to HRP alone. The maximum denaturation of enzyme in loaded particles, prepared using both the methods, took place within first day 30 days and later stabilized with time whereas for free HRP the activity kept on decreasing gradually over time at all temperatures. Its hypothesized that the initial loss of HRP enzyme activity seen in the profiles might be due to the denaturation of enzyme loosely bound to the surface of the particles. Higher stabilization of enzyme activity in encapsulated form at different storage temperatures might be due to the increased conformational stability provided by the protective sheath of the polymer surrounding the enzyme molecule (Temocin and Yigitoglu 2009).

4.3.3 Release Studies

Figure 4.3 shows the activity of released HRP encapsulated Ac-DEX particles prepared by both techniques at pH 5.0 and pH 7.4. Due to acid sensitivity of Ac-DEX, the HRP was released at a faster rate at low pH when compared to physiological condition.
Faster degradation of particles at lower pH indicates ensures maximum amount of drug being delivered intracellularly to the macrophages and dendritic cells with a minimum amount of drug being released to extracellular spaces, thus maximizing the efficacy of the drug and minimizing the systemic and local toxicity. A thorough examination of activity of released HRP and degradation profiles reveals that the trends and rates are quite similar at a low pH condition. However, at a physiological pH, 60% release of HRP was observed after one day even though only 40% of polymer had degraded. This supports the typically understood phenomena that release of protein from degradable polymeric particles depends on two factors: 1) polymer degradation and 2) diffusion/swelling. Polymer degradation appears to be the major factor affecting HRP release under acidic conditions, since 90% of the polymer degrades within 3 hrs. However, at pH 7.4, both polymer degradation and diffusion/swelling, appear to influence the activity of HRP released as indicated by a much higher release of drug than predicted based on degradation profile.

4.3.4 Physical and Chemical Characterization studies of particles

A. Scanning Electron Microscopy

Figure 4.4 and 4.5 show the SEM images of HRP encapsulated Ac-DEX particles prepared by probe sonication and homogenization methods. All the particles were stored at different temperatures (-20°C, 4°C, 25°C, 45°C) and imaged on day 0, 30, 60, and 90. For day 0 data, the particles were imaged just after taking them out of the lyophilizer. Both of the methods produced spherically shaped particles. However, the particles
prepared with the sonication method were smaller when compared to the homogenization technique. High energy produced during the probe sonication is most likely responsible for this observation. The particles prepared using homogenization technique were porous in nature. This may be due to the fusion of the water droplets trapped inside the particles during the stirring phase of emulsion preparation followed by evaporation, which in turn leaves empty spaces that result in the formation of pores. This coalescence of internal water droplets might occur because of the high surface energy (72.8 ml/m²) of water (Yang et al., 2000; Yang et al., 2001). This porous nature might be present even in probe sonicated particles but cannot be seen with the current SEM imaging technique. In general, all the particles maintained spherical morphology over the entire duration of study without any signs of agglomeration or degradation. The homogenized particles maintained their porosity till day 90.

Additionally, the stability of blank PLGA particles and blank Ac-DEX particles prepared by homogenization techniques stored at 45°C (Figure 4.6) was also investigated. On day 0 particles prepared by both methods were spherically shaped but by day 28, PLGA particles lost their spherical shape and attained a sheet-like structure because the glass transition temperature for PLGA is around 45 - 50°C (Singh, et al. 2004). On the contrary, Ac-DEX particles maintained their spherical nature even at high temperatures. Also, addition of HRP didn’t have any effect on the structure of Ac-DEX.
B. Particle Size and Zeta Potential

Table 4.2 shows the particle size (nm) and polydispersity index values for HRP encapsulated Ac-DEX particles measured using DLS. DLS measurements do not align with the sizing observed in SEM micrographs. Because increases in size over time across all the temperatures and fabrication methods were observed, particle size is not consistent with SEM micrographs. Also, a positive control was not used in the experimental runs and hence the data obtained with particle size analysis might not reflect the actual particle size.

Zeta potential values for HRP encapsulated Ac-DEX particles prepared by probe sonication and homogenization technique is shown in Table 4.4. Similar to particle size, even zeta potential values do not show any significant trend with storage temperature but displayed a marginal decrease with time. Again, addition of HRP did not have an effect on the zeta potential values for blank acetalated dextran particles prepared by probe sonication or homogenization techniques as shown in Table 4.5.

C. Differential Scanning Calorimetry

DSC thermograms of blank acetalated dextran particles prepared by probe sonication and homogenization methods are shown in Figure 4.7. Measurements were performed on days 0, 45 and 90 for the particles stored at different temperatures. The data showed no significant differences in endothermic peaks for blank Ac-DEX particles stored at different temperatures. Since the Tg for the polymer is high, the particles maintained their stability even at high temperatures.
Figure 4.1: Degradation profiles of horseradish peroxidase encapsulated acetalated dextran particles prepared by (A) probe sonication (B) homogenization techniques under acidic (pH 5.0) and neutral conditions (pH 7.4). Each data point is presented as the mean ± standard error (n=3).
Figure 4.1

A

B
Figure 4.2: Influence of the particle preparation method on the stability of horseradish peroxidase encapsulated acetalated dextran particles over time. Percent change in enzyme activity was measured with time (up to 90 days) for (A) particles prepared by probe sonication, (B) particles prepared by homogenization, and (C) unencapsulated horseradish peroxidase only, at various temperatures (-20°C, 4°C, 25°C, 45°C). Each data point is presented as mean ± standard error (n=3).
Figure 4.2

A

% Change in Activity

Time (Days)

50℃  4℃  25℃  45℃

B

% Change in Activity

Time (Days)

C

% Change in Activity

Time (Days)

-20℃  4℃  25℃  45℃
Figure 4.3: Release profiles of horseradish peroxidase encapsulated acetalated dextran particles prepared by (A) probe sonication (B) homogenization techniques under acidic (pH 5.0) and neutral conditions (pH 7.4). Each data point is presented as the mean ± standard error (n=3).
Figure 4.3

A

% Release

Time (Days)

B

% Release

Time (Days)
Figure 4.4: Scanning electron micrographs of horseradish peroxidase encapsulated acetalated dextran particles prepared by probe sonication method. The particles were stored at different temperatures (-20°C, 4°C, 25°C, 45°C) and imaged on day 0, 30, 60, 90. Scale bar = 1 µm.
Figure 4.5: Scanning electron micrographs of horseradish peroxidase encapsulated acetalated dextran particles prepared by homogenization method. The particles were stored at different temperatures (-20°C, 4°C, 25°C, 45°C) and imaged on day 0, 30, 60, 90. Scale bar = 10 µm.
Figure 4.6: Scanning electron micrographs of blank acetalated dextran and poly(lactic-co-glycolic acid) (PLGA) particles prepared by homogenization method. The particles were stored at 45°C temperatures and imaged on day 0, 3, 6 and 28. Scale bar = 2 µm
Figure 4.7: Differential scanning calorimetry thermograms for (A) probe sonicated and homogenized blank particles on day 0 (B) probe sonicated blank particles on day 45 (C) homogenized blank particles on day 45 (D) probe sonicated blank particles on day 90, and (E) homogenized blank particles on day 90.
Figure 4.7

A

Probe Sonication
Homogenization

B
-20°C
4°C
25°C
45°C

(Figure 4.7 continued)
(Figure 4.7 continued)
(Figure 4.7 continued)
Table 4.1: Drug loading, yield, baseline enzyme activity (BEA), and degradation half lives at pH 5.0 and 7.4 for horseradish peroxidase encapsulated acetalated dextran particles prepared by probe sonication and homogenization techniques.

<table>
<thead>
<tr>
<th></th>
<th>Drug loading (w/w) %</th>
<th>Yield (%)</th>
<th>BEA (%)</th>
<th>Degradation half life (hr)</th>
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<tr>
<td></td>
<td></td>
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<td></td>
<td>pH 5.0</td>
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<tr>
<td>Sonicated HRP loaded particles</td>
<td>1.0</td>
<td>55.0</td>
<td>54.0</td>
<td>&lt; 3</td>
</tr>
<tr>
<td>Homogenized HRP loaded particles</td>
<td>1.0</td>
<td>89.0</td>
<td>86.0</td>
<td>&lt; 3</td>
</tr>
</tbody>
</table>
Table 4.2:
(A) Particle size (nm) and polydispersity index values for horseradish peroxidase encapsulated acetalated dextran particles prepared by probe sonication and homogenization methods.
(B) Particle size (nm) and polydispersity index values for blank acetalated dextran particles prepared by probe sonication and homogenization techniques
<table>
<thead>
<tr>
<th>Days</th>
<th>0</th>
<th>15</th>
<th>30</th>
<th>60</th>
<th>90</th>
<th>0</th>
<th>15</th>
<th>30</th>
<th>60</th>
<th>90</th>
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</thead>
<tbody>
<tr>
<td>Temp</td>
<td>-20° C</td>
<td>1394</td>
<td>2456</td>
<td>2487</td>
<td>2111</td>
<td>4027</td>
<td>0.125</td>
<td>0.242</td>
<td>0.321</td>
<td>0.242</td>
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<tr>
<td></td>
<td>4° C</td>
<td>1394</td>
<td>2123</td>
<td>2108</td>
<td>2896</td>
<td>3815</td>
<td>0.125</td>
<td>0.332</td>
<td>0.238</td>
<td>0.332</td>
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<tr>
<td></td>
<td>25° C</td>
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<td>2649</td>
<td>2977</td>
<td>3424</td>
<td>0.125</td>
<td>0.284</td>
<td>0.289</td>
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<td>45° C</td>
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<td>1972</td>
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<td>4206</td>
<td>0.125</td>
<td>0.328</td>
<td>0.352</td>
<td>0.328</td>
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<table>
<thead>
<tr>
<th>Days</th>
<th>0</th>
<th>15</th>
<th>30</th>
<th>60</th>
<th>90</th>
<th>0</th>
<th>15</th>
<th>30</th>
<th>60</th>
<th>90</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temp</td>
<td>-20° C</td>
<td>1920</td>
<td>2314</td>
<td>1972</td>
<td>1591</td>
<td>3695</td>
<td>0.005</td>
<td>0.005</td>
<td>0.015</td>
<td>0.050</td>
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<td></td>
<td>4° C</td>
<td>1920</td>
<td>1997</td>
<td>2608</td>
<td>2174</td>
<td>3716</td>
<td>0.005</td>
<td>0.005</td>
<td>0.035</td>
<td>0.095</td>
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<tr>
<td></td>
<td>25° C</td>
<td>1920</td>
<td>1997</td>
<td>1139</td>
<td>1926</td>
<td>4559</td>
<td>0.005</td>
<td>0.005</td>
<td>0.091</td>
<td>0.121</td>
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<tr>
<td></td>
<td>45° C</td>
<td>1920</td>
<td>2277</td>
<td>1125</td>
<td>2038</td>
<td>4229</td>
<td>0.005</td>
<td>0.005</td>
<td>0.090</td>
<td>0.018</td>
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Table 4.2a
<table>
<thead>
<tr>
<th>Days</th>
<th>Temp</th>
<th>Particle Size (nm)</th>
<th>Polydispersity Index</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>0  15  30  60  90</td>
<td>0  15  30  60  90</td>
</tr>
<tr>
<td></td>
<td>-20° C</td>
<td>1389  2225  2060  2400  4122</td>
<td>0.09  0.3  0.35  0.42  0.38</td>
</tr>
<tr>
<td></td>
<td>4° C</td>
<td>1389  1796  2042  2788  3723</td>
<td>0.09  0.29  0.38  0.48  0.55</td>
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<tr>
<td></td>
<td>25° C</td>
<td>1389  1796  2239  2101  4884</td>
<td>0.09  0.54  0.035  0.262  0.331</td>
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<tr>
<td></td>
<td>45° C</td>
<td>1389  1249  2721  1040  3827</td>
<td>0.09  0.38  0.482  0.268  0.384</td>
</tr>
<tr>
<td></td>
<td>4° C</td>
<td>1389  2225  2060  2400  4122</td>
<td>0.09  0.3  0.35  0.42  0.38</td>
</tr>
<tr>
<td></td>
<td>25° C</td>
<td>1389  1796  2042  2788  3723</td>
<td>0.09  0.29  0.38  0.48  0.55</td>
</tr>
<tr>
<td></td>
<td>45° C</td>
<td>1389  1249  2721  1040  3827</td>
<td>0.09  0.38  0.482  0.268  0.384</td>
</tr>
</tbody>
</table>

Table 4.2b
Table 4.4: Zeta Potential (mV) for horseradish peroxidase encapsulated acetalated dextran particles prepared by probe sonication and homogenization technique. Values are presented as the mean ± standard error (n=3).

<table>
<thead>
<tr>
<th>Days</th>
<th>Temp</th>
<th>0</th>
<th>15</th>
<th>30</th>
<th>60</th>
<th>90</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-20° C</td>
<td>-7.7 ± 1.1</td>
<td>-6.2 ± 0.5</td>
<td>-7.1 ± 1.6</td>
<td>-8.1 ± 2.3</td>
<td>-6.9 ± 1.2</td>
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<td></td>
<td>4° C</td>
<td>-7.7 ± 1.1</td>
<td>-6.6 ± 0.6</td>
<td>-5.4 ± 1.6</td>
<td>-4.2 ± 0.6</td>
<td>-3.8 ± 1.3</td>
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<tr>
<td></td>
<td>25° C</td>
<td>-7.7 ± 1.1</td>
<td>-6.2 ± 1.7</td>
<td>-5.8 ± 0.3</td>
<td>-5.0 ± 0.9</td>
<td>-4.2 ± 2.3</td>
</tr>
<tr>
<td></td>
<td>45° C</td>
<td>-7.7 ± 1.1</td>
<td>-6.9 ± 1.1</td>
<td>-4.1 ± 1.2</td>
<td>-3.5 ± 2.7</td>
<td>-3.2 ± 1.0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Days</th>
<th>Temp</th>
<th>0</th>
<th>15</th>
<th>30</th>
<th>60</th>
<th>90</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-20° C</td>
<td>-5.0 ± 0.9</td>
<td>-6.2 ± 1.0</td>
<td>4.5 ± 0.3</td>
<td>-5.7 ± 2.7</td>
<td>-7.3 ± 2.8</td>
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<td>4° C</td>
<td>-5.0 ± 0.9</td>
<td>-4.9 ± 0.7</td>
<td>-4.6 ± 0.1</td>
<td>-5.1 ± 2.8</td>
<td>-3.7 ± 2.1</td>
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<tr>
<td></td>
<td>25° C</td>
<td>-5.0 ± 0.9</td>
<td>-6.2 ± 1.2</td>
<td>-6.1 ± 1.0</td>
<td>-6.5 ± 2.5</td>
<td>-4.9 ± 2.9</td>
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<tr>
<td></td>
<td>45° C</td>
<td>-5.0 ± 0.9</td>
<td>-7.5 ± 0.6</td>
<td>-4.6 ± 0.6</td>
<td>-3.5 ± 2.8</td>
<td>-5.6 ± 3.2</td>
</tr>
</tbody>
</table>
Table 4.5: Zeta Potential (mV) for acetalated dextran particles prepared by probe sonication and homogenization techniques. Values are presented as the mean ± standard error (n=3).

<table>
<thead>
<tr>
<th></th>
<th>Sonicated Blank Particles</th>
<th>Homogenized Blank Particles</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Days</td>
<td>Temp</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-20° C</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4° C</td>
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<tr>
<td></td>
<td></td>
<td>25° C</td>
</tr>
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<td></td>
<td></td>
<td>45° C</td>
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</tbody>
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CHAPTER 5

5.1 SUMMARY, CONCLUSIONS AND FUTURE DIRECTIONS

The main aim of this dissertation work was to develop a tolerogenic vaccine for the treatment of multiple sclerosis. Novel tolerogenic vaccines were developed by generating microparticles encapsulating a glucocorticoid and an autoimmune antigen into an acid sensitive polymer. On administration, this drug delivery system can act by generating antigen specific iT-reg by passive targeting of DCs.

In an effort to generate a tolerogenic vaccine for MS, dexamethasone and peptide were encapsulated into an acid-sensitive material, Ac-DEX, through emulsion techniques. Physical characterization studies showed that the particles formed by encapsulating dexamethasone (with or without peptides) into Ac-DEX were spherical, negatively charged and with a mean particle size suitable for passively targeting DCs. Moreover, DSC studies have shown no effect of microencapsulation on the physical nature of Ac-DEX, with the drug existing in a distorted crystalline or amorphous nature. Chemical characterization studies showed higher encapsulation of dexamethasone into Ac-DEX when compared to PLGA microparticles, as well as pH-sensitive drug release. The quicker release of dexamethasone in acidic conditions is attributed to the Ac-DEX
sensitivity at lower pH, demonstrating the potential for elicited release of encapsulated agents into the phagosomal compartments of phagocytes upon uptake. After successful encapsulation of active ingredients into Ac-DEX and their characterization studies, in vitro cell culture experiments were done to assess the immunosuppression potential of dexamethasone loaded Ac-DEX microparticles. In comparison to free dexamethasone, a dose sparing phenomenon was observed with microparticles when treating LPS stimulated RAW macrophages and BMDCs. Furthermore, when Ac-DEX microparticles encapsulating peptide and dexamethasone were given prophylactically to mice with EAE, there was a significant reduction in EAE score over time and also generation of a systemic iT-reg population. This iT-reg population trafficked to the brain (flow cytometry results of CNS infiltrating cells) where it is hypothesized that EAE progression was significantly affected. Overall the preliminary data shows good progress toward the development of a tolerogenic vaccine with Ac-DEX microparticles for the prophylactic treatment of MS. To confirm the hypothesis of generating iT-regs by giving tolerogenic vaccines is beneficial as a treatment; additional in vivo studies need to be performed.

Secondly, the development of tolerogenic vaccines with Ac-DEX polymer was further extended by examining different parameters; to optimize the microparticle preparation for better drug encapsulation efficiency, particle size, and degradation rates. Microparticles synthesized from 71k Ac-DEX (prepared from 71k dextran) exhibit better encapsulation efficiency of rapamycin, an immunosuppressant, than 10k Ac-DEX (prepared from 10k dextran), possibly because of the polymer's higher viscosity and longer chain length. Also, 71k Ac-DEX microparticles require approximately three times
longer for complete degradation in pH 5 conditions, and had larger particle diameters than 10k Ac-DEX microparticles. These results add to the versatility of Ac-DEX microparticles as a drug-delivery vehicle. Degradation times can now be controlled through both reaction time and molecular weight of dextran used, allowing for greater flexibility, which may be tailored to specific applications. Furthermore, this study demonstrated that immnosuppression of macrophages could be achieved with rapamycin-loaded Ac-DEX microparticles at levels similar to free drug in culture. Similar to dexamethasone, rapamycin-loaded Ac-DEX microparticles could potentially be passively targeted to phagocytes and subsequently phagocytosed in the acidic environment of the phagolysosome; this intracellular environment would trigger the intracellular burst release of the drug payload due to the acid-sensitivity of the Ac-DEX polymer with minimal drug released in extracellular pH-neutral environments. Because of their immnosuppressive capabilities, it is possible that immunosuppressant-loaded Ac-DEX microparticles could be applied in vivo to treat autoimmune diseases or prevent organ rejection while reducing the toxic side-effects of the drug. Future work with this line of investigation could include investigating scalable particle production methods and evaluating particle trafficking in vivo in both immune cells and tissues.

Finally after successful microencapsulation of active ingredients into Ac-DEX polymer and optimization of the parameters required for better encapsulation and particle preparation, the stability of Ac-DEX particles (with or without HRP) stored at various temperatures was observed, to develop a thermostable vaccine. These particles were prepared using two commonly used particle preparation techniques, namely, probe
sonication and homogenization. Unlike PLGA microparticles, particles prepared using Ac-DEX and stored at different temperatures maintained their spherical structure for 90 days. Ac-DEX microparticles prepared by homogenization showed better shielding of HRP over probe sonication particles at elevated temperatures. After 90 days, sensitivity of HRP in homogenized particles was approximately 2 fold higher than HRP alone at elevated temperatures (25°C and 45°C). Hence microencapsulation of HRP into Ac-DEX polymer increased its stability and also decreased the impact of storage temperature on vaccines. These studies suggest that Ac-DEX particles could be used in preparation of thermostable vaccines and provided a framework for future studies involving additional vaccine elements. Also addition of cryoprotectants before lyophilization and preparation of Ac-DEX particles using high-throughput and mass-production techniques such as electrospray could be used to prevent particle aggregation and to increase its stability. Future studies could involve loading of vaccine into Ac-DEX polymer using electrospray technique and studies of physiochemical behavior of both polymer and encapsulated ingredient at different storage conditions over time (1-3yrs).
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