Poly(β-Amino Ester)s as pH Sensitive Biomaterials for Microparticulate Genetic Vaccine Delivery

by

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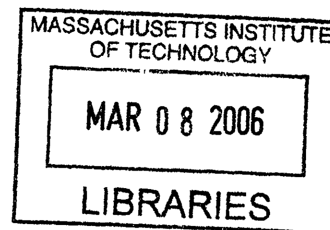

Submitted to the Department of Chemical Engineering in partial fulfillment of the requirements for the degree of

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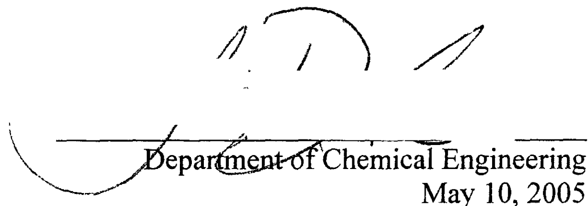

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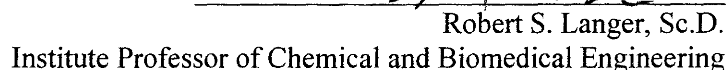

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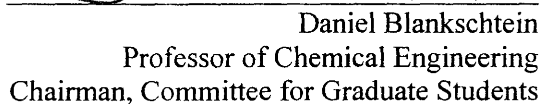

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Abstract

Genetic vaccination is the administration of nucleic acids to induce cellular expression of antigens, leading to an immune response. Unlike traditional vaccines, this technology has tremendous potential for treating or preventing diseases such as HIV, malaria, and cancer. However, this potential is currently unrealized because of the safety concerns which plague viral vaccine carriers and the inefficiency of nonviral delivery systems when compared to their viral counterparts. A promising and versatile nonviral delivery method for genetic vaccines involves microencapsulation of antigen-encoding DNA, because such particles protect their payload and target it to phagocytic, antigen-presenting immune cells. However, the biomaterial conventionally used in these microparticle formulations, an FDA-approved polyester called poly lactic-co-glycolic acid (PLGA), was not designed specifically to deliver DNA, takes too long to release encapsulated payload, and therefore fails to induce high levels of target gene expression. A new class of novel biomaterials have been synthesized called poly(β-amino ester)s which are biodegradable and can have similar physical properties to PLGA, but are pH-sensitive and have gene delivery functionalities. Using these materials we can fabricate microparticle-based delivery systems which have relatively high DNA loadings and can significantly buffer the destructive acidic pH microenvironment created by ester bond degradation. These formulations generate an increase of up to 5 orders of magnitude in DNA delivery efficiency when compared to PLGA alone and can be potent stimulators of antigen presenting cells *in vitro*. We have also demonstrated that incorporating these new biomaterials into microparticulate genetic vaccines can lead to antigen-specific, immune-mediated rejection of a lethal tumor dosage *in vivo*, a significant advance over conventional formulations. Finally, with the synthesis of libraries containing thousands of structurally diverse PBAEs, it is warranted to develop new methods of fabrication which enable the high-throughput screening of such libraries. Herein, we describe, for the first time, such a rapid fabrication technique and demonstrate that plasmid encapsulated in these formulations is transcriptionally active.

Thesis Advisor: Robert S. Langer, Sc.D.
Institute Professor of Chemical and Biomedical Engineering

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Foremost, I would like to thank my Lord Jesus for his unchanging love and the opportunity to wonder at his magnificent creation.

I would like to thank my excellent advisor Robert Langer for not only providing me with an opportunity to work in one of the greatest research laboratories in the world, but also for creating an environment which is so conducive to learning and research. I would also like to thank my committee members Doug Lauffenburger, Paula Hammond, and especially, Herman Eisen who have been so instructive and kind to me during my time at MIT.

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I would like to acknowledge my parents, Ronald and Debra Little, for their constant love and support.

Finally, I would like to thank my beautiful wife Heather, whose unbreakable spirit sustained me constantly. Thank you.

"The man who is thoroughly convinced of the universal operation of the law of causation cannot for a moment entertain the idea of a Being who interferes in the course of events... He has no use for the religion of fear and equally little for social or moral religion. A God who rewards and punishes is inconceivable to him for the simple reason that a man's actions are determined by necessity, external and internal, so that in God's eyes, he cannot be responsible, any more than an inanimate object is responsible for the motions it undergoes... A man's ethical behavior should be based effectually on sympathy, education, and social ties and needs; no religious basis is necessary."

Albert Einstein.....From: "Religion and Science"- New York Times

Some say... "all ideas of good and evil are hallucinations – shadows cast on the outer world by the impulses which have been conditioned to feel."......But then they must stick to it;...A moment after they have admitted that good and evil are illusions, you will find them exhorting us to work for posterity, to educate, revolutionize, liquidate, live and die for the good of the human race.... They write with indignation like men proclaiming what is good in itself and denouncing what is evil in itself, and not at all like men recording that they personally like mild beer but some people prefer bitter. Yet if the "oughts" of Mr. Wells and, say, Franco are both equally the impulses which Nature has conditioned each to have and both tell us nothing about any objective right or wrong, whence is all the fervour? Do they remember while they are writing thus that when they tell us we "ought to make a better world" the words "ought" and "better" must, on their own showing, refer to an irrationally conditioned impulse which cannot be true or false any more than a vomit or a yawn?
My idea is that sometimes they do forget. That is their glory. Holding a philosophy which excludes humanity, they yet remain human. At the sight of injustice thy throw all their Naturalism to the winds and speak like men.

C. S. Lewis.......From his book: "Miracles"

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1. Introduction

1.1. Overview

In the early 90's, a discovery was made which would revolutionize scientists' perception of prophylactic and therapeutic vaccines: the simple injection of plasmid DNA in saline into the muscle of mice led to the expression of a target gene[1]. Shortly thereafter, it was demonstrated that DNA vaccination could produce antibodies against an encoded antigen in 1992[2], and then cytotoxic T-cell responses and protection from lethal doses of influenza in 1993[3, 4]. These seminal studies were the first to show elicitation of an immune response using the genetic information for an antigen rather than the protein itself.

The concept of genetic vaccination has tremendous implications ranging from prevention and treatment of numerous diseases, to the screening of pathogenic genome libraries for the determination of protective antigens [5], and even to the high throughput generation of monoclonal antibodies with higher specificities to a target than the protein generated counterparts[6]. This has spurred an insurgence of publications, from just over

400 in the first decade to almost 1300 in the last four years (from a search of Web of Science for "DNA vaccines" and "Genetic vaccines"). However, the inherent ability of naked plasmid DNA to gain access to the nucleus of a cell seems counterintuitive. Early experiments which led to the discovery of naked plasmid expression in the muscle of mice were initially intended to demonstrate the superior ability of liposomal delivery systems over plasmid alone[7]. Surprisingly, this naked plasmid was actually capable of inducing more target gene expression than the liposomal formulations. However, the amount of uptake and expression, especially in muscle, dwarfs in comparison to the total amount of plasmid administered (less than 0.01%) [8-10].

The three problems here seem to be essentially the same as gene therapy as stated by Inda M. Verma as "delivery, delivery, delivery." Verma went on later to say, "the next battle will be "immunology, immunology, immunology"....Let's face it; we are trying to do with the viruses and vectors things that nature has designed to prevent." Viral vectors are by nature far more effective as delivery vehicles and can be powerful activators of the immune system. However, potential toxicity and immune rejection issues related to viral vectors can be limiting, especially in immunocompromised patients such as persons undergoing chemotherapy and the elderly population. An example of this shortcoming was apparent in research which demonstrated that re-stimulation with DCs transduced with an adenoviral genetic vaccine decreased the antigen-specific immune response in favor of a overbearing anti-adenovirus response in human melanoma patients[11]. Pre-existing immunity to a viral delivery vector can also be a limiting factor. Attenuated bacterial vectors have also shown promise for DNA delivery, but fall under similar safety constraints[12].

Synthetic, non-viral DNA vaccine delivery avoids many of the potential downsides of viral vaccines related to safety and immune rejection. However, these vaccines are far less effective at eliciting prophylactic immunity. A primary focus of these delivery systems has been to increase this level of expression, and possibly even more importantly, target its delivery to the appropriate cell type. Designing an appropriate synthetic vector to perform this function requires an understanding of both the specific cellular venue to be targeted and material properties of the delivery vehicle. To date, many approaches at designing new synthetic delivery vehicles have taken a "black box approach" at eliciting immune response due partially because of a lack of the said understanding, and partially due to a desire to use FDA approved materials on a fast-track towards the clinic. The goal of my thesis was to investigate new materials better suited for the delivery of plasmid DNA. Furthermore, I wanted to understand why these materials led to a better delivery vehicle and subsequently, a better genetic vaccine formulation.

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2. Background

2.1. Immunobiology of genetic vaccines

2.1.1. The role of dendritic cells in immunity

An understanding of the complex multi-cellular milieu of the immune system and the genetic vaccine mechanism is critical to the development of successful DNA vaccine delivery technologies. The primary activators of adaptive cell mediated immunity are professional antigen presenting cells (APC) called dendritic cells (DC). DCs, depending on their state, are the most powerful APC, capable of inducing naïve T-cell activation, or "priming". Only one DC is required to activate 100-3000 T-cells[1]. In the absence of antigen, DCs have been shown to contact approximately 500 individual T-cells per hour and can interact with greater than 10 antigen-specific T-cells simultaneously[2]. However, initially, DCs are thought to exist in an "immature" state in which they are relatively incapable of activating T-cells, yet fully competent to survey the peripheral environment by capturing antigen at the tremendously high rate of approximately one cell volume per hour[3]. Upon capture of antigen, these cells are thought to go through phenotypic changes in which their rate of antigen uptake briefly increases and then almost completely ceases.

The cell then goes through morphological changes and down-regulation of chemokine receptors responsible for the migration of the cell to the site of inflammation, and up-regulation of receptors that would mediate translocation to the lymph nodes where T-cells are waiting for activation. Surface expression of "co-stimulatory" molecules is up-regulated as well. These receptors (Signal 2) are required for the activation of T-cells in tandem with the presentation of antigen on MHC Class I and II (Signal 1) which is also up-regulated upon "maturation" of a DC.

Once a DC is in the lymph nodes, where the majority of T-cells reside, surface co-stimulatory and secreted cytokine signals between the APC and T-cell convey the location and type of antigen that was captured. T-cells, specific to the peptide-MHC complex through a highly variable T-cell receptor (TCR), will then proliferate into clones capable of either destroying a target cell which expresses that same complex on its surface ($CD8^+$ T_c), or in the case of helper T-cells ($CD4^+$ T_H), capable of secreting soluble signals to B and T_c cells to direct the type of immune response elicited. This complex dialogue between lymphocytes can polarize the immune response to react through a cytotoxic cell mediated response such as in viral infections called a Th1 response (characterized by secretion of cytokines such as IFN-γ, IL-2, and IL-12 along with IgG2a antibody), or by B-cell secretion of antibody which can bind and direct clearance of exogenous pathogens called a Th2 response (characterized by secretion of IL-4 and IL-10 along with IgG1 antibody). The rapid generation of $CD8^+$ T_c and $CD4^+$ T_H clones is thought to involve genetic alterations in certain persisting T-cells that enhance the magnitude of cellular proliferation in response to a second encounter with a pathogen. This effect is called a memory response, the basis of prophylactic immunity.

2.1.2. Genetic vaccine mechanism

The mechanism of immune induction for a traditional vaccine depends on its classification. Attenuated bacterial or viral vaccines are still competent to infect target cells, but are incapable of inducing a full-blown infection in persons with normal immune systems. Because of this, antigenic peptide is produced intracellularly and processed by the MHC Class I pathway to elicit a Th1 response. At the same time, the pathogen can be taken up and degraded by the MHC Class II pathway for elicitation of a Th2 response. These vaccines are extremely effective at inducing memory responses and usually result in lifetime immunity. However, for particularly dangerous pathogens, the risk of reversion to a virulent state is too great, especially in immunocompromised individuals. Therefore, a vaccine with fewer safety concerns is required. Completely inactivated, or subunit vaccines cannot infect a cell, and are mainly processed by the MHC Class II pathway. This results in activation of only the humoral arm of the immune system (characterized by an antibody response), and the lack of necessary T_c immunity required by certain infections. Currently, there are no vaccines capable of safely stimulating the immune system to battle certain diseases such as HIV, hepatitis C, herpes, malaria, and cancer.

Genetic vaccines, however, can enter a cell and cause expression of a target gene intracellularly, mimicking viral infection, and allowing for both arms of the immune system to be activated (Figure 2.1.). Most importantly, plasmid DNA vaccines achieve this feat without the use of any infectious agent. However, just like an inactivated or subunit antigen, one would surmise (as did scientists that pioneered genetic vaccination in the late 80's) that plasmid DNA alone would have no way of gaining entry into the

cytoplasm of a cell. However, it seems that by some unknown mechanism this does occur, albeit to an extremely small extent. The exact mechanism for immune induction via genetic vaccines is still unknown (Figure 2.2.).

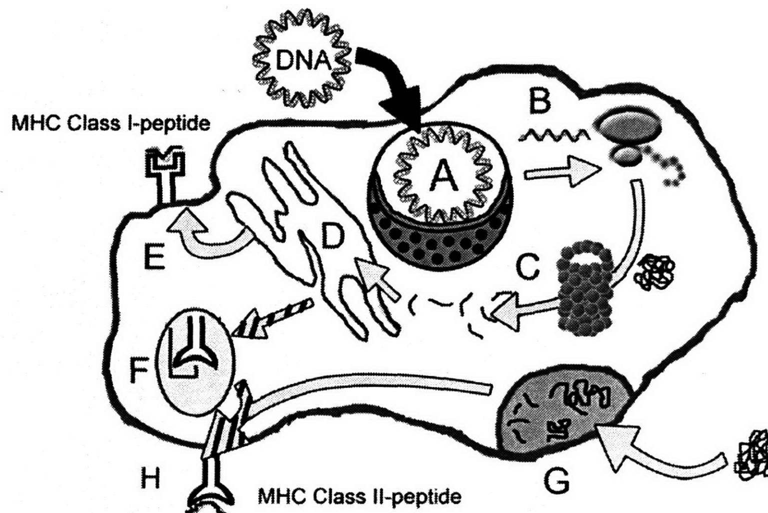

Figure 2.1. Antigen processing and presentation in a cell. Upon cellular uptake of DNA from a genetic vaccine or viral infection (A), the gene is transcribed and translated (B) intracellularly to create a full length protein. This "antigen" is subsequently marked for degradation into smaller peptide fragments by the proteosome (C), and then transported into the endoplasmic reticulum for loading onto MHC Class I molecules (D). This MHC-peptide complex is then transported to the surface for presentation to antigen specific CTLs (E). This complex, along with the appropriate co-stimulatory signals on the surface of an APC, would result in the activation and proliferation of the CTL (Tc). Alternatively, in the case of somatic cell presentation without co-stimulatory expression, the CTL would mediate lysis of the infected target cell. In APCs only, a MHC Class II complex is transported from the endoplasmic reticulum (along with an invariant chain to forbid Class I restricted peptide complexation) (F) in vesicles destined to fuse with endosomes containing exogenously captured antigen (G). The antigen is degraded in the late endosomes along with the invariant chain to allow MHC Class II – peptide loading, which is then transported for expression on the surface in tandem with co-stimulatory molecules for activation of helper T-cells (T_H) (H). The exclusivity of these pathways for processing and presentation of an endogenous or exogenous antigen to a Tc for a cell mediated response (Th1) or a T_H for an antibody response (Th2), respectively, is under debate due to the apparent ability of some exogenous antigens to bypass endosomal trafficking into the cell cytoplasm.

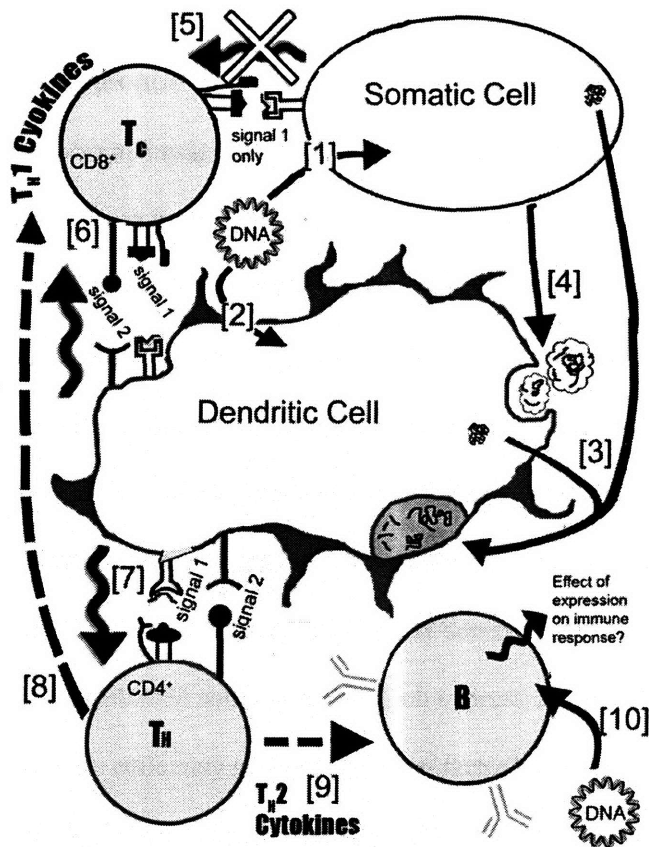

Figure 2.2. Activation of cellular immunity by genetic vaccines. Plasmid DNA is taken up by somatic cells[1] and/or DCs [2] for intracellular expression of antigen. This antigen can be secreted [3] and subsequently taken up by DCs. Somatic cells may also, in certain circumstances, secrete or present antigen in a way so that it is taken up into the cytoplasm of a DC [4]. Importantly, there is no empirical evidence that somatic cells can activate $CD8^+$ T-cells due to a lack of co-stimulatory expression [5]. Antigen expressed intracellularly by a DC [2] or taken up through cross-priming [4] can be presented by MHC Class I along with co-stimulatory signal 2 to a $CD8^+$ T-cell to initiate priming [6]. Furthermore, antigen taken up exogenously [3] may be processed by the MHC Class II pathway and presented alongside signal 2 to $CD4^+$ T_H cells [7] which can subsequently secrete soluble cytokine signals such as IL-12 back to the DC, proliferative signals such as IL-2 and IFN-γ to T_c cells [8], or signal directed toward B-cells such as IL-4 to induce B-cell proliferation and antibody secretion [9]. The role of directly transfected B-cells is still yet to be determined [10].

Speculation has occurred as to the potential route of plasmid internalization of myocytes (the cell primarily transfected using i.m. vaccination) such as the linking of membrane invaginations called caveolae to plasmid uptake[4]. However, myocytes themselves do not express co-stimulatory molecules which are necessary (Signal 2) to induce activation of naïve T-cells upon expression of processed peptide associated with surface MHC Class I (Signal 1). It seems as though these cells may have a greater capacity to tolerize an antigen specific T-cell due to the lack of Signal 2 in the presence of Signal 1[5-7] (with the exception of their presence in the lymphoid organ where another cell may deliver Signal 2[8]). Furthermore, studies have shown that immune responses are restricted to the haplotype of parent bone marrow derived cells and not to the haplotype of transplanted somatic cells which express antigen from a genetic vaccine[9-12]. These somatic cells may serve as "antigen factories" to produce and secrete large amounts of antigen which then may be taken up and processed by DCs. However, this secreted antigen, under normal circumstances, is thought to only be processed by the MHC Class II pathway, similar to the way an inactivated or subunit vaccine is capable of only eliciting a Th2 type response.

Another possibility is that the APC itself takes up the antigen encoding DNA and presents the peptides on its surface in the context of both Signal 1 and Signal 2. This seems to be the simplest explanation and has been backed up empirically by several groups who have demonstrated APC expression of a reporter gene administered in a DNA vaccine by several delivery routes in the draining lymph nodes [13-18] in very small numbers of cells. However, some have questioned the extent to which this limited number of cells contributes to the observed immune response. It should be noted that as

few as 500-1000 DCs transfected *in vitro* and administered to a mouse have induced immune responses equivalent to standard genetic immunization (i.e. comparable to transfecting only 0.5-1% of the cells in the target area) [19]. Another consideration is that dendritic cells have proven to be a particularly difficult cell to transfect with plasmid DNA, as will be discussed later in this review. This cell seems to be a logical target for delivery technology.

A more complex mechanism of immune activation using genetic vaccines involving both somatic and APCs has also been proposed[10, 11]. The theory of "cross-presentation" suggests that antigen secreted under special circumstances or associated with proteins that mediate cytoplasmic transport (e.g. heat shock proteins, apoptotic/necrotic bodies) could be processed by the MHC Class I pathway of an APC. Evidence of this cross-priming mechanism has been demonstrated[20-22] and seems to involve phagocytosis by an immature DC through $\alpha_v\beta_5$ integrins and CD36. Furthermore, studies have shown that this process requires $CD4^+$ T-cell help[23], either through secretion of soluble T_c proliferation signals such as IL-2 and/or interacting with the DC in such a way as to make it more capable of mediating T_c priming. Importantly, this is most likely in the context of some sort of "danger signal", otherwise the body would presumably react to self antigens that it would theoretically capture and survey at all times[24]. It is thought that without this "danger signal", an antigen which is processed by an APC (such as the ones it encounters regularly under normal conditions) does not alarm the immune system, and may even create peripheral tolerance[25].

Finally, a recent report by Coelho-Castelo et al. demonstrated that B-cells express a reporter gene after i.m. injection of plasmid DNA, contrary to previous reports[26]. The

contribution of transfected B-cells to the immune responses elicited by DNA vaccines remains uncertain, however, B-cells may be an interesting future target for DNA vaccine delivery.

2.2 Using genetic vaccines to treat cancer

Inflammation is a direct result of tissue damage due to the invasive growth typical of cancer. Therefore, it is logical to think that the immune system would be alerted to malignant tumor cells. Apparently, however, cancer is capable of evading this immune recognition. Cancer cells may accomplish this feat through several mechanisms[27]: 1) down-regulated presentation of certain tumor associated antigens (TAA) that may alert the immune system, 2) complete loss of some TAA, and 3) secretion of soluble signals which can diminish the ability of APCs to initiate immune rejection. Examples of such secreted factors include IL-10 and TGF-β, which have both shown to dampen immune activation [27, 28]. Though it is still unclear as to what extent these mechanisms contribute to tumor persistence, it is certainly clear that tumor cells have developed ways to avoid recognition by the immune system.

Since tumor cells seem to evade immune recognition, it is believed that activation of these so called "non-responsive" TAA specific T-cells is the key to effective cancer immunotherapeutics. The choice of antigen in which the immune system should be alerted is a major consideration in this process and is different for different types of cancer. This process of choosing the appropriate TAAs is beyond the scope of this review and is described in detail elsewhere[29, 30]. Despite the optimization of responses to different TAAs and the large number of clinical trials genetic vaccine therapeutics, DNA vaccination has not produced the caliber of immune responses in humans that has been

shown in small animals. For instance, as much as 5 mg of plasmid is required for effective DNA vaccination without a delivery vehicle (naked DNA vaccination) in non-human primates [31] while only 50-300 μg are required in mice [32]. Although large amounts of DNA have been shown to be tolerated by humans[33], technologies to increase the potency of DNA vaccines are certainly welcome.

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3. Non-Viral DNA Vaccine Delivery

3.1. Delivery Route

3.1.1. Parenteral Delivery

The administration route of a genetic vaccine may be the single, most broadly applicable way to alter the way in which a target antigen is presented to the immune system. Initially, two methods of DNA delivery were employed, intramuscular delivery of naked DNA, and "gene gun" administration to the skin. Gene gun administration was the first delivery strategy for antigenic DNA and involves the coating of plasmid onto the surface of tiny gold beads which are then accelerated to a high speed into the skin by a high pressure helium source[1]. Expression of genes administered in this manner are mainly restricted to keratinocytes and fibroblasts,[2] but migratory APCs have been demonstrated to be transfected as well[3,4]. This direct transfection of APCs is facilitated by the large amount of resident DCs, called Langerhans cells, in the skin (~1000 cells per mm^2 in mice or 1-3% of the cells in the epidermis[5]). This may seem like a relatively small population of cells, but due to the large surface area of a Langerhans cell, this translates to approximately 20% of total skin surface. Immune responses elicited by

vaccination using gene gun to the skin seem to inherently require much less plasmid DNA, on the order of several hundred to several thousand times less[6, 7].

Interestingly, responses elicited by gene gun vaccinations are mainly Th2 in nature, whereas direct i.m. injection of naked plasmid DNA results in Th1 response. This may be due to several reasons, some of which pertain to the structure of bacterial plasmid DNA. This sequence of the plasmid, when compared to vertebrate DNA, contains much higher levels of unmethylated CpG motifs[8]. These CpGs are optimally flanked by two 5prime purines and two 3prime pyrimidines[9]. These immunostimulatory motifs have been associated with the secretion of Th1 cytokines such as IFN-γ and IL-2[10], and elimination of these CpG motifs has resulted in reduced cytokine production and Ab responses in vivo [11]. Later, these motifs were shown to bind to a toll-like receptor (TLR9) on the surface of DCs, which was required for the immunostimulatory effect of bacterial CpG[12]. These bacterial derived patterns in the plasmid DNA may be responsible for the Th1 response seen in i.m. injection where extracellular CpG is present in large quantities, verses gene gun administration where much less plasmid is used and DNA is forced into cells ballistically, possibly bypassing the effects of CpG-TLR9 signaling.

Finally, due to the inherently large number of DCs which reside in the skin, immunization via this route most likely directly transfects more DCs than in i.m. immunization, where the number of DCs is extremely low and cross-priming of antigen may occur more frequently. Support of this theory has been put forth by demonstrating that removal of the immunized muscle site minutes after immunization does not affect the magnitude of immune response[13]. Conversely, removal of the dermal site up to 24 hours

from the time of gene gun vaccination completely abrogates immune induction[13, 14]. This demonstrates the fundamental differences between delivery of genetic vaccines by various routes of administration.

3.1.2. Mucosal Delivery

In contrast to parenteral delivery, mucosal delivery is thought to induce immune responses to both the mucosal and the systemic immune system. The lining of the respiratory and gastrointestinal tract consists of mucosal associated lymphoid tissue (MALT) which is thought to be lined with epithilim containing specialized microfold cells (M-cells). These highly phagocytic cells are thought to transport antigen to APCs from the apolateral to basal surface. Access to these tissues is obtained by delivery of plasmid intratrachially, intranasally, orally, rectally, or intravaginally and successful immunization via this route is characterized by secretory IgA instead of IgG antibody.

This method of immunization is non-invasive, simple to perform, and can induce immunity at distant mucosal sites. However, there are many barriers to mucosal transfection such as enzymes, acidic pH, and removal by ciliated epithilium[15]. Also, it is believed that there is a need for high levels of stimulation for the maturation of mucosal DCs when compared to other sites[15]. Also, mucosal vaccination has been generally aimed at creating mucosal immunity towards pathogens which may enter the body through this route. and this delivery of tumor associated antigens mucosally isn't as extensively employed. Recent progress in this field has been extensively reviewed elsewhere[15, 16]. It will be interesting to see if the barriers to transfection of DNA mucosally can be overcome by advances in delivery technologies aimed at mucosal vaccination.

3.2. Methods of genetic vaccine delivery

3.2.1. Gene Gun

The first attempt to increase potency in DNA vaccines by delivery of plasmid DNA delivery is the use of a gene gun. This method involves delivery of DNA coated onto tiny gold beads which are accelerated to high velocities in order to penetrate into the skin [1]. Compared to direct i.m. injection of plasmid, gene gun vaccination requires 2 to 3 orders of magnitude less DNA [6, 7]. This may be due to the direct insertion of a plasmid coated bead into skin cells, bypassing the needs for uptake, but may be partially due to the large amount of dendritic cells present in the dermis (Langerhans cells)[5]. However, a downside to gene gun vaccination is that immune responses elicited are usually Th2 polarized and the commercial viability of this technology is under question.

3.2.2. Electroporation

Elecroporation is most commonly known as a effective *in vitro* transfection procedure. Electric pulses temporarily disrupt the cellular membrane and also physically translocate plasmid DNA into the cell due to its anionic charges (a process known as ionophoresis). The application of electroporation, *in vivo,* requires the use of probes or clamp electrodes to the site of DNA administration resulting in a non-specific post-injection delivery method to the surrounding cells. Modification of process parameters from low voltage, long pulses to high voltage, can significantly increase the effectiveness of this procedure for gene delivery [17].

Research into the delivery of TAA genes including electroporation has shown promise, but has its downsides. Electroporation enhanced vaccination with both plasmid encoded human GP100 and mouse TPR2 antigen was shown to elicit protection from

melanoma challenge in mice[18]. It was also demonstrated that introduction of plasmid encoding inflammatory cytokine signals by electroporation at tumor sites induced transduction and tumor growth inhibiation without the systemic cytokine levels seen using adenoviral delivery methods [19]. Futhermore, Kalat et al used electroporation to facilitate the discovery of novel TAA plasmid construct variations by optimization of tyrosinase related protein-2 antigens, leading to inhibition of tumor growth in two separate models[20]. This same group later demonstrated that electroporation could induce immune responses which were comparable to those induced by viral infection[21].

Although it has been suggested that the increase in transfection efficiency afforded by electroporation is responsible for the observed immune activation, it is also possible that tissue damage caused by electroporation at the immunization site may be responsible for the recruitment of APCs and inflammation, leading to a more effective response [22]. Electroporation can be destructive to tissues, and it has been reported that pain has occurred in patients during administration of electroporation during clinical trials[23].

3.2.3. Lipoplexes and Liposomes

Cationic lipids are arguably the most common non-viral transfection reagent. These lipid formulations condense anionic plasmid DNA through the formation of lipid bi-layers and through their cationic charges, leading to the formation of a lipoplex. Examples of common lipids used in these formulations are DOTAP, DOPE, and cholesterol, both of which can also have endosomal disruption properties[24] (Figure 3.1.). The mechanism of cellular uptake of lipoplexes was originally believed to be that of cell membrane fusion, however it is now widely accepted that this process occurs through

endocytosis or phagocytosis of the lipoplex. Access to the cytoplasm thought to be mediated by destabilization of the endosomal membrane[25].

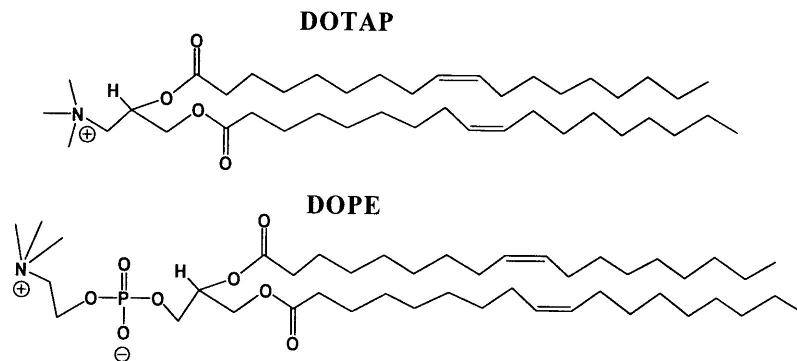


Figure 3.1. Chemical structures of DOTAP and DOPE, two commonly used lipids in liposomal formulations

Additionally, plasmid DNA can be encapsulated within a lipid vesicle called a dehydrated-rehydrated vesicle (DRV)[26]. DRVs are produced through the process of freeze drying lipoplexes (which is thought to increase the association of plasmid DNA with the flattened liposomal vesicles). Subsequent rehydration leads to the formation of a DRV with entrapped plasmid (Figure 3.2.). DRVs, compared to naked DNA and cationic lipoplexes, have shown the ability to generate improved cellular immunity and secretion of greater IgG1 levels in mice[26]. Various lipids have been shown to increase vaccine potency when added to these formulations[27] and have also been shown to enhance oral delivery of DRV formulations [28].

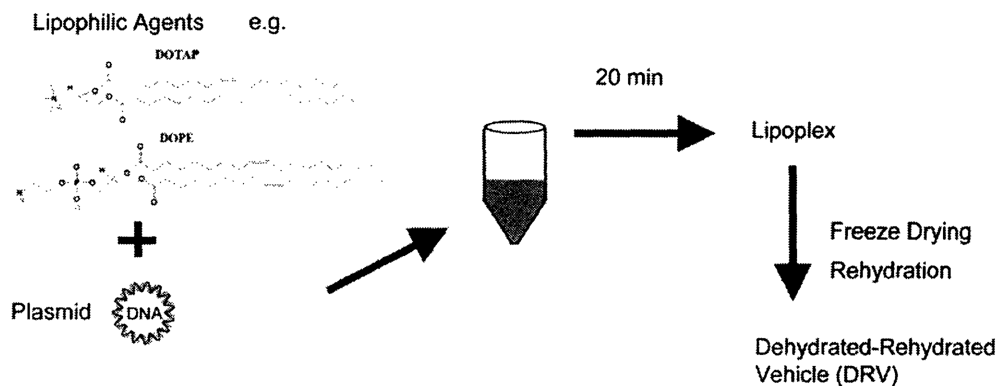


Figure 3.2. Formation of lipoplexes and DRVs.

Another strategy using cationic lipids involves the incorporation of viral fusogenic peptides. These added components may impart advantageous viral properties to the complexes and have enhanced responses against tumor associated antigens. Okamoto et al. demonstrated increased antibody response when using Hemaglutinating Virus Japan (HVJ) fusogenic peptides to delivery plasmid DNA i.m. in a mouse model while naked DNA was ineffective[29]. Zhou et al. demonstrated later that these same HVJ peptides used with cationic lipids i.m. could induce cellular immune responses and protection against melanoma along with antibody responses in a gp100 TAA model[30]. This delivery vector has also been used with i.n. administration but has shown to be less effective than i.m. injection in a gp100/TRP2 TAA model[31]. Influenza fusogenic peptides have been used for the treatment of prostrate carcinoma with the successful induction of CTL responses using a parathyroid hormone-related peptide[32].

TAA	Liposome type	Delivery Route	Ab	CTL	Protection	Ref
MAGE 1&3	HVJ Fusogenic	i.m.	+	ND	ND	29
gp100 melanoma	HVJ Fusogenic	i.m.	+	+	+	30
Hsp65 (for mesothelioma)	Cationic Lipoplex	i.p.	ND	+	*90% (AC29) *40% (AB12)	33
gp100 + TRP2 melanoma	HVJ Fusogenic	i.m.†, i.n.	+Th1	+Th2	+	31
PTH-rP Prostate carcinoma	Influenza Fusogenic	i.n.	ND	+	ND	32, 34

Table 3.1. Examples of cancer models using liposomal delivery of DNA vaccines.
+ = positive response,
ND= experiment not performed.
* % of long term survivors post-tumor challenge (~150 days) AB12 more aggressive than AB12
†i.m. route demonstrated better CTL response than i.n.

Cationic lipid formulations are easy to prepare, can protect the plasmid DNA from nucleases in the extracellular environment, and are promising candidates for genetic vaccination *in vivo*. However, the poly-cationic nature of the lipoplex/liposomal formulations impart a degree of cellular promiscuity and a pronounced tendancy to bind to proteins in serum. This can severely limit the ability to target particular cells of the immune system and maintain stable plasmid/lipid formulations. Creating targeted liposome vectors with improved serum stability could be the key to a successful cationic lipid delivery vehicle for genetic vaccines.

3.2.4. Particulate encapsulation and adsorption

Polymeric encapsulation or binding of DNA vaccines may be one of the most promising non-viral delivery methods for several reasons: 1) these particles protect plasmid payload from extracellular degradation[35], 2) these formulations are capable of

carrying large payloads, making co-delivery of many plasmids and other immunostimulatory agents possible, 3) these particles can offer a phagocytosis-based passive targeting to APCs in the range of 1-10 μm (Figure 3.3.), surface modifications are easily made which can further enhance targeting and uptake[36], 5) these particles are widely believed to be adjuvants themselves.

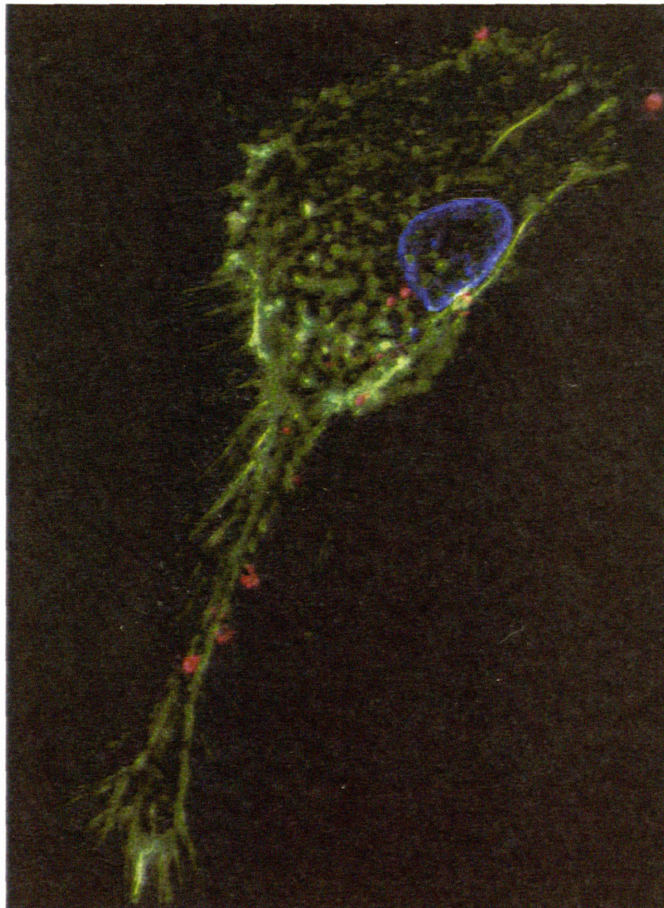


Figure 3.3. Phagocytosis of 5μm fluorescently labeled microparticles(red) by a primary, human peripheral blood mononuclear derived DC which has been fixed and stained for actin (green) and nuclear material (blue).

The reason for the adjuvancy of microparticles alone is unknown but several mechanisms have been proposed. The physical size of microparticles are characteristic of some pathogens[37] and the immune system may somehow mistakes a particle for a potentially dangerous microorganism. It has been demonstrated that uptake of 1 μm latex microparticles by monocytes leads to the differentiation of DCs and their migration to lymph nodes[38]. Futhermore, phagocytosis of latex beads by DCs in-vitro induces phenotypic maturation of the DCs, as shown by CD83 up-regulation[39]. Another potential explanation is the microparticle mediated, controlled release of antigen or plasmid DNA containing immunostimulatory bacterial CpG motifs which are recognized by TLR9 on the surface of a DC [12].

3.2.4.1. Microencapsulation of plasmid using PLGA

Traditionally, the polymer most often utilized in antigen and plasmid encapsulation is poly lactic-co-glycolic acid (Figure 3.4.). This biodegradable and biocompatible polymer is already approved by the US Food and Drug Administration, making it more easily advanced to the clinic than new polymers. It was originally used for degradable sutures[40] but has found application from everything from delivery of narcotic agonists[41], contraceptives[42], pesticides[43], and the healing of bone fractures[44] and ligaments[45]. It decomposes by acid and base hydrolysis or enzyme catalyzed degradation[46] and ultimately leaves the body as carbon dioxide. This polymer's application to the delivery of protein or peptide antigen vaccines are beyond the scope of this report, and the curious reader is directed to more comprehensive reviews[37, 47].

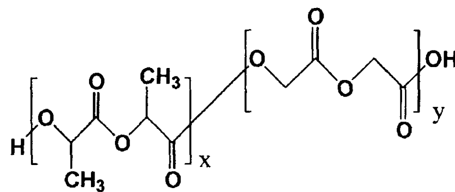
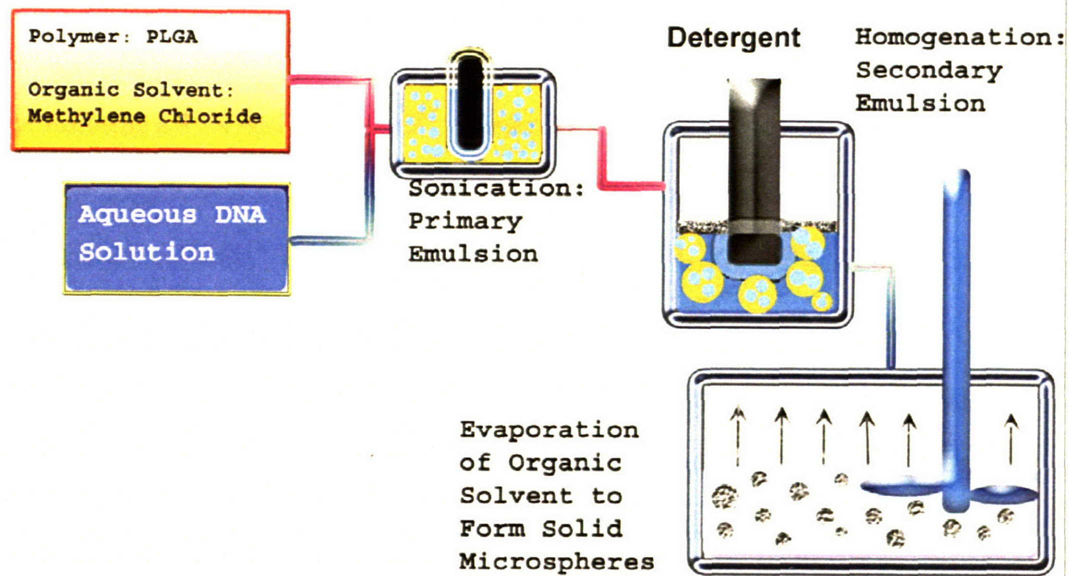


Figure 3.4. Structure of poly lactic-co-glycolic acid. (Ratio of lactide to glycolide is x:y).

The most common methods for encapsulation of drugs with PLGA are the double-emulsion procedure and spray drying. Double emulsion (reviewed in [48]) is the most common method for the encapsulation of plasmid (Figure 3.5.). Spray drying is used less frequently for plasmid microencapsulation [49], but in both procedures, an aqueous solution containing plasmid is emulsified with an organic phase containing the polymer to serve as the controlled release agent (e.g. PLGA). The release of plasmid from PLGA microparticles typically occurs through burst phases due to a property of PLGA and other similar polymers which allow diffusion of water into the interior of the particle (aptly named "bulk erosion") [50, 51]. This plasmid release is tunable using various molecular weights and polymer monomer ratios made up of hydrophobic lactide and hydrophilic glycolide.

A.



B.

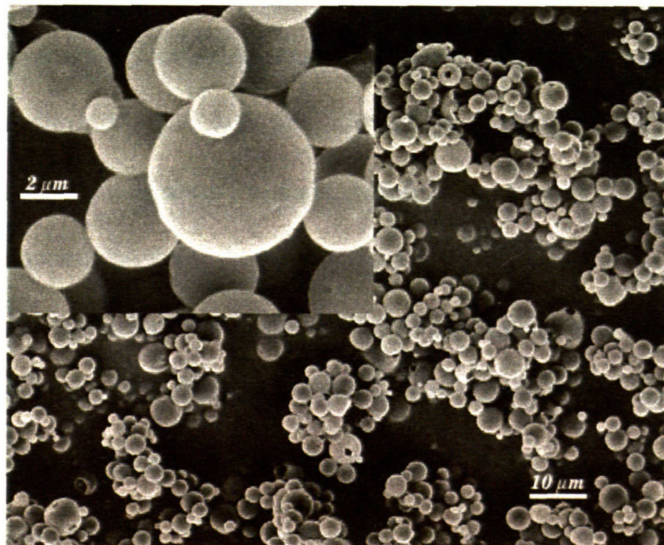


Figure 3.5. A. Schematic deptiction of the double emulsion procedure. **B.** PLGA microparticles made by the double emulsion/solvent evaporation technique. Body is 1000X and inset is 5000X magnification.

The first attempt to deliver plasmid as a vaccine using PLGA microencapsulation was by Hedley et al. who showed that stronger CTL responses were elicited using microencapsulated plasmid delivery s.c. and i.p. when compared to naked plasmid injections using a VSV antigen system[52]. Clinical trials using the PLGA microparticle delivery system showed 83% of patients demonstrated an immune response which persisted 6 months using a plasmid encoding (HPV-16 E7) [53]. Furthermore, Phase 1 clinical trials for cervical intraepithelial neoplasia using PLGA delivery of plasmid indicated that no adverse side effects were observable, and 73% of patients exhibited a specific immune response[54]. Furthermore, 33% of patients exhibited a complete histologic response[54]. Later, it was demonstrated that oral immunization with plasmid DNA led to protective immunity against rotavirus challenge when PLGA microparticles were used for delivery[55].

The attractiveness of PLGA has generated a great deal of effort to advance this technology into the clinic. Although these studies demonstrate the ability of PLGA to function as a genetic vaccine delivery vehicle, this polymer was never designed for this particular application and has several limiting disadvantages. Particularly, acidic degradation products which build up in the microparticle interior can severely stunt or permanently damage the activity of plasmid DNA. This can be attributed to PLGA ester bond degradation leading to acids which cannot easily diffuse out and away from the particle interior. It has been demonstrated using pH sensitive fluorescent probes and microscopy that the pH can drop to as low as 2 after three days of incubation[56]. Although this internal pH microclimate can stabilize some drugs[57], low pH has been shown to completely abolish plasmid transfection activity below a pH of 4[51]. In addition, the

amount of time needed for quantitative release of plasmid DNA from even low molecular weight PLGA microparticles is on the order of 2 weeks[58], while the lifespan of the majority of DCs after activation is approximately 10 days[59].

Addition or replacement of PLGA with agents aimed at enhancing the immunogenicity of the formualations, such as lypophilic molecules (taurocholic acid (TA) and polyethylene-glycol-distearoylphosphatidylehanolamine (PEG-DSPE)), can increase both CTL and antibody response and can protect mice against tumor challenge i.v. [60]. Potential mechanisms for the observed heightened activity as a vaccine could involve membrane disruption upon uptake by a cell, or plasmid binding which may protect plasmid inside the particle or after release.

Besides microclimate pH deactivation of supercoiled plasmid, the process of fabrication itself can substantially damage DNA because of high sheer stresses encountered during sonication and homogenization, as these are required in the double emulsion procedure. It is also possible that organic/aqueous interfaces, which tend to denature proteins, have a deleterious effect. Furthermore, freeze drying is commonly used prior to isolation of powdered microparticles and can also damage plasmid. To address these issues, Ando et al. put forth a technique for fabrication of plasmid microparticles which virtually eliminated the loss of supercoiled plasmid during fabriction[61]. This process involves the freezing of the internal aqueous phase containing plasmid DNA as to shield it from sheer stress[61]. Also stabilization agents, such as lactose have been shown to eliminate damage to plasmid during freeze drying.

3.2.4.2. Cationic particles

3.2.4.2.1. Absorption via cationic surfactants

To completely avoid the deleterious effects of processing plasmid DNA during the double emulsion procedure, cationic microparticles can be fabricated which retain a cationic surface to which polyanionic plasmid DNA could be bound. Addition of a cationic surfactant called cetyltrimethylammonium bromide (CTAB) (Figure 3.6.) produces this positively charged surface in contrast to the use of conventional detergenst such as poly-vinyl alcohol which impart a negatively charged surface (PVA). These cationic microparticles are capable of eliciting humoral responses 250X greater than naked DNA and heightened CTL responses using a HIV p55 gag model with a relatively small dose of DNA (1 μg)[62]. Furthermore, these microparticles can transfect primary DCs, albeit to a low extent[63], and have been found in draining lymph nodes 3 hours post injection[64]. Further studies showed that although naked DNA works better at higher dosages, this response is diminished upon injection of lower amounts of DNA[65]. Particles with surface adsorbed plasmid, however, maintain high levels of Ab and CTL response with 1000x less plasmid[65]. It is unknown as to the exact mechanism of these cationic microparticles and the effects of CTAB, however this system may allow for greater uptake by APCs, faster release of plasmid DNA, and endosomal release properties imparted by the cationic detergent.

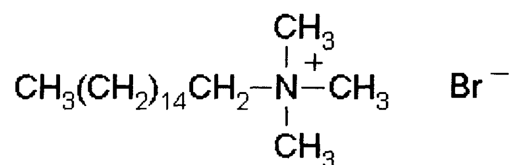


Figure 3.6. The structure of the cationic surfactant, Cetyltrimethylammonium bromide (CTAB).

Application of this genetic vaccine delivery system to cancer was first directed toward delivery of carcinoembryonic antigen (CEA) encoded plasmid. This formulation inhibited CEA expressing, adenocarcinoma cell growth in a population of vaccinated mice when used as a vaccine[66]. Furthermore, a boosting regimen with naked DNA i.m. encoding GM-CSF (a potent immunomodulatory cytokine), results in an increased frequency of responders and inhibition of tumor growth[66].

Recently, an extremely simple method of creating cationic nanoparticles using a hot cetyl alcohol-polysorbate 80 wax / aqueous emulsion formed by adding cationic surfactant and cooling the system to room temperature to create cationic microparticles approximately 100nm in diameter.[67] This method has several advantages including simplicity, uniform size of particles, cationic surface capable of binding plasmid DNA, and elimination of harsh environment present in double emulsion preparations, not to mention obviating the need for organic solvents. These particles were combined with formulations with DOPE to increase transfection, and cholesterol mannan on the surface to target DC mannose receptor (which was later determined to increase phagocytosis of nanoparticles by 200%[68]), and injected intradermally to elicit IgG titres 16X greater than naked plasmid DNA[69]. This group has used the plasmid coated nanoparticles to elicit immune induction by a variety of routes, all resulting in higher antibody and Th1 cell mediated responses[70-72].

3.2.4.2.2. Cationic chitosan nanoparticles.

Chitosan is a biodegradable polymer derived from chitin in the shells of crustaceans, the second most abundant polymer on earth behind cellulose (Figure 3.7.).

Chitosan is the deacetylated version of chitin, and has a variety of potential uses in textiles, water treatment, and biodegradable films to name just a few[73]. More importantly for genetic vaccine purposes, the mannose receptor commonly found on APCs interacts with acetylglucosamine, which is a repeating unit in chitosan[74]

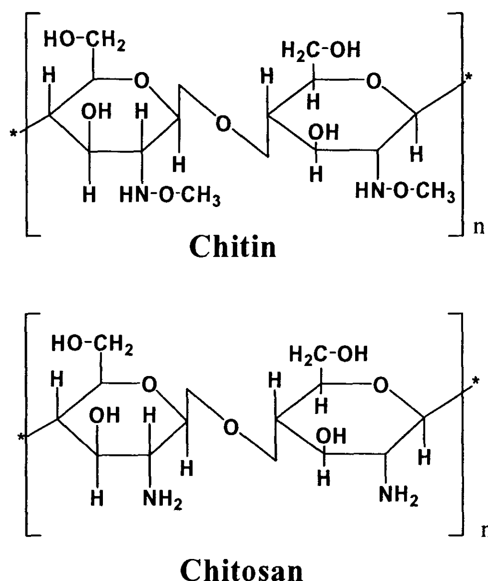


Figure 3.7. Structures of chitin and chitosan

Similarly to cationic lipoplexes, the simplest way to create plasmid / chitosan complexes is through simple incubation and condensation through charge neutralization. Alternatively, cationic nanoparticles can be prepared either by a coacervate method[75], or by addition of carboxymethylcellulose[76]. Generally, chitosan particles are directed toward the delivery of genetic vaccines to the mucosal tissue and are not used for cancer genetic vaccines. However, Kabbaj et al used chitosan nanoparticles to deliver tumor inhibitory mycobacterial DNA to reduce the level of degradation by nucleases[77]. The

ability of this plasmid / chitosan formulation was 20X more effective than naked DNA for inhibition of melanoma tumor cell growth.

3.2.4.3. Poly-ortho ester microparticles

Recently, Wang et al illustrated the use of biodegradable and biocompatible, poly-ortho-esters (POE) for microparticulate genetic vaccines (Figure 3.8.). Unlike bulk degradation of PLGA, POE's degrade by erosion of the surface. Because this polymer degrades by surface degradation, acidic byproducts can diffuse away rather than building up inside the polymer matrix. Particularly interesting is the ability of these polymers to degrade more rapidly at acidic pH in range of endosomes than at physiologic pH. One of these polymers led to higher levels of secreted antibody and greater $CD8^+$ T-cell responses than PLGA microparticle delivery. This polymer also showed the ability to inhibit the growth of tumor cells expressing the Class I restricted epitope in mice vaccinated with the POE formulations. This difference in immunogenicity of the formulations was attributed to the ability of the microparticles to release plasmid in a time frame that corresponds to the induction of immune response by processing and presentation of peptide on the surface of activated DCs.[78]

Poly(ortho ester)

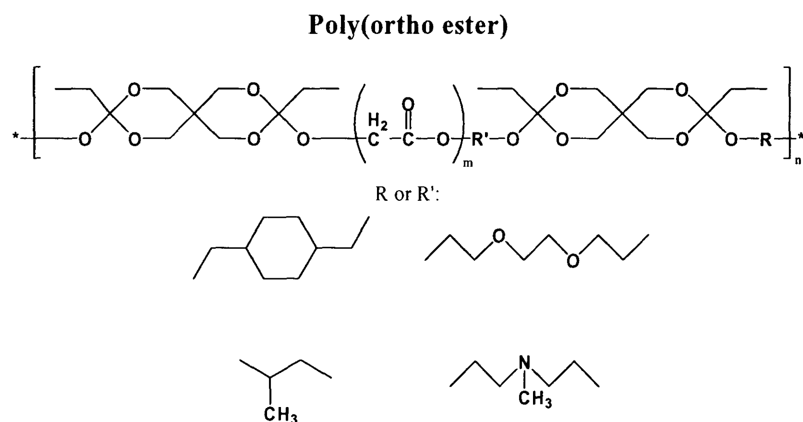


Figure 3.8. The structure of the poly (ortho ester) used by Wang et al[79]. R or R' is shown below the polymer chain.

3.2.5. Genetic Engineering of DCs *in vitro*

An alternative strategy to targeting transfection of DCs *in vivo* is the isolation of immature DCs from a patient for antigen loading *in vitro*. The cells could then subsequently be injected back into the patient to allow the DCs to prime naïve T-cells specific for the antigen. One method to pulse TAA to DCs is through tumor antigens or tumor lysates [80-83]. These have the disadvantages of limited duration of antigen expression[84] and in the case of tumor antigen pulsing, restriction of therapy to the haplotype of the antigen. Some groups have fused DCs with tumor cells by PEG co-culture to gain both the expression of correct tumor antigens with the costimulatory competency of DCs[85]. It has been demonstrated that these cells express surface molecules from both cells, and introduction back into mice results in antigen specific CTLs and rejection of established metastasis. Others have recently used electrofusion techniques to allow fusion of directly isolated tumor cells with DCs without the need for extended *in vitro* culture periods used with PEG co-culture[86].

Attempts to transfect DCs *in vitro* have resulted in only low levels of DC transfection using non-viral gene delivery. Primary DCs in culture have proven to be a particularly difficult cell to transfect [63, 87-91]. In one particular instance it was necessary to use RT-PCR to detect the low levels of transfection inducible using non-viral means[63]. However, some progress has been made in sufficiently transducing a DC *in vitro* as to render it capable of activating naïve, antigen specific T-cells *in vivo.* One study employed the use of a cationic peptide called CL22, which demonstrated the ability to transfect DCs *in vitro* and protected mice against melanoma challenge while peptide pulsing was ineffective[88]. Another group reported a peptide containing ornnithine and histidine DNA binding amino acids which were capable of transfecting a dendritic cell line. Injection of cationic peptide transduced cells induced secretion of IFN-γ, while naked DNA transduced cells were ineffective[92]. Also PEI complexes with mannose and adenoviral particle moieties have been shown to transfect DCs in vitro and stimulate proliferation in allogenic and autologous mixed lymphocyte reactions upon reinjection[87].

Transfection of DCs with RNA encoding reporter genes may be a more efficient alternative to plasmid, because unlike DNA transfection, RNA only needs to be delivered to the cytoplasm to be effective rather than the nucleus. In support of this concept, Strobel et al. reported the use of RNA to transduce primary human DCs resulted in 2 fold better expression than DNA when using liposome delivery[93]. Furthermore, these cells, when reinjected, elicited stronger antigen specific influenza matrix protein antigen memory T cell responses than DNA transfected cells.[93] Importantly, DCs retained their immunological phenotype after transfection, which may be crucial factor in DC migration to the lymph nodes upon reintroduction.

Use of "whole tumor RNA" to transfect DCs via electroporation may be the best way to allow for natural immunodominance in processing and presenting the antigen optimally. This obviates the need for discovery of haplotype restricted antigens in each patient. Resected tumors can be sampled and RNA can be amplified without loss of function to obtain complete RNA from tumor cells for transfection of DCs[94]. Using this technology, studies have been preformed using whole tumor RNA for myeloma[95], breast carcinoma[96], colorectal cancer (renal cell carcinoma)[97-99], and chronic lymphocytic leukemia[100]. In addition, antisense oligonucleotides specific for inhibition of invariant chain expression have been delivered to DCs *in vitro*. Theoretically, loss of invariant chain expression would lead to the complexation of otherwise MHC Class I restricted epitopes on MHC Class II molecules. Using this technique, increased magnitude of immune response was observed along with persistence of $CD8^+$ Tcell responses in an ovalbumen model system.[101]

3.3 References

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4. Improving Genetic Vaccines

4.1. Enhancing the immunogenicity of genetic vaccines

4.1.1. Adjuvants and co-stimulation

Numerous attempts have been made to increase the potency of non-viral genetic vaccines through genetic modifications, targeting strategies, and boosting regimens, to name just a few. Adjuvants are defined as anything added to a vaccine that increases the immune response in terms of magnitude, duration, or time of onset[1]. By this definition even micro-injury during inoculation with vaccine formulations[2-4] or the haplotype of an individual[5] can be conceivably called an adjuvant. As it relates to DCs, adjuvancy can be more tightly defined as anything that induces progression toward an optimal level of signal 1 (antigen presentation enhancements such as in delivery systems) and signal 2 (such as co-stimulatory molecule and cytokine up-regulations by using "immunostimulatory adjuvants"). Enhancing the presentation to signal 1 seems fairly straightforward by increasing expression by the antigen in the proper cell type. However, methods for enhancing the optimal presentation of Signal 2 remain unclear, due in part to the complex dialogue between lymphocytes.

It is clear, however, that Signal 2 requires the up-regulation of co-stimulatory molecules and the secretion of Th1 and Th2 cytokines. What causes this reaction to a stimulus is not fully understood. One theory states that the immune system is finely tuned to react to "danger signals"[6]. These signals distinguish between when to mount an attack, in the case of an invading pathogen, and when to suppress immune rejection, in the case of regularly surveyed "self / non-dangerous" antigens. The current dogma is that the immune system induces tolerance to some antigens in certain circumstances (e.g. without Signal 2 or in the presence of some other signal), and that tumor cells may have the ability to down-regulate this signal[7]. Attempts to modulate the immunostimulatory properties of genetic vaccines have resulted in incremental increases in potency and understanding of the immune system.

4.1.2. Traditional and genetic "adjuvants"

Perhaps the most straightforward way to facilitate T-cell stimulation during DNA vaccination is to deliver genes encoding for the known co-stimulatory and secreted cytokine signals. The numerous types and variations of these signals are too many to discuss here, but are reviewed thoroughly elsewhere[8]. Examples of secreted cytokines signals are the Th1 cytokines such as IL-2, and IL-12, Th2 cytokines such as IL-4 and IL-10, and the seemingly non-polarized GM-CSF, the most commonly used genetic adjuvant. The timing and administration in respect to antigen plasmid administration can significantly affect the outcome of a genetic cytokine vaccination. Also, combinations of two or more of these cytokine signals can have a more pronounced effect than either of the two alone. There are indications that modifications can be made to certain known immunostimulatory cytokines which can alter their systemic toxicity profile while still

retaining their anti-tumor effects[9]. Genes encoding for the T-cell activating co-stimulatory molecules such as B7.1 and B7.2 are promising candidates for increasing potency, but results have been conflicting[8, 10].

Some fusion partners have an inherent immunogenicity to which they can impart upon an antigen. Examples of this are tetanus toxoid[11], plant viral proteins[12], and HSP70[13]. Addition of these fusion constructs is associated with large increases in potency. Mechanisms of this increased immunogenicity are thought to involve induction of helper T-cell responses through processing of the fusion proteins by the MHC Class II pathway.

A range of traditional adjuvants have also been explored by co-administration with the genetic vaccine formulation. As previously discussed, the delivery systems themselves can have adjuvant properties. Even the gold beads used in gene gun immunization have adjuvant properties. A recent study has shown gold beads in tandem with *in vivo* electroporation led to an increase in observed immune responses[14]. Importantly, this did not enhance gene expression, but may have acted as a recruiting factor for DCs[14]. Other examples include alum (aluminum salts) and Freund's oil-in-water adjuvant (a powerful, yet toxic adjuvant containing mycobacterial materials). It is doubtful that the latter will ever be used in humans despite the fact that modifications have been made to decrease toxicity of this system. A cationic emulsion, called MF59, has been used to adsorb and increase the persistence of plasmid DNA encoding HIV p55 gag at the injection site, which resulted in increased serum IgG titers when compared to naked plasmid in mice and rabbits[15].

4.2. Targeting genetic vaccines

There are three primary ways to target an antigen to a particular cell or organ: 1) targeting the delivery system for uptake by a specific cell, 2) linking the antigen to a targeting protein or 3) using DNA that is transcriptionally regulated and only active in the target cell. For a specific cell type, modifications can be made to the antigen to direct it to different pathways of antigen processing and presentation.

4.2.1. Targeting Uptake

There are a variety of surface receptors that are potential targets for APC specific DNA or post-transcriptional antigen delivery. Fc receptors are thought to bind immune complexes and opsonize particulates. This binding activates DCs by up-regulation of co-stimulatory molecules[16]. CTLA-4 is another ligand that has been used to target DCs, and is thought to bind B7.1/B7.2 at a high affinity. Some chemokines act through binding to DC cell surface receptors and can be employed as well. Certain proteins such as CD36 and αβ5 integrins are involved with receptor mediated phagocytosis[17]and others such as DEC205 (or the human homolog LY75) and DC-SIGN, which are DC markers mediate receptor mediated endocytosis[18]. All of these are potential targets for use in directing DNA or antigen specifically to APCs (Table 2). A good example is the addition of mannose or mannan to a delivery system to target the mannose receptor on the DC surface. Targeting this receptor has led to 2-fold increase in phagocytosis of particle formulations by APC *in vitro*[19] and has also been used to increase transfection of cultured DCs[20]. The addition of mannan to the surface has also been associated with an increase in antibody and cell mediated immune responses *in vivo*[21].

Ligand	Target	Ref
IgG F_C Fragment	DC F_C Receptor	22
CTLA-4	DC B7.1 & B7.2	23-25
L-selectin	Endothelial CD34 (Lymph Node)	23, 24
RANTES chemokine	DC Chemokine Receptor	26
IP-10 chemokine	DC Chemokine Receptor	27
MCP-3 chemokine	DC Chemokine Receptor	27
Mannose/Mannan	DC MR	20, 21
DEC205 mAb	DC Receptor Mediated Endocytosis	ND
CD36 / αβ5 integrin Ligands	DC Receptor Mediated Phagocytosis	ND

Table 4.1. Some examples of the genetic vaccine targeting strategies. ND=These targets, have not been investigated in genetic vaccine formulations.

4.2.2. Intracellular Targeting

Targeting of antigen to different cellular compartments may influence the way that antigen is processed and presented. Conceivably, an antigen normally processed by the MHC Class II pathway that is presented on MHC Class I pathway (or vice-verse) could lead to immune responses that primarily elicit CTL activity instead of antibody secretion (isotype switching), and possibly a more relevant therapy to a particular disease.

One of the most common cellular localization sequences used for targeting an antigen fusion partner to the MHC Class I pathway is ubiquitin. Ubiquitin marks proteins for degradation by the proteosome into small peptides which are then transported to the endoplasmic reticulum for loading onto MHC Class I molecules. Addition of ubiquitin to

plasmid fusion constructs usually increases CTL responses at the cost of humoral responses[28-32]. However, in one study, a ubiquitin fusion construct demonstrated a decrease in humoral response while CTL response remained unchanged[33]. Further examination of ubiquitin fusion constructs will be required for generalization of this strategy. Calreticulin (CRT) is a particularly interesting candidate for cancer vaccines because it has both MHC Class I targeting capacity and anti-angiogenesis properties (the ability to inhibit blood vessel growth to the site of a tumor)[34-37]. Addition of CRT to fusion constructs has shown to exhibit notable anti-tumor activity when given as a DNA vaccine for HPV-16 E7 antigen. It is believed that the anti-angiogenesis properties of CRT are involved in this observed response[38].

Targeting the MHC Class II pathway may also be a logical strategy if a humor response is desired. This pathway can be targeted through fusion with lysosomal associated proteins such as LAMP-1[39-41] or LIMP II[28]. An antigen can also be targeted to the cell surface[42]. Another apparent mechanism for increasing MHC Class II processing is targeting antigen for secretion. This antigen could then be taken up by a DC and associated with Class II molecules in the lysosome. Interestingly, both humoral and/or cell mediated immunity are increased by using this strategy[43, 44]. This phenomenon may involve a cross-priming mechanism to allow antigen to enter the cytoplasm.

4.2.3. Transcriptional Targeting

One of the most commonly used mammalian promoters in genetic vaccines is the cytomegalovirus promoter (pCMV). This is an extremely strong viral promoter that is capable of mediating high levels of antigen expression in many cell types. However, some of the expression products in a genetic vaccine, such as the immunomodulating

cytokines discussed earlier, may generate toxicity if expression is not controlled. Also, persisting expression of low levels of antigen after vaccination may induce tolerance to the expressed antigen[45]. Alternatively, one strategy is to use transient promoters capable of transfecting a targeted subset of cells, such as DCs. One such DC specific promoter is the lectin promoter, which was used with GFP plasmid to demonstrate transfection of DCs *in vivo* and anti-GFP CTL response[46]. Another example is the mature DC specific fascin promoter, which demonstrated a distinct Th1 response compared to Th2 responses observed when using pCMV[47, 48]. The isotype switching of responses by transcriptional targeting may prove to be a powerful method to alter the way the immune system reacts to an antigen.

4.3. <u>Increasing gene expression</u>

DCs have proven to be a notoriously difficult cell to transfect[20, 49-53]. Increasing transfection of these cells seems to be a logical way to increase vaccine potency. Although some evidence suggests that greater antigen expression does not always lead to greater immune responses[54], others have shown that increasing the magnitude and duration of antigen expression is a viable way to increase the immunogenicity of genetic vaccines. Some examples of these strategies are: 1) Optimization of the plasmid construct, 2) Avoiding degradation in the lysosomes, 3) Increased DC lifespan, and 4) Self replicating antigen constructs.

4.3.1. Plasmid Modifications

One of the most straightforward ways to increase gene expression is through the addition of multiple gene expression cassettes in the same plasmid vector. Sasaki et al used these dual antigen expression vectors to generate significantly higher expression

than that obtained by using 2X the amount of single expression vector cassettes. Vaccinations with these plasmids correspondingly led to increased IL-4 and IFN-γ secretion by isolated splenocytes[55]. Haas et al demonstrated that optimizing codon usage, which can be significantly different in mammals relative to bacteria, led to increases in antibody and CTL responses in mice using a HIV gp120 antigen construct[56]. Another example is codon optimized plasmid encoding for a MHC class I restricted listeria antigen, which showed increases in CTL responses and partial protection from listerial challenge while unoptimized plasmid remained ineffective[57].

4.3.2. Avoiding lysosomal degradation of plasmid

Other attempts to increase gene expression are aimed at avoiding lysosomal degradation of the plasmid DNA. Trehalose 6,6'-dimycolate (TDM) has been shown to cause inhibition of fusion between the lysosome and phagosome[58] and this inhibition may allow more time for the transfer of DNA from phagosomal compartments to cytoplasm of APCs before lysosomal degradation. Inclusion of TDM in PLGA microparticle vaccine formulations induces stronger resistance to mycobacterium tuberculosis in mice[59]. Other strategies attempt to avoid the lysosomal pathway altogether by adding mechanisms for traversing the plasma membrane[60]. Using a plasmid encoding either the protein transduction domains for HSV-1 (VP-22)[61], or Pseudomonas aeruginosa exotoxin A (ETA(dII))[62], fused with HPV type 16 E7 antigen, Hung et al observed a 50X increase in the amount of responding $CD8^+$ T-cells along with the increased ability of vaccinated mice to react to E7 expressing tumors.

4.3.3. DC lifespan

Increasing the lifetime of an antigen expressing DC *in vivo* is yet another strategy to increase the immune presentation. Kim et al. investigated the effect of including a plasmid encoding anti-apoptotic proteins such as Bcl-x_L[63] and Serine Protease Inhibitor 6 (SPI-6)[64] to antigen fusion constructs with MHC Class II targeting signals. These anti-apoptotic proteins increased avidity of T-cells and elicited stronger tumor protection. Interestingly, co-vaccination with genes such as Fas[65] and caspases 2 or 3[66] (apoptotic proteins) can also increase the potency of genetic vaccine formulations. While the exact mechanism of immune stimulation is unclear, it is possible that cross presentation of antigen from the apoptotic cells to a DC may serve as an appropriate "danger" signal.

4.3.4. Replicons

Self-replicating RNA antigen constructs, or replicons, are based on alpha viruses such as the Venezuelan equine encephalitis virus, Sindbis Virus, and Semliki Forest Virus. Plasmid replicons contain the information for the transcription of a positive strand of RNA, which in turn encodes for both a 5' replicase complex, and a negative strand of antigen encoding RNA (Figure 4.1.). These vectors do not produce viral structural proteins, leaving no possibility for recombinant events. This is accomplished by replacing the viral gene for the structural proteins with a heterologous gene. Replicons have also been called "suicide vectors" because the presence of large quantities of dsRNA is thought to induce apoptosis shortly after transfection. Due to the infection process occurring in the cytoplasm, there is little possibility of chromosomal integration.

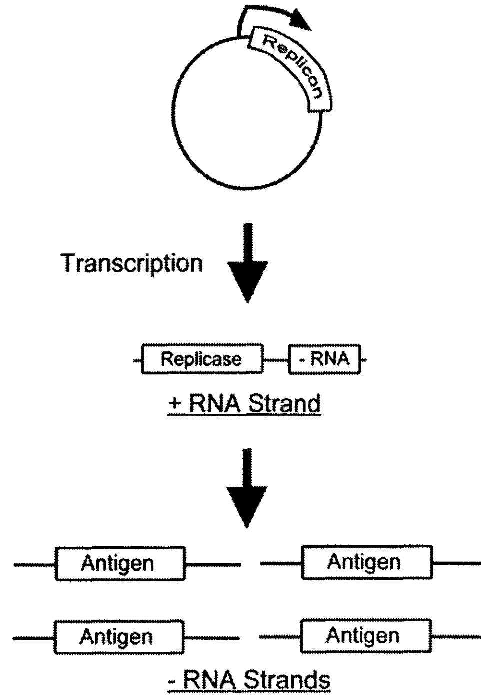

Figure 4.1. Self-replicating plasmid replicons.

It should be noted that by using a defective helper gene encoding structural proteins, an infection competent, but replication incompetent, viral particle can be produced. These particles can target DCs[67, 68] and have higher gene transfection efficiency than replicon plasmids alone. However, there is a small probability that recombination events could occur, leading to infectious particles. The reader is directed to a recent review on alphaviral vectors for more detail on this topic[69].

Replicons have proven to be powerful enhancements to DNA vaccination, and are capable of eliciting antibody and tumor protective responses at up to 1000 times lower titers than conventional naked DNA vaccines in a β-gal expressing tumor model[70]. Vaccination with replicons has also induced protective immunity to melanoma challenge

in a TRP-1 expression system, unlike conventional DNA vaccines[71]. Although it is logical to infer that increased antigen expression is the reason for this enhancement, it is widely accepted that is rather due to the presence of dsRNA. The formation of dsRNA can activate antiviral apoptosis pathways, which subsequently lead to cross-priming of antigen in the presence of a danger signal[71].

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5. Fabrication and Characterization of Microparticles Containing pH Sensitive Poly(β-Amino Ester)s

5.1 Introduction

Along with the discovery of antigens, the primary focus of research in the field of genetic vaccines has been increasing immunological potency. To this end, the goal of cancer immuno-therapeutics has been to stimulate tolerant or non-responsive antigen-specific T-lymphocytes to attack cells expressing tumor associated antigens (TAA)[1]. This has proven difficult, in part, because cancer cells are thought to evade immune recognition by down-regulating the antigen processing and presentation mechanisms of dendritic cells (DC)[2]. Adjuvants, cytokines, and self-replicating RNA vectors have been implemented to further stimulate the immune system to recognize a TAA as representative of a "dangerous" target[3-6]. Still another approach is to improve delivery of the antigen encoded genes because uptake and expression is extremely inefficient using plasmid injections without delivery vectors(i.e. naked DNA vaccines)[7-9].

Viral vectors are naturally the most efficient method for gene delivery. However, the same shortcomings experienced in gene therapy applications (e.g. toxicity, immune

rejection) limit the implementation of viruses for genetic vaccines. For example, it was recently reported that re-stimulation with adenovirally transduced DCs actually decreased the antigen specific immune response in favor of strong anti-adenovirus specific responses in human melanoma patients[10]. In addition, pre-existing immunity to viruses can limit applicability to the general population.

Non-viral DNA delivery systems lack the drawbacks of viral vectors, but at the cost of gene delivery efficiency. Advances in potency have been made with the use of electroporation[11, 12], gene gun[11, 13], and liposomes[14, 15], but much progress still remains to be made to obtain viral functionality and the effectiveness that follows. A particularly promising non-viral delivery system for vaccine use is degradable microparticulate DNA delivery formulations made from the FDA approved material poly lactic-co-glycolic acid (PLGA). The ability to passively target antigen presenting cells such as dendritic cells and macrophages, along with the adjuvant qualities that complement their characteristic pathogenic size, makes microparticle systems an interesting candidate for vaccine formulations[16]. This strategy is also appealing because of its versatility in delivering both extremely large plasmid payloads and inclusion of virtually any immunomodulating agent in the same package.

Although PLGA microparticles can protect DNA payload in a physiological environment, and facilitate APC targeting, this material was never meant to mediate intracellular delivery of DNA. Even low molecular weight PLGA systems require up to 13 days to fully release encapsulated DNA after dendritic cell uptake *in vitro*[17]. This seems an excessively long period of time given evidence that most dendritic cells die within 7 days after activation and migration to draining lymph nodes[18]. Furthermore,

PLGA microparticles can produce an extremely low pH microclimate (pH < 3.5) after only 3 days in an aqueous environment[19]. This level of acidity has been shown to severely reduce the activity of plasmid DNA[20]. PLGA microparticles have also been shown to remain confined to phagolysosomal vesicles, and generate only low levels of gene expression in antigen presenting cells[21]. Improvements have been made to PLGA microparticles for delivery of plasmid DNA with promising results[22-26], but novel delivery strategies are still needed to advance the potency of non-viral genetic vaccines for use in the clinic.

Recently, a degradable poly(β-amino ester) was reported for use as a polymeric gene transfer vector[27]. This polymer has been used to form microspheres and release encapsulated rhodamine conjugated dextran upon pH stimulus[28]. We hypothesize that the properties of this polymer that allow for 1) triggered release of encapsulated contents upon phagosomal acidification, 2) lysosomal bypassing functionalities, and 3) the ability to condense and protect plasmid DNA intracellularly will lead to an increase in vaccine potency. The formulation and characterization of these microparticles encapsulating plasmid DNA is, of course, critical to their successful use, and it is these issues that are examined in this chapter.

5.2. Materials and Methods

5.2.1. Materials

Poly(*d,l*-lactic-co-glycolic acid) polymer (PLGA, RG502H Resomer 50:50) was purchased from Boehringer Ingelheim (Ingelheim, Germany). Poly(β-amino ester) (PBAE) was synthesized as previously reported ($M_n \approx$ 7-10 kD) [28]. Plasmid DNA

encoding firefly luciferase (pCMV-Luc) was obtained from Elim Biopharmaceuticals (Hayward, CA).

5.2.2. Cells

The P388D1 macrophage cell line was obtained from ATCC (Manassas, VA). Cells were cultured in RPMI 1640 media (Gibco Life Technologies; Carlsbad, CA) containing 10% FBS, 0.1 M HEPES, 1 mM Sodium Pyruvate, and 100 U/ml Penicillin/Streptomyocin.

5.2.3. Preparation of microparticles

Plasmid containing microparticles were prepared by the following modification of the double emulsion technique previously described[29]. Varying amounts of PLGA blended with PBAE were explored in microsphere formulations to determine optimal payload release. Lyophilized plasmid DNA (1 mg) was added to an aqueous solution (100 μL) of EDTA (1 mM) and D(+)-Lactose (300 mM). This solution was then emulsified with an organic solution of PLGA and PBAE at varying degrees of composition (200 mg) in CH_2Cl_2 (4 ml) using a probe sonicator (Sonics and Materials Inc; Danbury, Conneticut). The resulting emulsion was then immediately added to a homogenized solution of poly(vinyl alcohol) (50 ml, 5% PVA (w/w), 5000 rpm) and NaCl. After 30 seconds, the final water-oil-water mixture was added to a second PVA solution (100 ml, 1% PVA, (w/w)) and allowed to stir for 3 hours at room temperature and then 1 hour at 4°C. Microspheres were washed and centrifuged 4X (rcf < 150 x g) to remove PVA prior to lyophilization for 48 hours. Yields were commonly 50-75% by weight of a white, fluffy powder. Microparticles and polymers were stored at -20°C in a desiccated chamber.

5.2.4. Characterization of microparticles

Encapsulation efficiency of the DNA microparticles was determined by dissolution in CH_2Cl_2 and extraction into 1X TAE buffer (pH = 8.0) over a 2 hour period. DNA concentration was detected using PicoGreen (Molecular Probes) and the Mithras plate reading fluorimeter (Berthold Technologies; Bad Wilbad, Germany). DNA integrity was determined using standard gel electrophoresis (1% agarose) comparing sample band integrity with unprocessed plasmid DNA standards using Image J software. Osmolality of the internal aqueous phase (i.e. solution with the same concentrations of EDTA, lactose, and plasmid used for the internal aqueous phase during the double emulsion procedure) and external aqueous phase (i.e. solution with the same concentrations of PVA and NaCl used for the external aqueous phase during the double emulsion procedure) was determined using a Vapro vapor pressure osmometer (Wescor: Logan, Utah) via vapor pressure depression. Microsphere size distributions were measured via volume displacement impedance using a Multisizer 3 using 30-200 μm orifice tubes (Beckman Coulter; Miami, FL). Zeta potentials were obtained using a ZetaPALS analyzer (Brookhaven Instruments; Holtsville, NY) with 10mM HEPES buffer at pH=7.4. Morphology of microsphere surfaces was imaged using scanning electron microscopy (SEM). Microparticle samples were certified low endotoxin level (<0.50 EU/mg) by the Cambrex LAL testing service (Walkersville, MD).

5.2.5. pH microenvironment measurements

Internal hydrogen ion concentration was determined as previously described[30]. Briefly, varying weights of microparticles were carefully weighed out into pre-weighed microcentrifuge tubes. Particles were incubated with 1ml of 50 μM HEPES (pH = 7.4)

for 24 or 72 hours. Tubes were centrifuged at 5000 rpm for 10 minutes, the supernatants were discarded, and the total weight of the microparticles and aqueous microenvironment was determined. Particles were then dissolved in acetonitrile (ACN) by vigorous vortexing. Tubes were centrifuged a second time to remove any remaining material and 0.7 ml of this ACN solution was added to 0.175 ml of deionized water prior to pH measurement using a micro probe reader. This measurement determines the total number of moles of free hydrogen ion in the microenvironment, and along with the total weight of water, the pH of the microclimate could be estimated.

5.2.6. Release profiles

Microparticles were incubated in Tris HCl (pH 7.5) at 37 ° C for 24 hours in triplicate. The samples were centrifuged briefly and the supernatant was transferred to a new tube and stored at -80° C to prevent DNA degradation. Fresh Tris HCl was added to the pelleted spheres and the tube vortexed gently to resuspend particles. This process was repeated for Days 2-7. On Day 7 following the above process, each sample tube from each day was analyzed for double stranded DNA content using Pico Green fluorescence in a black, polypropylene 96 well plate and a fluorescence plate reader at 488 nm (Berthold Technologies). Concentrations were determined with the use of a standard curve.

5.2.7. Reporter gene transfection

To obtain a full expression profile for the P388D1 macrophage cell line, we modified the 6-well plate protocol used by Hedley *et al.* to a 96 well plate format[31]. Briefly, P388D1 macrophages were seeded at $5x10^4$ cells/well in fibronectin coated, white polystyrene 96 well plates and allowed to achieve 75% confluence. Media was then replaced with a

suspension of pCMV-Luc plasmid DNA containing microspheres in cell media and allowed to incubate for 20 hours. A titration of the soluble, lipid-based transfection agent, Lipofectamine 2000 (Invitrogen), was prepared with DNA as a positive control. At several time points, the media was aspirated from the samples and cells were washed with PBS. The cells were lysed by incubation for 10 minutes at room temperature with Glo Lysis Buffer (Promega, 100 μl, 1X). The wells were then analyzed for luciferase protein content using the Bright Glo Luciferase Assay System (Promega) and a Mithras plate reading luminometer (Berthold Technologies). Alternatively, cells were treated with Cytochalasin-D (10 μm, Sigma) to inhibit phagocytosis along with transfection agents. Groups were compared using ANOVA and t-test analysis for significance ($\alpha = 0.05$).

Total well protein content was determined using a micro-BCA assay (Pierce Biotechnology; Rockford, IL) in tandem with the bioluminescence assay. After the lysis step, BCA reagents were added and the cells were incubated for 3 hours at 37°C and absorption was read at 562nm using the Spectra Max 384 Plus multi-well plate reader (Molecular Devices; Sunnyvale, CA).

5.2.8. Toxicity

Microparticle toxicity was determined by using a standard MTT assay (ATCC) using P388D1 macrophages. Briefly, 50,000 cells were plated into a 96 well plate and allowed to recover overnight. Supernatants were removed and replaced with a suspension of microparticles or DNA/Lipofectamine complexes in P388D1 media. Non-treated wells were used as controls and were titrated to give optimal signal as suggested in the manufacturer's instructions. After 24 hours of incubation with formulations, cells were

assayed for metabolic activity after addition of reagent, lysis buffer, and measuring absorbance at 570 nm with a plate reading spectrophotometer (Molecular Devices).

5.3. Results

5.3.1. Effect of the incorporation of PBAE on particle formation

As described here, and in previous work[28], addition of PBAE (structure shown in Figure 5.1.A.) into the microparticle formulation with PLGA (structure shown in Figure 5.1.B.), introduces several issues that need to be addressed during fabrication. PBAE is generally tackier than PLGA polymers and high speed centrifugation at temperatures above 4°C can cause the fusing of particles and extensive aggregation[28]. Another caveat to the addition of PBAE is its sensitivity to differences in internal vs. external aqueous phase osmolality. For this reason, the osmolality of the solution making up the internal aqueous phase (drug compartment) was determined along with the solution making up the outer aqueous phase for the homogenization step and stirring step (including PVA) using a titration of NaCl (0.1-0.5M) (Figure 5.2.A). This data suggest that approximately 0.2 M provides osmotic balance.

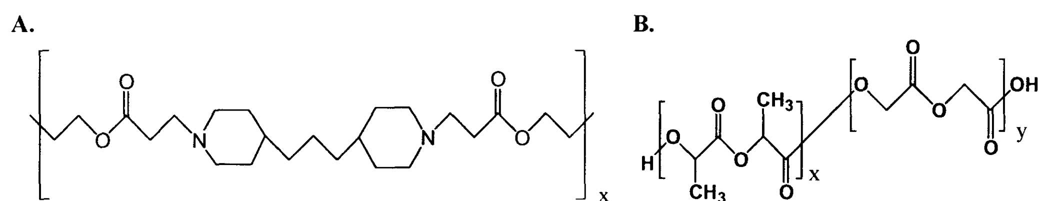


Figure 5.1. Molecular structure of **A.** PBAE and **B.** PLGA

To demonstrate the resulting osmotic effect on microparticle integrity and loading, particles were prepared using pCMV-Luc plasmid using 0, 0.2, and 0.5 M NaCl. Determination of total amount of plasmid encapsulation using CH_2Cl_2/TAE buffer

extraction and Pico-Green detection indicate that 0.2 M (92%) and 0.5 M NaCl (73%) in the external aqueous phase provides greater encapsulation than no salt at all (55%). SEM analysis of microparticle surface integrity demonstrates the effect of osmotic imbalance when PBAE is present in the microparticle polymer matrix. The surface of particles fabricated in the presence of 0.2 or 0.5 M salt in the external aqueous phase is of high integrity with very little flaws while the surface of particles prepared with no NaCl is covered with large cavities, presumably due to rupture of the polymer matrix above the internal aqueous compartments due to water influx (Figure 5.2. C-E). However, upon increasing salt concentration to 0.5 M, aggregation of particles was apparent after stirring. This aggregation was greater than in the case of particles prepared using 0 and 0.2 M NaCl (Figure 5.2.B).

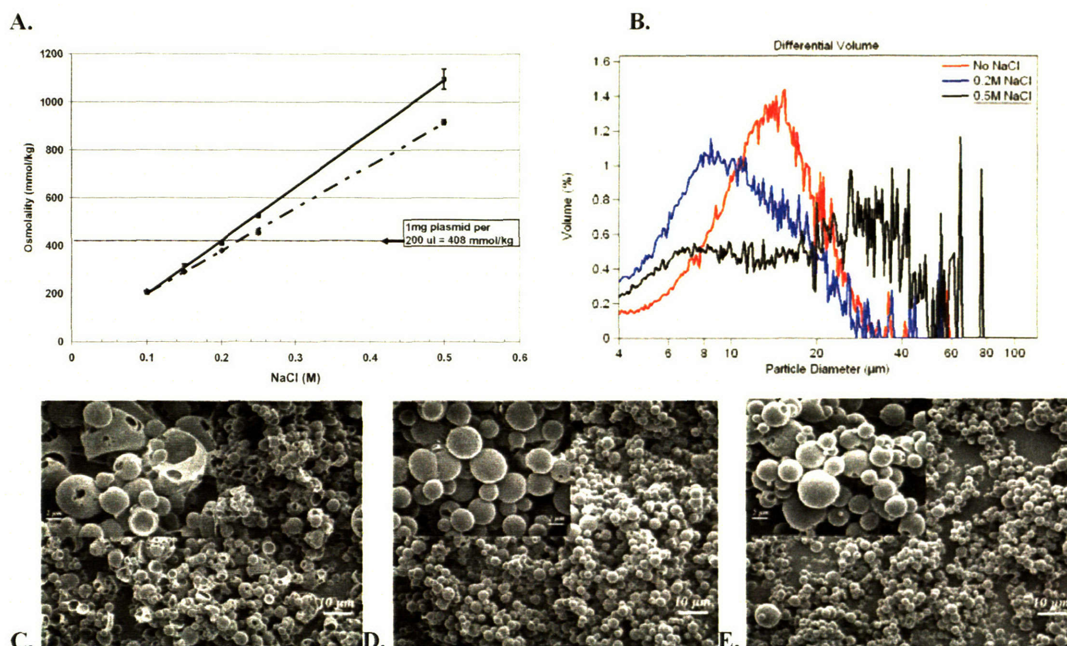


Figure 5.2. The effect of osmolality balance during fabrication of 25% PBAE microparticles. **A.** The osmolality (mmol/kg) of the outer aqueous phases (5% PVA w/v=bold line, 2.3% PVA w/v=dashed line), with varying amounts of NaCl. The osmolality of the internal aqueous phase was recorded at 408 ± 3 mmol/kg which corresponds to approximately 0.2M NaCl in the PVA solutions. **B.** The effect of salt addition to the outer aqueous phase on aggregation during microparticle fabrication. Microparticles that were prepared using an outer aqueous phase which osmotically matched the internal aqueous phase had the lowest diameter measured during fabrication when particles are partially swollen with solvent (blue line). In contrast, particles fabricated using no salt in the external aqueous phase generated slightly more swollen particles. Particles made with 0.5 M salt in the external aqueous phase were smaller, but heavily aggregated. Scanning Electron Micrographs of microparticles were taken after lyophilization and are represented above for use of (**C.**) 0 M salt (**D.**) 0.2 M salt, and (**E.**) 0.5 M salt. Magnifications are 1000X (body) and 5000X (inset) and size bars are included. Encapsulation efficiencies were 55% for 0M salt, 92% for 0.2M salt, and 73% for 0.5M salt.

5.3.2. Physical properties

The physical properties of lyophilized microparticles prepared from PBAE and PLGA were examined (n=3) to determine suitability for targeted delivery of plasmid DNA to phagocytic antigen presenting cells. Diameters obtained by volume displacement for all microparticle formulations were between 1 and 10 μm allowing for a passive targeting by phagocytosis. There did not seem to be a correlation between size or aggregation and PBAE content when using refrigerated washing and stirring processing steps.

Quality and quantity of plasmid DNA content seemed to favor formulations with PBAE included when compared to PLGA alone. On average, PLGA loadings were approximately 50% efficiency, while particles prepared from 15-50% PBAE had much higher encapsulation of plasmid (Table 5.1.). Supercoiled content directly after the encapsulation and lyophilization process was also generally higher for microparticles with PBAE in the formulation (Table 5.1.). Plasmid integrity after incubation in buffer at 37 °C is discussed below.

Formulation (% by weight)	Volume % Mean Diameter (μm) ± SD	Encapsulation Efficiency % ± SD	% Supercoiled Content ± SD	Mean Zeta Potential (mV) ± SD
100% PLGA	5.0 ± 0.6	50.6 ± 9.3	36 ± 8%	-3.4 ± 0.3
5% PBAE/95% PLGA	8.0 ± 1.4	52.0 ± 3.9	N/A	-7.3 ± 1.4
15% PBAE/85% PLGA	6.6 ± 0.5	68.0 ± 6.1	72 ± 5%	-1.0 ± 0.2
25% PBAE/75% PLGA	6.6 ± 0.9	82.3 ± 6.0	68 ± 5%	-0.8 ± 1.4
25% PBAE/75% PLGA No DNA	5.8 ± 0.9	-----	-----	0.0 ± 0.4
35% PBAE/65% PLGA	6.8 ± 0.5	83.5 ± 0.8	N/A	1.9 ± 0.7
50% PBAE/50% PLGA	6.3 ± 1.3	77.0 ± 4.2	N/A	8.6 ± 1.2

Table 5.1. Population properties of microparticles containing varying amounts of PBAE with respect to PLGA. All particles shown were prepared with 0.2 M salt in the external aqueous phase. Population values for the physical properties of microparticles are shown above for dry, lyophilized formulations resuspended in buffer at physiologic pH (n=3).

Zeta potential analysis indicated that particles prepared with PLGA alone had slightly negative values due to residual PVA as previously reported[32]. Interestingly, particles prepared from 5% PBAE exhibited more negative zeta potentials than pure PLGA microparticles. Other than this value, increasing the amount of charge inducible PBAE in the particle formulation increased the zeta potentials proportionally (Table 1 and Figure 5.3.).

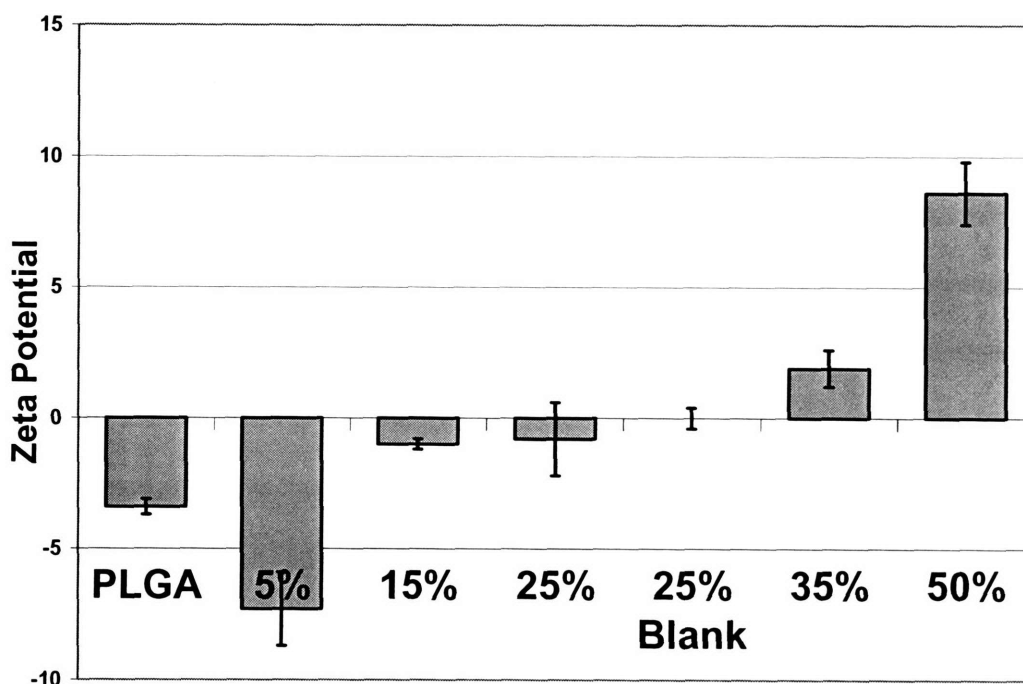


Figure 5.3. Zeta potential of microparticles with varying amounts of PBAE with respect to PLGA.

5.3.3. Effect of pH microclimate

To observe the effect of PBAE buffering on internal microclimate pH, we utilized a previously described procedure in which particles (n=3) were incubated at 37 °C in buffer followed by dissolution in acetonitrile and physical measurement of pH to calculate the resulting microclimate[30]. We determined that 24 hour (Fig 5.4.A) and 72 hour (Fig 5.4.B) incubation of microparticles resulted in a significantly lower microclimate pH for 100% PLGA microparticles when compared to PBAE microparticles. This effect was especially pronounced at 72 hours where the pH of PLGA microparticles was approximately 2.75 while PBAE buffered the microclimate to pH > 4.

Plasmid DNA was extracted from these microparticles after aqueous incubation using the same technique described above. The integrity of the extracted plasmid was examined using 0.8% agarose gels (24 hours, Fig 5.4.C and 72 hours, Fig 5.4.D, n=3). Integrity of plasmid encapsulated in PBAE microparticles was found to be substantially higher when compared to plasmid extracted from particles prepared from PLGA alone, especially after 72 hours (3-4X greater), corresponding to the data obtained for the low microclimate pH at this time. Supercoiled DNA content did not decrease greatly from 24 hours to 72 hours for 15 and 25% PBAE microparticles.

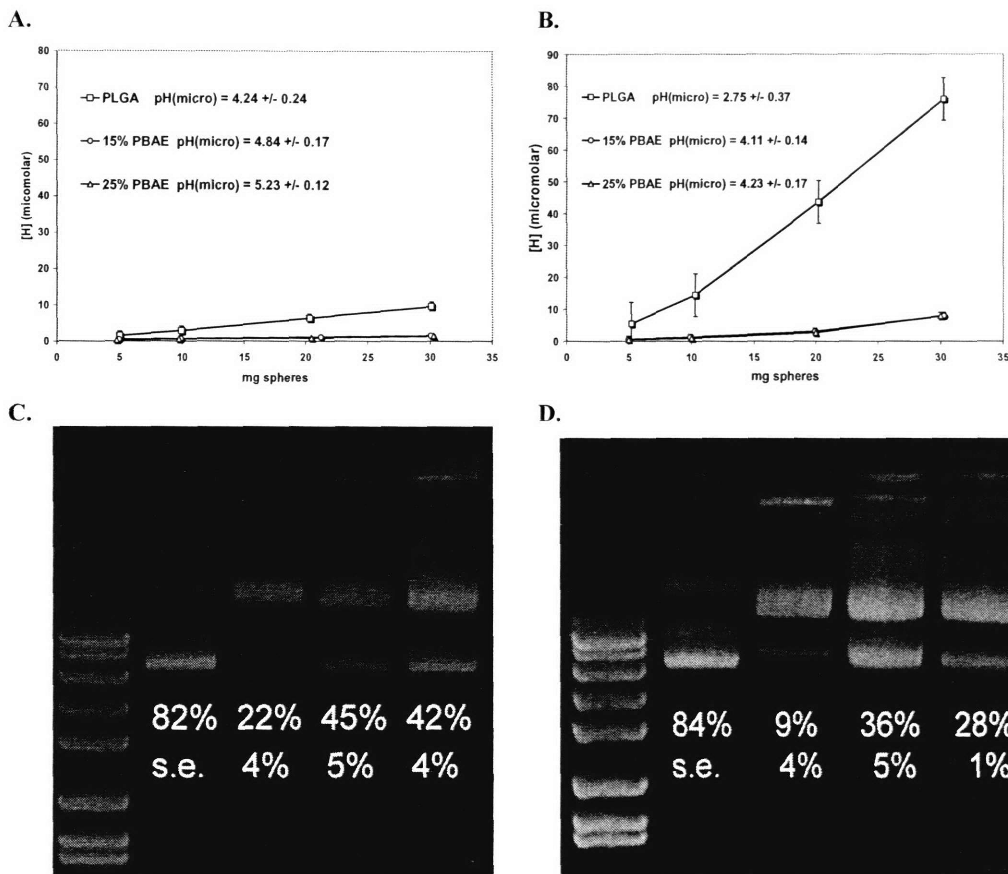


Figure 5.4. Effect of pH microenvironment on PLGA and PBAE microparticles containing plasmid DNA. Microparticle samples were carefully weighed and incubated for **A.** 24 hours and **B.** 72 hours at physiological pH. Samples were centrifuged and supernatants removed to allow weighing of the pellet followed by addition of acetonitrile:water (0.7 ml:0.175 ml) and measurement of pH. Plots represent measured hydrogen ion concentration vs. amount of microparticles incubated and then dissolved in ACN:H_2O. Calculated microenvironment pH [pH(micro)] is reported in the legends. Supercoiled DNA content of the microparticles are shown for **C.** 24 hour incubation, and **D.** 72 hour incubation for microparticle samples composed entirely of PLGA (Lane 3), 15% PBAE (Lane 4), or 25% PBAE (Lane 5). A DNA ladder (Lane 1) and unprocessed plasmid DNA (Lane 2) were used as controls. Supercoiled plasmid percentages are shown for each lane of the representative gel with standard errors for comparison (n=3).

5.3.4. Plasmid release

To determine the effect of increasing amounts of PBAE on release of plasmid, particles (n=3) were incubated in buffer for 1-7 days and supernatants were removed daily to be assayed for plasmid concentration using Pico Green. Particles prepared with the lower amounts of PBAE (5%, 15%) exhibited a larger burst phase than those composed of PLGA alone (Figure 5.5.). PBAE content of 15% and 25% most closely resembled that of 100% PLGA. Larger amounts of PBAE (35% and 50%) seemed to delay release of plasmid for several days before burst phase release. Plasmid release studies were also attempted in which the pH of the media was reduced to 4.7, simulating the low pH environment in phagosomes. However, dissolution of PBAE causes extensive binding to the DNA resulting in a failure to detect plasmid using Pico Green, which yields no signal, and standard UV detection, which yields abnormally high signals possibly due to contribution by PBAE or PBAE/DNA complexation to the absorption at 260 nm.

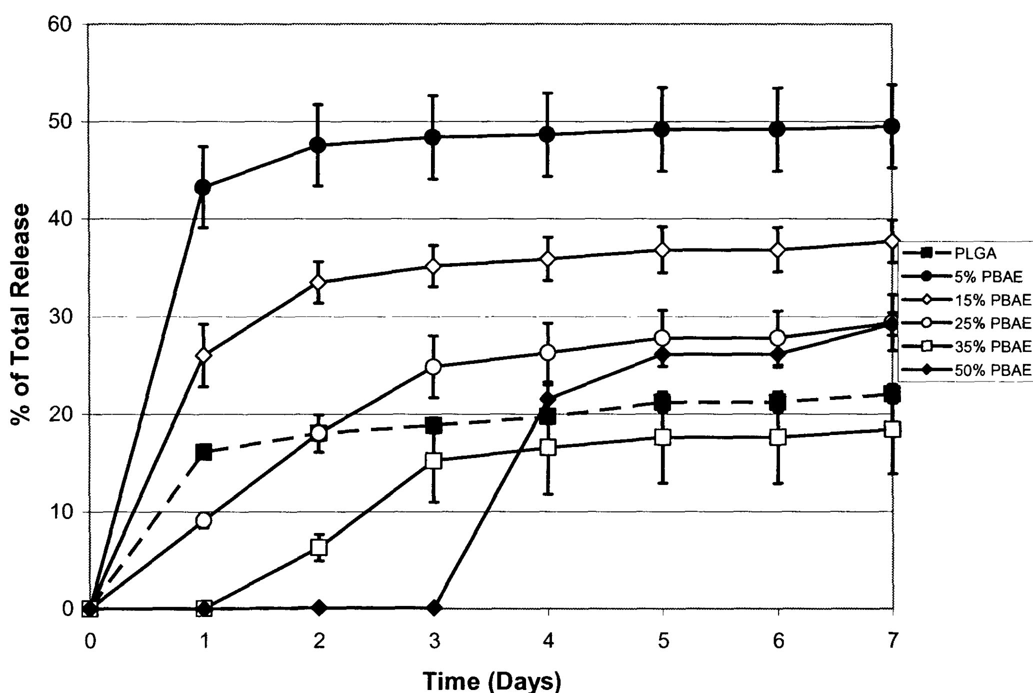


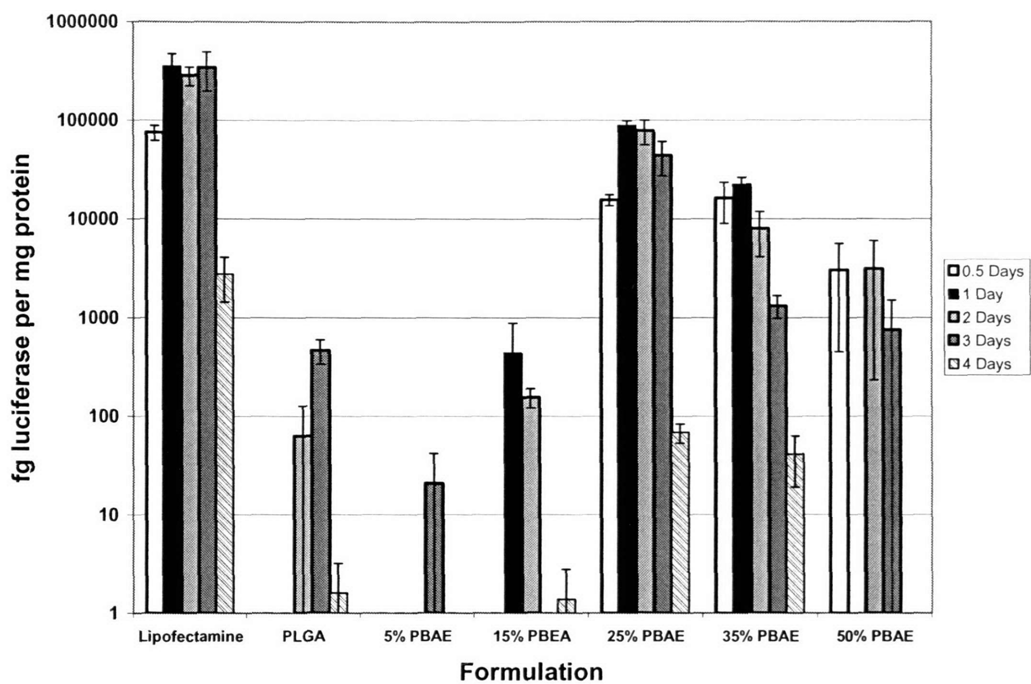
Figure 5.5. Release of plasmid DNA from PBAE/PLGA microparticle formulations. Microparticles were incubated for 1 week with supernatants removed and replaced every 24 hours. Supernatants were tested for DNA concentration using Pico Green fluorescence in a plate reading fluorimeter and standard curves were used to generate DNA concentration shown above as % of total release from the microparticle sample. Release is shown above for PLGA (■, dashed line), 5% PBAE (●), 15% PBAE (◇), 25% PBAE, (O), 35% PBAE (□), 50% PBAE (◆). Error bars represent standard error at each timepoint (n = 3).

5.3.5. Transfection of P388D1 macrophages using pCMV-Luc containing microparticles

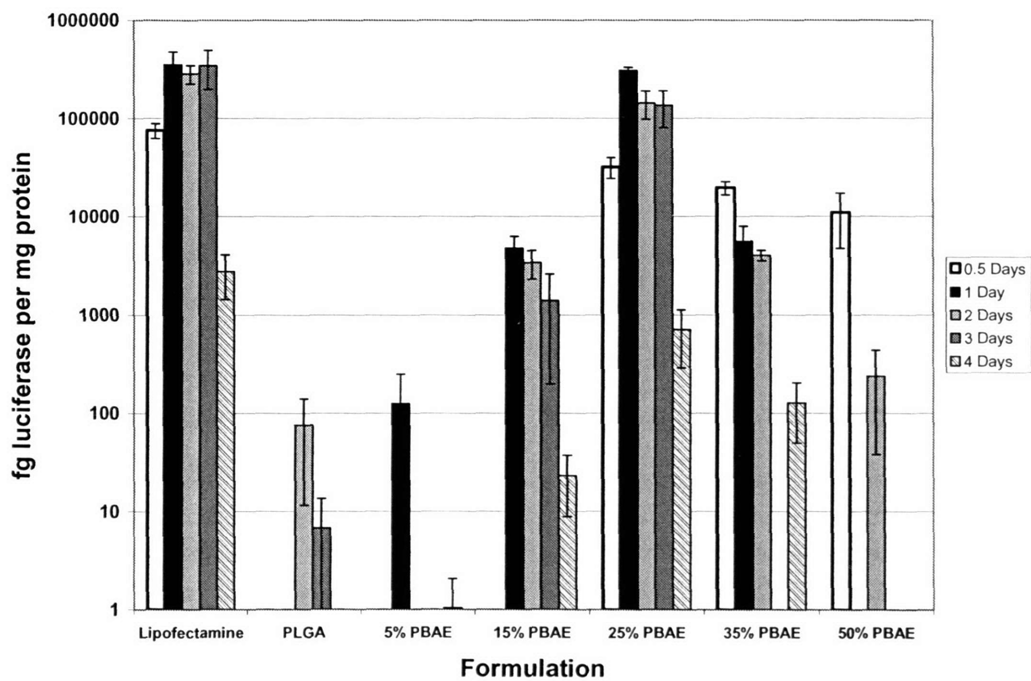
Varying amounts of PBAE in microparticles were tested for the ability to enhance plasmid delivery to the nucleus in a P388D1 macrophage cell line (Figure 5.6.). P388D1 cells were chosen as they have previously been shown to be amenable to transfection by PLGA to compare relative magnitudes[31]. Lipofectamine 2000 was used as a positive

control. It was found that increasing the amount of PBAE in the particle makeup increased transfection up to 5 orders of magnitude for 25% PBAE. Increasing the levels further to 35 and 50% PBAE caused a decrease in detected transfection. Optimal formulations of 25% PBAE performed equivalently to 1 log unit below that of an optimal formulation of Lipofectamine 2000 despite the 20X greater level of plasmid needed for this level of transfection with Lipofectamine. With 35% and 50% PBAE microparticles, the observed transfection was greatest across the board with lower microparticle concentrations, especially at the later time points. However, 15% PBAE microparticles achieved the highest transfection at the higher microparticle concentrations.

A.



B.



C.

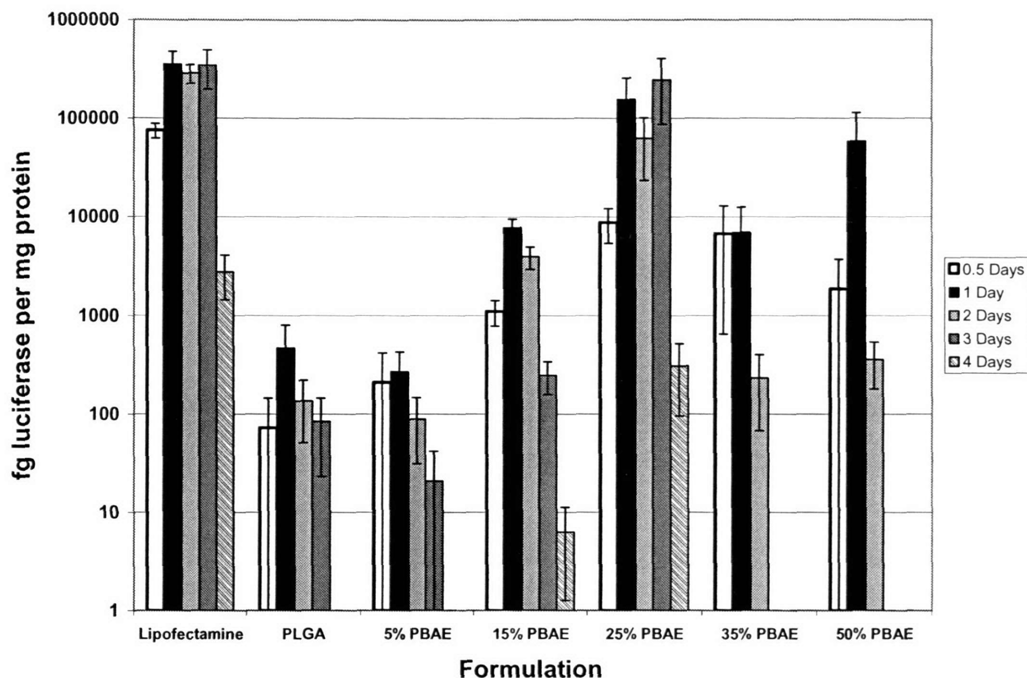


Figure 5.6. Transfection of P388D1 macrophages using microparticles with increasing amounts of PBAE. P388D1 macrophages were incubated with microparticle formulations containing pCMV-Luc in a 96 well plate for up to 4 days at suspended microparticle concentrations of **A.** 10 μg/ml **B.** 30 μg/ml **C.** 100 μg/ml. Wells were analyzed for luminescence after adding luciferin and ATP and were normalized using total protein content in each well by BCA assay. Results show expression levels of luciferase after 0.5 days (white), 1 day (black), 2 days (light grey), 3 days (dark grey), and 4 days (diagonal striped). Standard deviations are included (n=4).

Transfection of these cells should be mostly due to phagocytosis of the particles if they are to passively target APCs in a complex *in vivo* cellular milieu. To verify this was the case in our system, we transfected P388D1 macrophages in the presence of cytochalasin-D to inhibit phagocytosis but not endocytosis. Addition of 10 μM cytochalasin-D completely abolished transfection of macrophages using all microparticle

formulations tested (Figure 5.7.). However, Lipofectamine 2000 transfection of P388D1's did not change upon addition of this phagocytosis inhibitor.

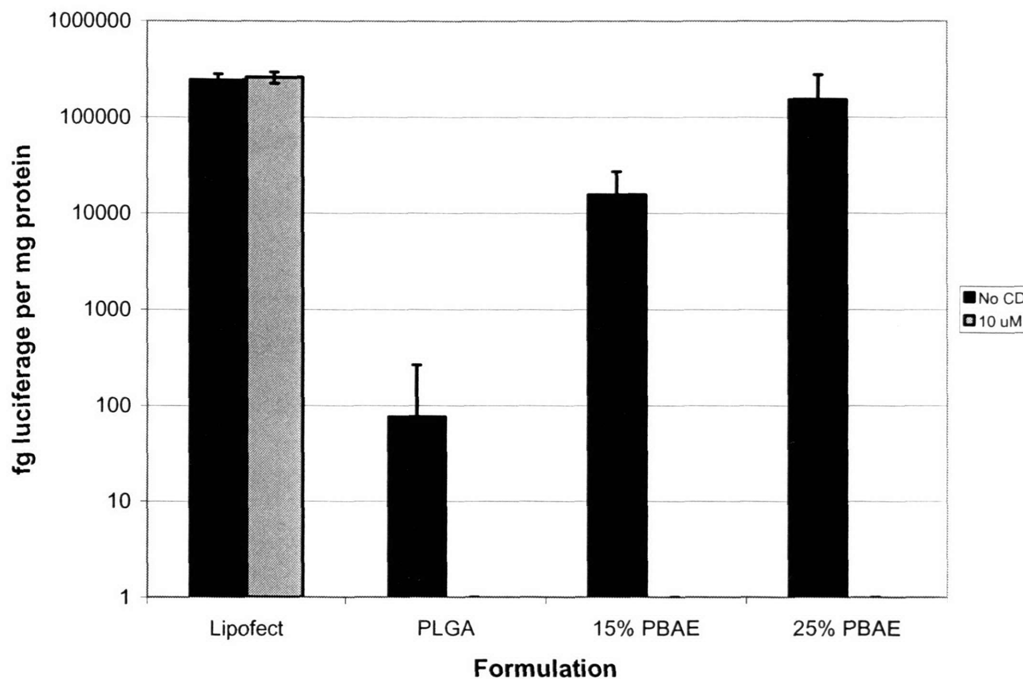


Figure 5.7. Effect of Cytochalasin-D on transfection of phagocytic cell line. Results shown above indicate luciferase transfection after 1 day incubation with microparticles containing pCMV-Luc, with or without the presence of Cytochalasin D (10 μM) in the media to inhibit actin mediated phagocytosis. Data is representative of 4 averaged experiments with included standard deviation bars.

5.3.6. Toxicity of PBAE microparticles

Toxicity associated with larger amounts of PBAE may be partially responsible for the observed decrease in transfection using larger amounts of particles. An MTT assay was employed to examine this effect on the P388D1 line. Non-treated cells were used as a negative control and Lipofectamine 2000 was used as a positive control for toxicity.

Cells were incubated for 24 hours with microparticle formulations, or Lipofectamine 2000, and then tested for metabolic activity. It was confirmed that addition of PBAE to microparticles increased toxicity as did increasing total dosage of particle to cells in the case of 25% PBAE particles (Figure 5.8.). Lipofectamine 2000 also demonstrated significant toxicity as expected. However, both PLGA and 15% PBAE showed no detectable levels of toxicity in all dosages used.

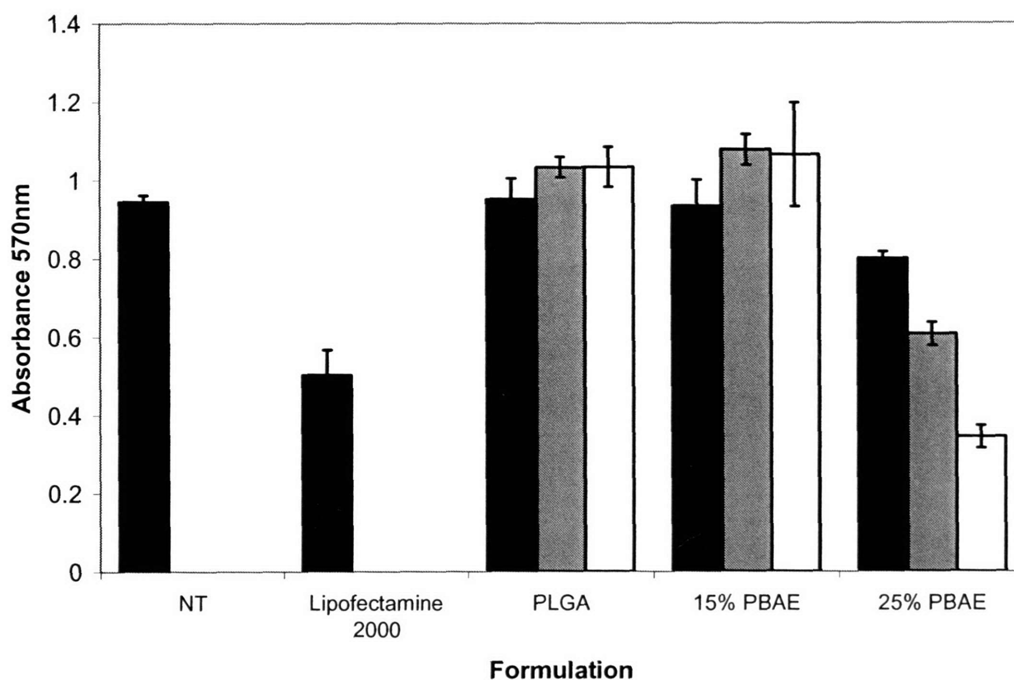


Figure 5.8. Toxicity of PBAE microparticles. P388D1 macrophages were incubated with microparticle formulations for 24 hours and then analyzed for viability using a standard MTT assay. Results above show absorbance at 570 nm of a solubilized precipitate indicating level of metabolic activity for microparticle concentrations of 10 μg/ml (black), 50 μg/ml (grey), and 100 μg/ml (white).

5.4. Discussion

Disadvantages to using PLGA in plasmid containing microparticles have prompted investigation into additives and replacements which are better suited for the delivery of genetic vaccines[22-26]. It is thought that these systems are more appropriate because they either deliver the DNA in a fashion which is timelier to the induction of an immune response or in a form which is more amenable to the transfection of targeted cells. However, switching from a pure, biocompatible and FDA approved material may introduce undesirable side effects such as cellular toxicity, which may or may not be avoidable if the system is to be highly efficient. Therefore, it is imperative that new systems are investigated to allow for optimization of immunogenicity afforded by a delivery system with low toxicity. We recently employed a pH sensitive PBAE polymer which has shown to responsibly release encapsulated material in response to pH[28]. This new biomaterial has also been shown to have minimal toxicity at low amounts[27], however microparticles require the use of larger amounts of this polymer per unit mass of DNA. Here, we consider the formulation and characterization of polymer microparticles prepared with varying amounts of PBAE added to PLGA in a microparticle encapsulating plasmid DNA.

The physical properties of PBAE require that it be treated differently during the microparticle fabrication procedure[28]. As stated before[28], refrigerated centrifugation steps are required to minimize aggregation and particle deformation when PBAE is present in microparticle formulations. Another special consideration when using PBAE is the osmotic balance between the internal versus external aqueous phases during homogenization and solvent evaporation steps. This may be particularly pronounced in

this system due to PBAE being partially charged in contact with an aqueous phase and therefore more conducive to water influx in the presence of an osmotic gradient (higher semi-permeable membrane effect). The presence of a high osmolality in the microparticle interior and a low osmolality in the outer stirring phases may cause an influx of water and the bursting of compartments. This would lead to the escape of entrapped plasmid and low effective loading. Pico Green analysis of DNA extracted from lyophilized 25% PBAE microparticles confirms that particles prepared with no osmotic matching exhibit lower encapsulation efficiencies and cavities in the microparticle surface (Figure 5.2.C-E.). In contrast, particles prepared using salt in the exterior stirring phase had higher encapsulation efficiencies and smooth surfaces. However, too much salt in the stirring phase caused extensive aggregation of the particles (Fig 5.2.B). It is possible that large amounts of salt may diminish any surface charge repulsion between particles which would tend to reduce such aggregation.

In general, addition of PBAE into the microparticle formulations seemed to increase the integrity and quantity of encapsulated DNA along with creating a more positive zeta potential (Table 5.1. and Figure 5.3.). However, the addition of 5% PBAE decreased the measured zeta potential when compared to that measured for 100% PLGA microparticles. This finding may be attributable to a more basic environment at the particle surface due to the weak bases in the PBAE backbone, as will be discussed further below. This basic microclimate may result in faster degradation of ester bonds, producing an abundance of anionic carboxylic acid groups. The competing effect of increasing zeta potential due to partially cationic PBAE becomes more significant at formulations of 15% PBAE and higher.

The more positive zeta potentials associated with PBAE may be responsible for the larger encapsulation efficiencies due to a reduction in hydrophobicity resulting from charged tertiary amines present in the polymer backbone which would interact with anionic DNA more favorably. The highly hydrophobic environment present in pure PLGA microparticles would not serve as an optimal retention environment for DNA during microparticle fabrication. The association of the negatively charged plasmid with either free PBAE in the particle interior or a positively charged particle surface may also be responsible for the higher integrity of plasmid DNA following fabrication. High shear stresses are present during sonication and homogenization which can diminish the amount of plasmid in a supercoiled form[33]. The complexation or association of plasmid with PBAE may serve to reduce this effect.

The tertiary amines in PBAE (Fig 5.1.A.) may be responsible in several ways for increased delivery capacity of microparticles containing this polymer. One of these gene delivery functions involves the absorption of protons in the acidic endosome which eventually could release the plasmid payload into the cytoplasm of a cell by a proton sponge mechanism[34]. Furthermore, we hypothesized that the tertiary amines in PBAE (which are absent in PLGA (Fig 5.1.B.) may also act as a weak base, absorbing the protons present in the pH microclimate originating from degradation of ester bonds. This effect was first demonstrated by Shenderova et al who determined that PLGA particle microclimate pH can be as low as 1.8[30]. Our data suggest that PBAE significantly buffers the acidic microclimate effect caused by ester degradation (Figure 5.4.). This effect is particularly pronounced after 3 days of incubation. Measurements obtained at a three day time point indicate that that the microclimate pH for PLGA microparticles is

approximately 2.75. The higher values obtained here than in previous results[30] may be due the larger sizes of the particles used in these studies which would hinder diffusion of acid from the interior even more so than a smaller particle. Microclimate pH after 3 days was measured at 4.11 and 4.23 for 15% and 25% PBAE, respectively. This is important because reduction of pH below 4 has been shown to severely reduce the supercoiled content and transfection activity of plasmid DNA[20]. Our data suggest that the supercoiled DNA content of PLGA microparticles is substantially lower than PBAE microparticles after 1 day of aqueous incubation and drastically lower after 3 days of incubation (Figure 5.4.C-D.). Although the buffering of pH microclimate by PBAE is most likely responsible for this stabilization, it cannot be ruled out that plasmid complexation by free or microparticle associated PBAE could be involved with higher levels of supercoiled plasmid.

Release data obtained from microparticles containing PBAE indicates that the amount of partially cationic polymer determines the release of plasmid. Data obtained for the release from PLGA particles correlates well with prior studies on a similar system[35]. Low amounts of PBAE, 5% and 15%, seemed to exhibit a larger burst phase than PLGA alone (Figure 5.5.). This may be due to a higher amount of plasmid residence on the surface of the particle as suspected earlier to explain zeta potential data. This burst phase could be diminished upon the addition of more cationic polymer which may more tightly bind anionic plasmid. Correspondingly, our data shows that upon increasing the amount of PBAE in the microparticle composition, this burst phase was reduced, and in the case of 35% and 50% PBAE, there is a delay of any release of up to 3 days. This delayed release associated with larger amounts of PBAE may be beneficial in smaller

particle systems aimed at targeting destructive genes to cancer cells as to avoid release before uptake.

Transfection of P388D1 macrophages was substantially increased upon addition of up to 25% PBAE (Figure 5.6.). This effect was less pronounced with 15% PBAE but was still 1-2 orders of magnitude greater at most time points. Larger amounts of PBAE seemed to decrease the transfection levels seen with 25% PBAE, and this effect was even more apparent using larger amounts of microparticles in the cell supernatant. This decrease in observed transfection is most likely due to toxicity associated with this amount of PBAE. However, it seems that at least some level of PBAE needs to be present for significantly enhanced transfection as in the case of 15% PBAE where lower levels of microparticles were not as effective as higher doses (Figure 5.6.). The toxicity effect of larger amounts of PBAE is apparent using 25% formulations which had toxicity equivalent to Lipofectamine 2000 above 50 μg/ml. In contrast, formulations containing 15% PBAE had no observable toxicity in any of the dosages tested, as did 100% PLGA formulations.

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6. *In vitro* and *in vivo* efficacy of particulate PBAE genetic vaccine delivery

6.1. Introduction

Genetic vaccination has tremendous potential for treating or preventing numerous diseases for which traditional vaccines are ineffective, but can be limited by low immunogenicity in larger animals (1, 2). This deficiency is particularly pronounced in non-viral genetic vaccine cancer therapies where epitopes can be weakly recognized, and tumors can down-regulate the ability of antigen presenting cells (APC) to process and present antigen efficiently to T-cells in an activated state(3). Current non-viral genetic vaccine systems are not designed to activate APCs (4), and lack the gene delivery capacity of viral vectors. In an attempt to increase the effectiveness of non-viral systems, focus has shifted towards exploring the use of adjuvants, cytokines, and self-replicating RNA systems (5-8). Ideally, delivery vectors would have the capability of altering both the extent of antigen expression as well as the level of immunogenicity.

Recently, we described the synthesis of a degradable, pH sensitive poly-β amino ester (PBAE)(9) and its application to microparticles capable of releasing fluorescently labeled payloads instantaneously upon pH changes in the physiological range(10). In this chapter, we report that these formulations are taken up and are potent activators of primary dendritic cells. To further examine the effectiveness of this delivery system, we utilized a plasmid that contains a sequence for a fusion protein containing an octapeptide epitope (SIYRYYGL, henceforth called SIY) which associates with MHC Class I (K^b) and can stimulate polyclonal $CD8^+$ T-cell responses in B6 mice (11). Primary dendritic cells, when treated with particles encapsulating this plasmid, are able to activate SIY specific T-cells *in vitro* and vaccinations with pCMV-SIY formulations activated naïve, specific T-cells *in vivo*. Furthermore, we demonstrate the ability of hybrid PBAE/PLGA microparticles to induce an antigen-specific, rejection of SIY expressing tumor cells *in vivo,* unlike conventional PLGA microparticle and naked DNA formulations.

6.2. Materials and Methods

6.2.1. Materials

Poly(*d,l*-lactic-co-glycolic acid) polymer (RG502H Resomer 50:50) was purchased from Boehringer Ingelheim (Ingelheim, Germany). Poly(β-amino ester) was synthesized as previously reported ($M_n \approx 5$ kD) (10). Plasmid DNA encoding β-galactosidase, or SIYRYYGL peptide/β-galactosidase fusion (pCMV-SIY) were obtained from Elim Biopharmaceuticals (Hayward, CA). Dextran conjugated tetramethyl rhodamine ($M_n \approx 70$ kD) was purchased from Molecular Probes (Eugene, OR).

6.2.2. Mice

C57BL/6 (B6, H-2 K^b) mice (6-10 weeks) were purchased from Taconic (Germantown, NY). 2C transgenic mice were raised in the MIT animal facility.

6.2.3. Cells and cell lines

The P388D1 macrophage cell line was obtained from ATCC (Manassas, VA) and cultured as recommended. Leukopaks were obtained from Massachusetts General Hospital and human peripheral mononuclear cells were isolated by adherence as described (12, 13). Human dendritic cells were differentiated in IMDM (Gibco) including 1% human serum (Valley Biomedical; Winchester, VA) along with 50 ng/ml GM-CSF and 20 ng/ml IL-4 (RnD Systems; Minneapolis, MN). Primary bone marrow-derived dendritic cells were isolated from B6 mice and cultured as described(14) before purifying with magnetic beads (CD11c MACS, Miltenyi Biotec; Auborn, CA) (98% measured by anti- CD11c mAb in flow cytometry analysis). EL-4 murine thymoma cells were obtained from ATCC and a transduced, SIY expressing EL-4 cell line were cultured in RPMI-1640 with 10% FCS with 1 mg/ml G418 (Gibco). Previous studies have indicated that the SIY-K^b peptide MHC complex is expressed in the transfected EL-4 cells(15).

6.2.4. Preparation of microparticles

Plasmid containing microparticles were prepared by double emulsion/solvent evaporation as described using varying amounts of PLGA blended with PBAE(10). Fluorescent microspheres were prepared similarly, but with dextran tetramethyl-rhodamine (200 μl, 1 mg/ml) in the primary emulsion. All *in vitro* cellular assays and *in vivo* tumor challenge experiments were performed by normalizing the microparticle amount to equalize plasmid DNA dosage.

6.2.5. Characterization of microparticles

Loading of DNA microparticles was determined by dissolution in CH_2Cl_2 and extraction into 1X TAE buffer (pH = 8.0). DNA concentration was detected using PicoGreen (Molecular Probes) and the Mithras plate reading fluorimeter (Berthold Technologies; Bad Wilbad, Germany). DNA integrity was determined using gel electrophoresis (1% agarose) and Image J software. Microsphere size distributions were measured using a Multisizer 3 (Beckman Coulter; Miami, FL). Zeta potentials were obtained using a ZetaPALS analyzer (Brookhaven Instruments; Holtsville, NY). All microparticle formulations were certified to have a low endotoxin level (<0.50 EU/mg) by the Cambrex LAL testing service (Walkersville, MD).

6.2.6. 3D imaging of antigen presenting cells

Human PBMC derived dendritic cells were seeded on glass coverslips at 4×10^5 cells/well in 6 well plates. Fluorescent microspheres were added (50 μg/ml cell media) and allowed to incubate for 4-6 hours. Cells were then washed, fixed with 3.2% paraformaldehyde solution in PBS, and permeated using 0.2% triton X 100 (Sigma). Actin filaments and nuclear materials were labeled using Alexa Fluor 488 conjugated phalloidin and Hoechst dye, respectively (Molecular Probes). Cells were imaged using the Zeiss Axiovert fluorescent microscope with an Apochromat 100X oil immersion lens (Carl Zeiss; Göttingen, Germany) and vertical slices (0.2 μm separation) were deconvoluted using Openlab software (Improvision; Lexington, MA).

6.2.7. Flow cytometry analysis of fluorescently labeled surface markers

Primary bone marrow dendritic cells were plated at 1×10^6 cells per well of a 6 well plate (BD Biosciences). Media was then replaced with a suspension of microspheres

(50 μg/ml) and incubated for 24 hours. Untreated cells were used as negative controls. Positive controls were prepared by adding LPS (100 ng/ml, Sigma). At several time points, cells were harvested and stained with antibodies for MHC Class II (Pharmingen; San Jose, CA), F4/80 (Caltag; Burlingame, CA), mCD40, mCD86, mCD80, and m41BBL (e-Bioscience; San Diego CA), hCD83, hCD14 and hCD11c(Immunotech, Miami, FL) at 4°C for 30 minutes. Cells were then analyzed with a FACScan flow cytometer (Benton Dickenson; San Jose, CA) with propidium iodide gating (5 μg/ml) collecting a total of 30,000 total events.

6.2.8. *In vitro* T-cell activation by DCs treated with pCMV-SIY containing particles

$2x10^4$ L3100, SIY antigen specific, T-cells(16) were added to wells containing $2x10^5$ (10:1) and $1x10^5$ (5:1) dendritic cells treated with microparticle formulations encapsulating pCMV-SIY plasmid DNA for 24 hours. Activation was measured by transferring the T-cells to an IFN-gamma ELISPOT plate (RnD Systems) after 18 hours of incubation with the dendritic cells and the plate was processed and analyzed the next day for number of IFN-gamma spots. Positive controls were performed with 10^{-6} M SIY peptide incubated with the dendritic cells 4 hours prior to L3100 addition. Negative controls were untreated dendritic cells and 25% PBAE microparticles containing pCMV-Luciferase.

6.2.9. Adoptive transfer and i*n vivo* 2C T-cell activation in vaccinated mice

B6 mice (n=2) were given $1x10^6$ adoptively transferred 2C T-cells via eye vein injection which were harvested from spleens and lymph nodes of 2C, $RAG^{-/-}$ knockout mice. Simultaneously, these mice were immunized intradermally, and then again 2 weeks later, with 1) PBS as a negative control, 2) naked pCMV- SIY plasmid (10 μg), 2) PLGA

encapsulated pCMV-SIY microspheres (10 μg plasmid total), 3&4) PBAE/PLGA (15% and 25% w/w PBAE) hybrid encapsulated pCMV-SIY microspheres (10 μg plasmid total in each case). One week following the last immunization, mice were given an i.p. dose of SIY peptide (1 μg), and 3 days later, spleens were harvested for isolation of 2C T-cells. These cells were analyzed by flow cytometry for CD69 upregulation using mAb conjugated to FITC (Pharmingen).

6.2.10. Immunization and tumor challenge

Mice were immunized intradermally as described (17) twice at 2 week intervals with 1) naked pCMV- SIY plasmid (10 μg), 2) PLGA encapsulated pCMV-SIY microspheres (10 μg plasmid total), 3&4) PBAE/PLGA (15% and 25% w/w PBAE) hybrid encapsulated pCMV-SIY microspheres (10 μg plasmid total), 5) a PBS control group, 6) PBAE/PLGA (25% w/w PBAE) microspheres with no encapsulated plasmid, and 7) encapsulated pCMV-β-galactosidase control groups. One week following the last immunization, mice were challenged subcutaneously with a lethal number (3×10^6) of EL-4 cells on the right flank and an equal number of SIY expressing EL-4 cells on the left flank. Beginning a week later, tumor size was measured with a caliper every other day in two dimensions for 9 days. Statistics were performed by comparative ANOVA (samples to PBS injected controls) using a Dunnett's error confidence interval of 95%.

6.3. Results

6.3.1. Hybrid polymeric microparticles have properties well suited for genetic vaccine delivery.

Plasmid DNA encoding for SIY-P1 antigen (pCMV-SIY) was encapsulated into polymeric microparticles as described in Materials and Methods. As demonstrated

earlier, formulations containing 15% and 25% PBAE showed rapid release and were therefore used in all subsequent studies. Scanning Electron Microscopy (SEM) analysis of microsphere preparations reveal a smooth, spherical surface on all microsphere preparations (Figure 6.1.), and all formulations ranged in average diameter between 1 and 10 micrometers (Table 6.1.) allowing for a passive, APC targeting mechanism by phagocytosis.

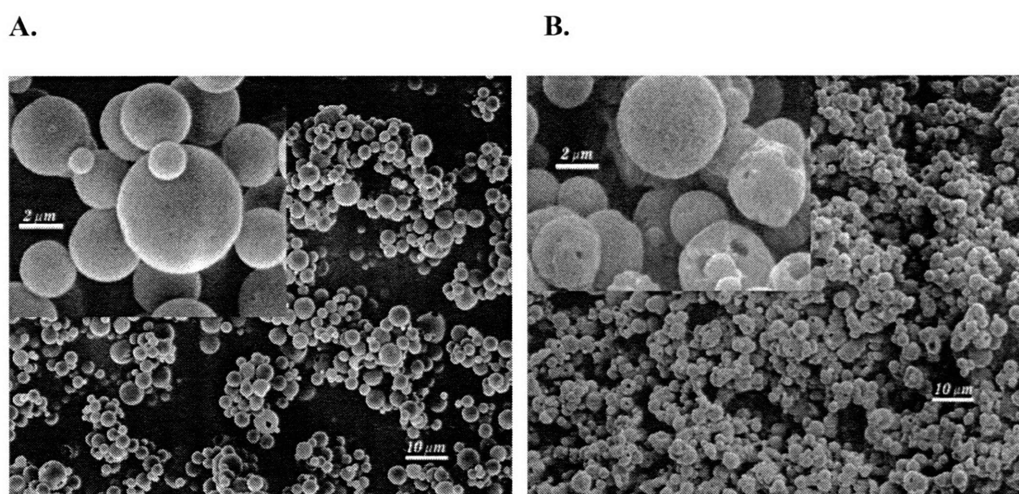


Figure 6.1. SEM micrographs of microparticles prepared from (A) PLGA and (B) 25% PBAE show high surface integrity as determined by scanning electron microscopy. Magnifications are 1000X (body) and 5000X(inset).

Formulation (% by weight)	Volume % D (µm)	Encapsulation Efficiency	% Supercoiled Content	Zeta Potential (mV)
100% PLGA	4.35±2.34	~69%	~45%	-3.76±0.40
15% PBAE/85% PLGA	6.01±2.06	~68%	~72%	-0.86±0.62
25% PBAE/75% PLGA	5.53±2.31	~78%	~64%	0.46±0.38
25% PBAE/75% PLGA No DNA	5.12±2.20	-----	-----	0.41±0.36

Table 6.1. Characteristics of microparticles containing pCMV-SIY made from PBAE and PLGA.

Microspheres incorporating PBAE (Fig 6.2; Lanes 6 and 7) had similar or greater encapsulation efficiencies when compared to PLGA microparticles (Table 6.1.) but exhibited higher supercoiled plasmid content than those prepared with PLGA alone (Fig 6.2; Lane 5). Zeta potential analysis of microspheres indicate a negative surface charge on PLGA micoparticles similar to those previously reported(18). In contrast, 15% and 25% PBAE preparations showed slightly more positive zeta potentials than PLGA formulations (Table 6.1.).

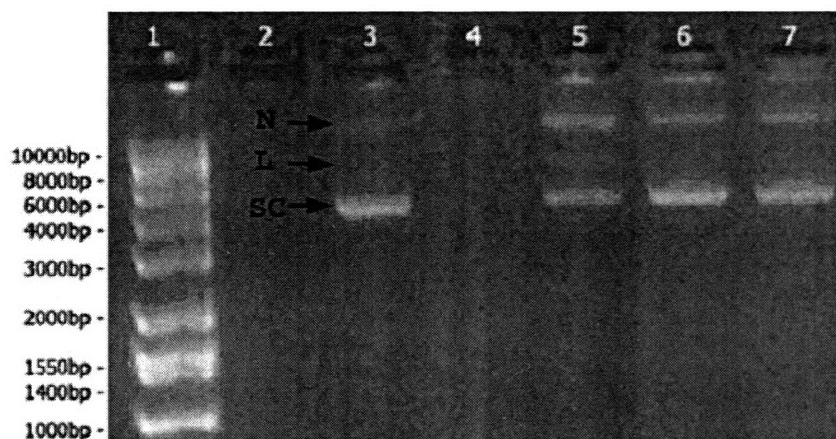


Figure 6.2. 1% agarose gel demonstrating integrity of DNA extracted from microparticles prepared by double emulsion. Lane 1: Ladder, Lanes 2 and 4: Empty, Lane 3: Unprocessed control (88% supercoiled), Lanes 5-7: Aqueous extract from PLGA, 15% PBAE, and 25% PBAE microparticles respectively after lyophilization. Labels indicate supercoiled (SC), linear (L), and nicked (N) forms of the DNA plasmid.

6.3.2. Uptake of PBAE/PLGA microparticles by dendritic cells *in vitro*.

To examine the effects of PBAE on antigen presenting cell phagocytosis, primary human dendritic cells derived from peripheral blood mononuclear cells or monocytes (PBMC) were incubated with particle formulations containing rhodamine-conjugated dextran, fluorescently stained, and examined by visual fluorescence microscopy. Three dimensional, overhead views of treated cells are shown in Fig 6.3.

Imaging of dendritic cells incubated 4-5 hours with PLGA and 25% PBAE particle formulations revealed substantial uptake of all microparticle formulations, even at microparticle concentrations as low as 1 μg/ml. In general, the intracellular distribution of the labeled dextran in PLGA microparticles remained sharp, bright, and spherical objects, as though restricted to phagosomal compartments (Fig 6.3.A). In contrast, a population of cells treated with 25% PBAE formulations demonstrated a dimmer, diffuse fluorescent signal, suggesting release from phagosomes (Fig 6.3.B).

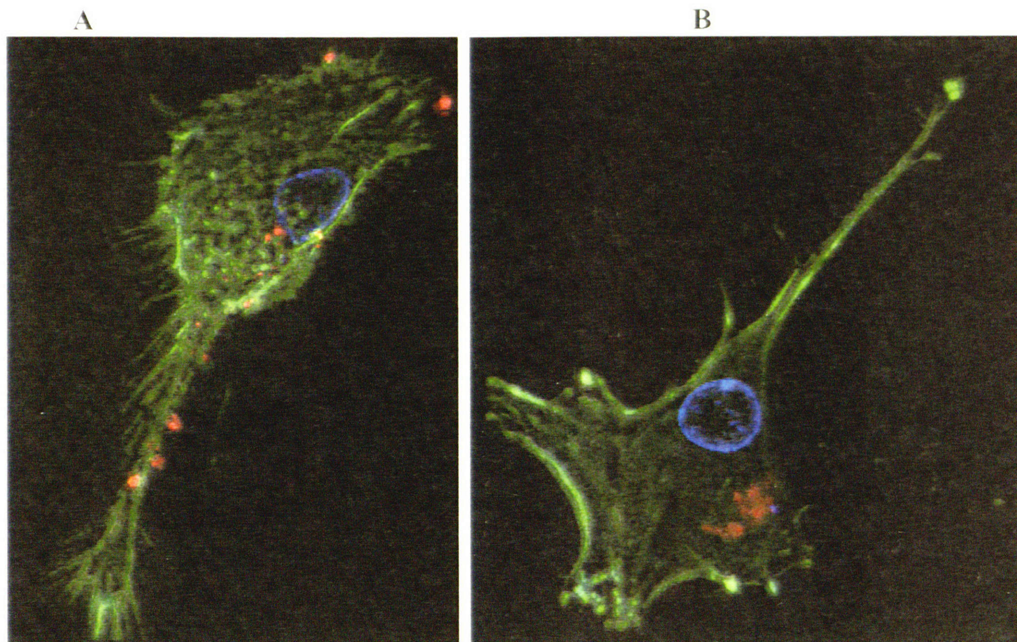


Figure 6.3. Dendritic cells phagocytose microparticle formulations of PLGA and PBAE *in vitro*. PBMC derived dendritic cells (**A** and **B**) were incubated with rhodamine conjugated dextran encapsulated microparticles (red) for 5 hours, fixed, and then stained with Hoechst dye for nucleus (blue), and Phalloidin-Alexa Fluor 488 for actin (green) . 3D fluorescent microscopy images indicate uptake in each cell for both PLGA microsphere formulations (**A**) and 25% PBAE / 75% PLGA microsphere formulations (**B**). Intracellular rhodamine signals were seen as bright, localized spheres in 100% PLGA treated dendritic cells (**A**). In 25% PBAE microsphere treated cells, rhodamine distributions were sometimes seen as dim and dispersed, as though in the cell cytoplasm (**B**).

6.3.3. PBAE containing microparticles activate primary dendritic cells.

Primary, bone marrow-derived murine dendritic cells (BMDCs) were analyzed for surface expression of co-stimulatory molecules, CD80 (B7-1), CD86 (B7-2), CD40, and 41BB Ligand (CD137L). F4/80 surface expression was also examined to demonstrate the absence of non-specific binding of antibody to PBAE microparticles on the surface of cells due to its characteristic down-regulation upon dendritic cell maturation (19, 20). Lipopolysaccaride (LPS, 100 ng/ml) treatment was used as a positive control. If the treatment activates a dendritic cell, the amount of surface co-stimulatory molecules would increase and correspondingly, the amount of detected fluorescently labeled mAb specific to these co-stimulatory molecules would also increase (depicted in Figure 6.4.A.).

After incubation with conventional PLGA microparticle formulations, the co-stimulatory profile changed slightly across the spectrum. (Fig 6.4.B.). However, with 15% and 25% PBAE formulations containing plasmid DNA, the amount of F4/80 greatly decreased and the surface expression of co-stimulatory molecules was markedly increased, indicating an activated, mature phenotype (Fig 6.4.B.). 25% PBAE formulations with no encapsulated DNA also activated the dendritic cells but to a lower extent in CD40 and 41BBL than 25% PBAE particles that included plasmid DNA. In addition, BMDC incubated with naked plasmid DNA in quantities equivalent to the total amount of DNA in microparticles (assuming 100% loading during encapsulation and instantaneous release) demonstrated costimulatory profiles similar to that of untreated cells (Fig 6.4.).

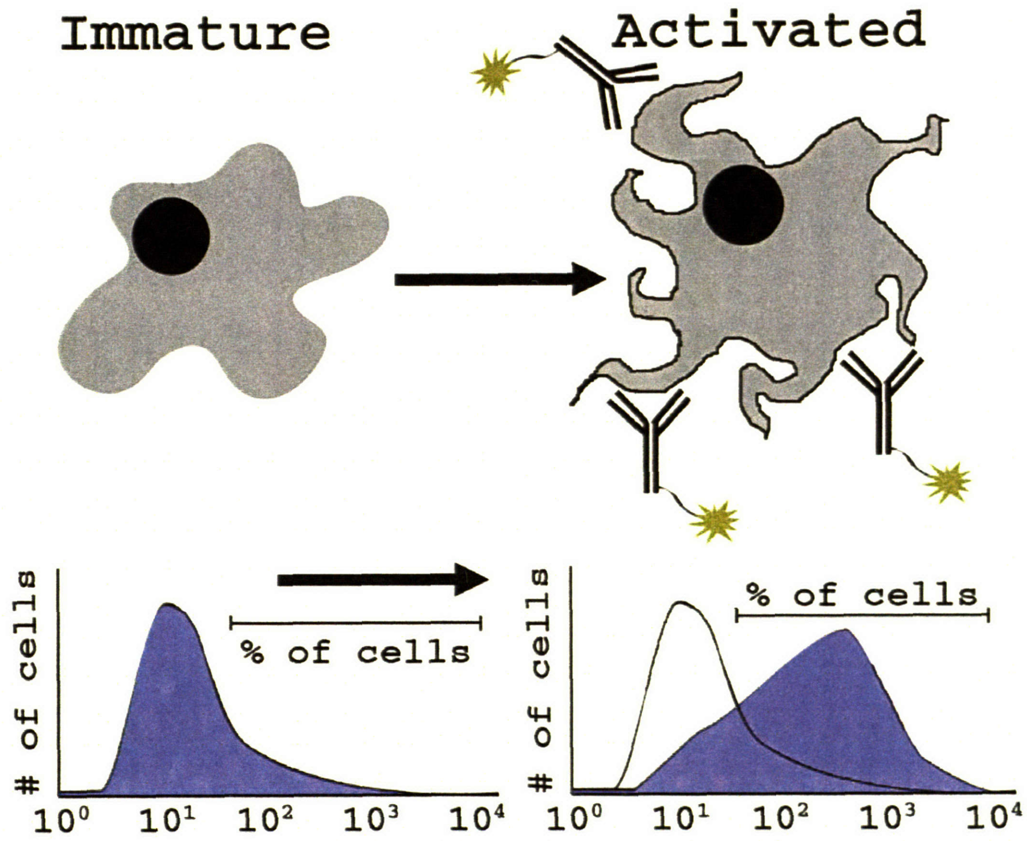


Figure 6.4.A. Schematic of dendritic cell activation (top) and corresponding flow cytometry readout (bottom).

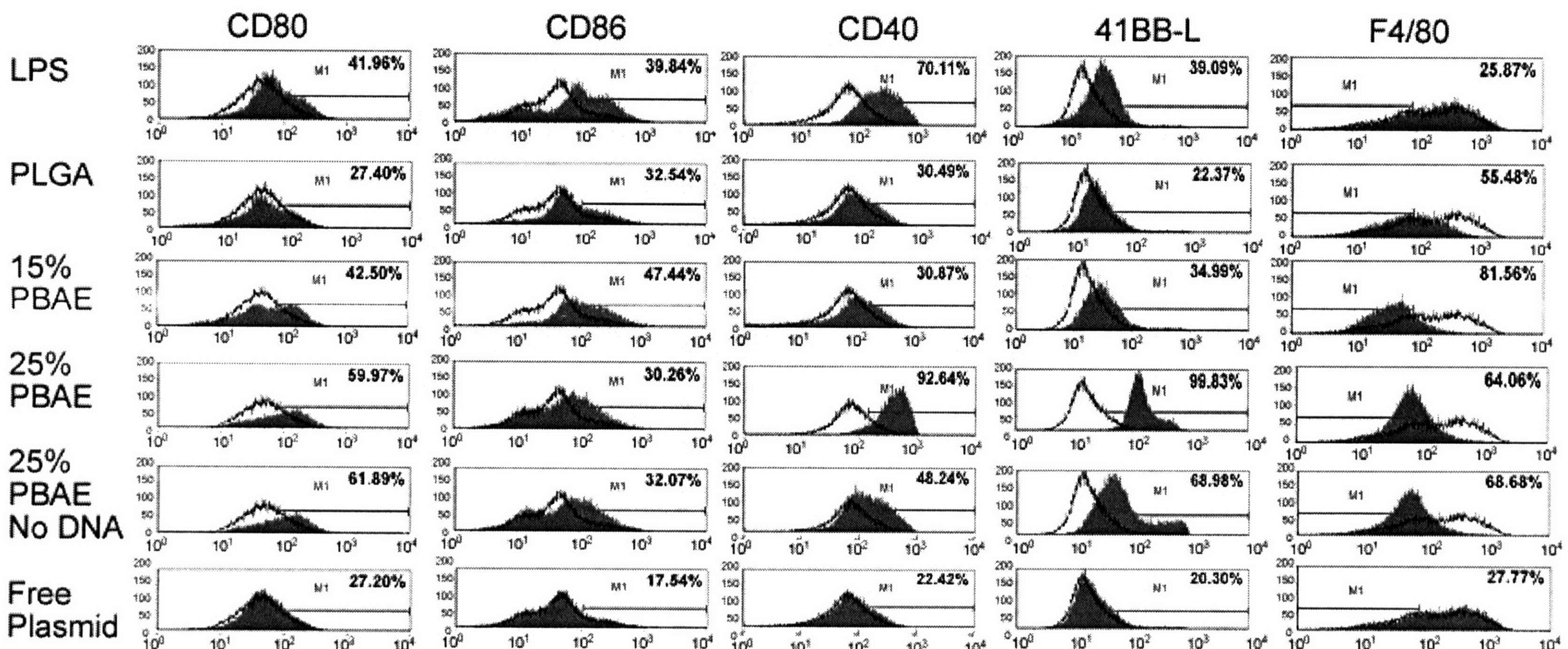


Figure 6.4.B. Activation of primary antigen presenting cells by incubation with PBAE microsphere formulations. Histograms show expression levels of the indicated co-stimulatory molecules after 18 hour incubation with LPS (100 ng/ml), PLGA, 15% PBAE, and 25% PBAE microparticles encapsulating plasmid DNA (50 μg/ml), 25% PBAE microparticles without encapsulated plasmid, and an amount of free plasmid equivalent to 100% theoretical loading of microparticle treatments and instantaneous release into the supernatant. Untreated controls are shown as the black background trace in each histogram. Cells incubated with LPS demonstrated higher expression of co-stimulatory molecules but unchanged F4/80 expression. PLGA microsphere treated cells appear to have a slightly activated phenotype, while cells incubated with 15% PBAE and also 25% PBAE microsphere formulations with and without plasmid DNA are activated as indicated by both down-regulation of F4-80 and up-regulation of co-stimulatory molecules. Results shown above are representative of 3 independent experiments.

6.3.4. Antigen specific T-cells are activated by DCs treated with PBAE microparticles containing pCMV-SIY *in vitro.*

To compare the microparticle formulations' ability to appropriately modulate dendritic cells to activate antigen specific T-cells, we performed an *in vitro* microparticle transfection of DCs followed by incubation of these cells with EL4 T-cells which have been transfected and express SIY (L3100). Two different ratios of dendritic cells to T-cells were used (10:1 and 5:1) and the mixed culture took place in a IFN-gamma ELISPOT plate. After incubation, the wells were processed for IFN-gamma spots and counted using a dissecting microscope. Averages of wells (n=4) are shown in Figure 6.5.).

Untreated controls, along with wells containing DCs which had been treated with 25% PBAE microparticles which did not contain pCMV-SIY gave the lowest number of identifiable IFN-gamma spots. SIY peptide treated DCs yielded the most IFN-gamma spots given that the peptide can directly bind to available DC surface MHC Class I without being processed intracellularly. PLGA treated DCs gave a slightly elevated number of spots, however this number was not statistically significant when compared to the untreated control. 15% PBAE and 25% PBAE treated groups, conversely, yielded more spots than the PLGA treated group and were statistically greater than the untreated control.

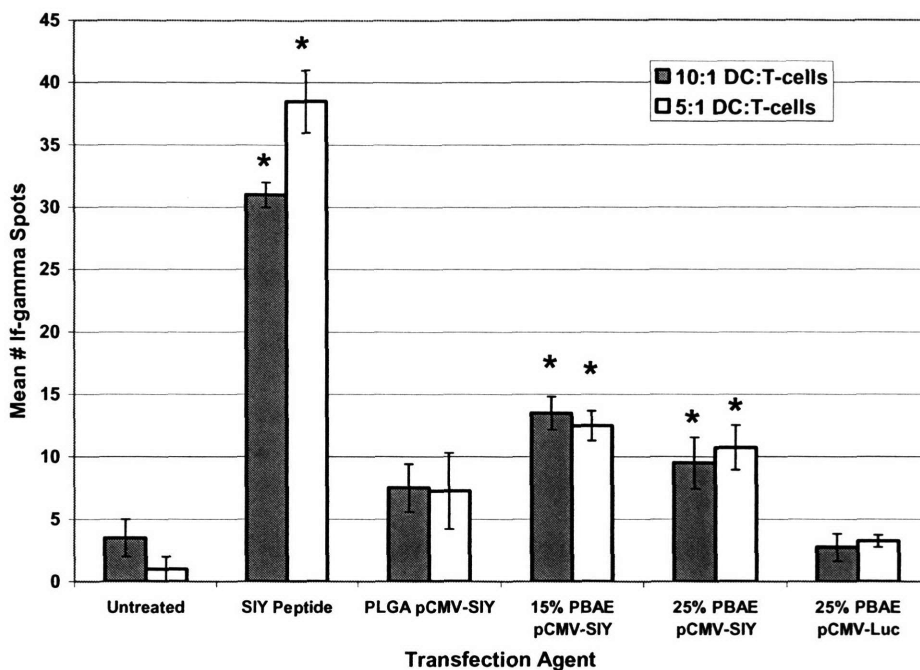


Figure 6.5. *In vitro* activation of T-cells using primary bone marrow dendritic cells treated with PBAE microparticle formulations. Number of IFN-Gamma spots were counted for wells containing T-cells treated with DCs at a ratio of (10:1, Grey) and 1×10^5 (5:1,White) for each group. Negative controls (1st column) were untreated dendritic cells and (6th column) 25% PBAE microparticles containing pCMV-Luciferase. The results shown above are averages of 4 repeats and standard error bars are included. Asterisks indicate a significant difference from the negative control at the same DC:T-cell ratio using comparative ANOVA.

6.3.5. SIY specific, 2C T-cells are activated in mice vaccinated PBAE microparticles containing pCMV-SIY *in vivo*.

To examine the effect of microparticle vaccinations on antigen specific T-cells *in vivo*, we administered pCMV-SIY vaccine formulations to B6 mice which had been adoptively transferred with cells harvested from 2C transgenic mice which recognize surface bound K^b-SIY peptide complexes. Another vaccination was given 2 weeks after the first, and SIY peptide was administered i.p. 1 week following the last immunization. 4 days after i.p. injection, spleens were harvested and 2C T-cells were isolated to examine activation by the amount of CD69 surface expression as shown in Figure 6.6.).

PLGA microparticle encapsulated pCMV-SIY vaccinated mice demonstrated a low level of $CD69^+$ cells comparable to that of the PBS vaccinated control (approximately 6% $CD69^+$). Naked pCMV-SIY vaccinated mice exhibited a higher level of $CD69^+$ 2C T-cells with one mouse yielding 12.23% and the other 38.57% $CD69^+$ T-cells. 15% PBAE encapsulated pCMV-SIY treated mice yielded the highest level of $CD69^+$ T-cells (38.5%, 61.3%) and the 25% PBAE encapsulated pCMV-SIY treated group yielded one mouse with 8.65% $CD69^+$ T-cells and the other with 49.57% $CD69^+$ T-cells.

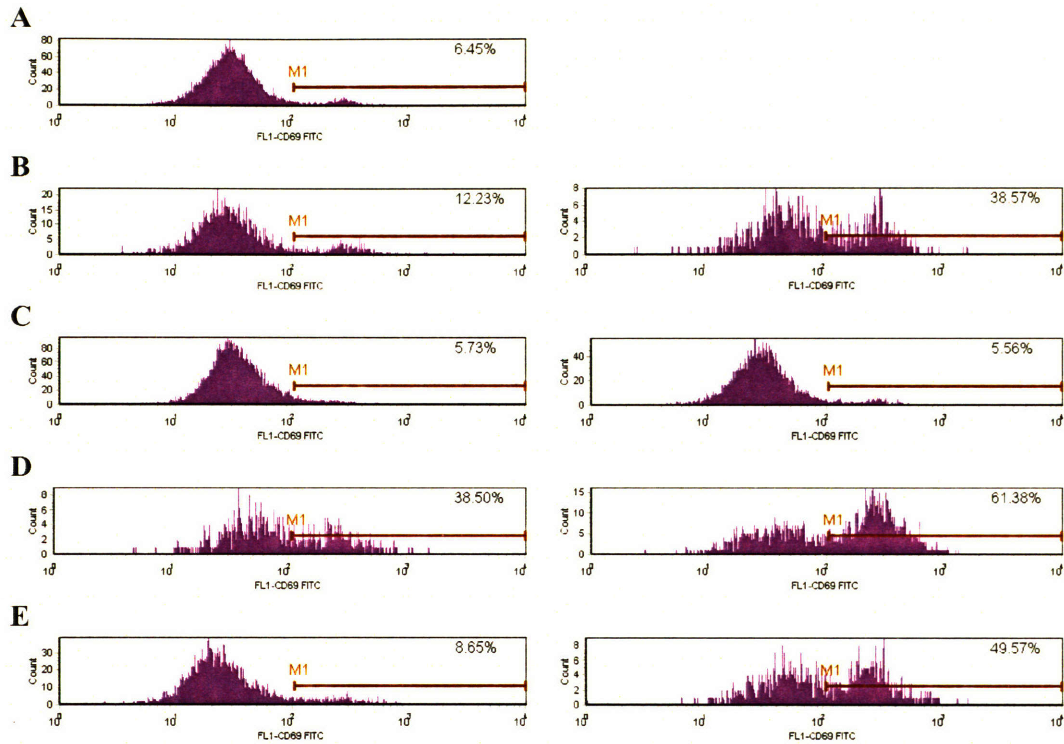
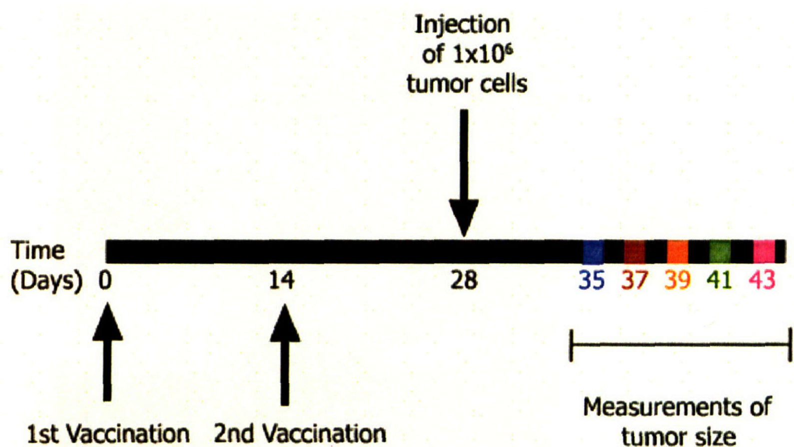
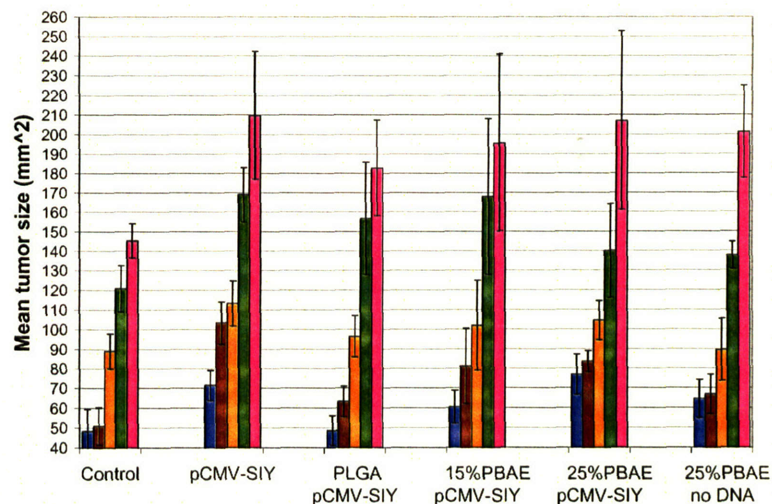


Figure 6.6. CD69 upregulation in transplanted, transgenic CD8$^+$ T-cells specific to SIY peptide isolated from mice vaccinated with microparticle formulations containing PBAE. **A.** Mice vaccinated with PBS as a non-treated control. {6.45% CD69$^+$} **B.** Mice vaccinated with naked pCMV-SIY (n=2). {12.23%, 38.57% CD69$^+$} **C.** Mice vaccinated with PLGA microparticles encapsulating pCMV-SIY (n=2). {5.73%, 5.56% CD69$^+$} **D.** Mice vaccinated with 15% PBAE microparticles encapsulating pCMV-SIY (n=2) {38.50%, 61.38% CD69$^+$} **E.** Mice vaccinated with 25% PBAE microparticles encapsulating pCMV-SIY{8.65%, 49.57% CD69$^+$} (n=2).

6.3.6. Vaccination with PBAE microparticle formulations containing pCMV-SIY results in antigen-specific rejection of SIY expressing tumor cells in B6 mice.

To compare the immunogenic efficacy of PBAE containing microparticle formulations, B6 mice (5 per group) were vaccinated with plasmid containing microparticles, naked DNA, empty microparticles, or PBS following the schedule represented in Fig 6A and described in the Materials and Methods section. The SIY-K^b complex is presented on the surface of EL-4 tumor cells transfected with SIY plasmid (administered left flank) but not presented on the surface of untransfected EL-4 tumor cells used as a control (administered right flank)(11, 15). SIY expressing EL-4 tumor cells on the left flank grew at similar rates in mice injected with PBS controls, naked DNA, PLGA/DNA microparticles, and blank 25% PBAE microparticle formulations. Conversely, in mice injected with formulations composed of 15 and 25% PBAE containing pCMV-SIY the average rate of growth of tumors expressing SIY were distinctly reduced (Fig 6.5.C.). Also, in two of the five mice in the 15% PBAE formulation group and in one of the five mice in the 25% PBAE formulation group, the SIY expressing tumors decreased in size and completely disappeared on the days indicated (* Fig 6.5.C.). On the right flank, control, untransfected EL4 tumors grew progressively in all groups (Fig 6.5.B.). Moreover, vaccination with a plasmid that exclusively expresses β-galactosidase (without the added SIY sequence) did not inhibit growth of the SIY expressing tumor cells (data not shown).





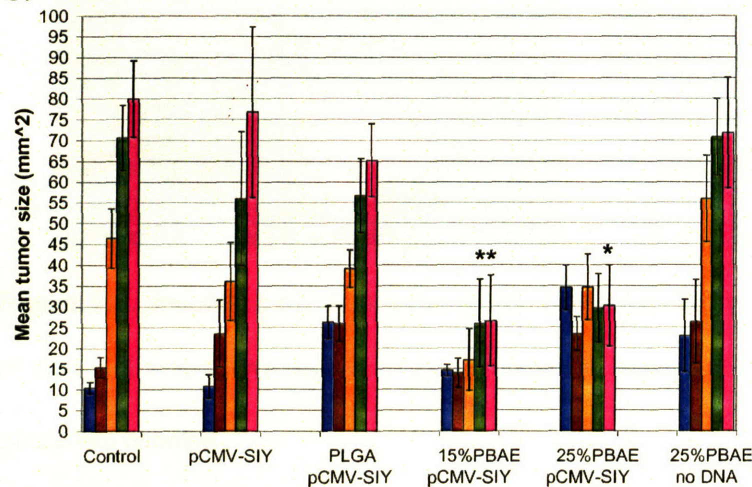


Figure 6.7. *In vivo* tumor rejection in B-6 mice after treatment with genetic vaccine formulations. B6 mice were vaccinated and challenged using the schedule shown in **(A)** and mean tumor size was measured using a caliper in 2 dimensions 7 (blue), 9 (red), 11 (orange), 13 (green), and 15 (pink) days after sub-cutaneous injection of $3x10^6$ normal EL4 thymoma cells **(B)** or EL4 cells which are transfected with SIY and express it on their surface **(C)** Standard error bars are shown for comparison. * indicates one mouse in which the tumor expressing SIY had completely regressed by this time point.

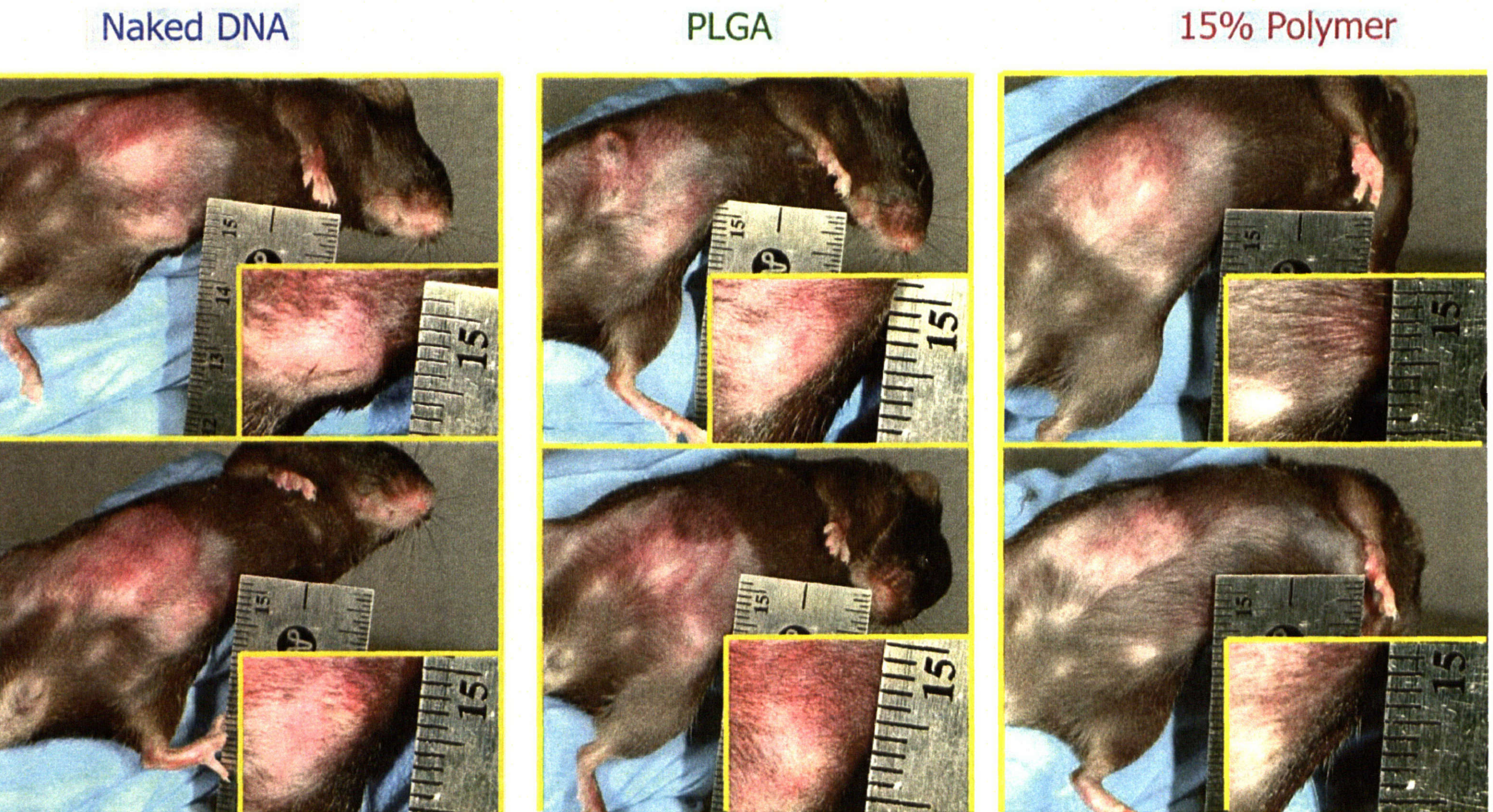


Figure 6.8. Smallest 2 EL-4 antigen specific tumors in each group shown above on the mouse's left side for naked pCMV-SIY, PLGA, and 15% PBAE on day 13 (inset = contrast enhanced zoom of EL-4 tumor). Note EL-4 tumor on both mice in the 15% PBAE group has receded.

Statistical analysis using comparative ANOVA showed that the 15% PBAE formulation was significantly different from the PBS injected control after day 11 and formulations containing 25% PBAE were significantly different after day 13. No other group showed significantly reduced tumor size when compared to the control group at any time point.

6.4. Discussion

Polymeric microparticles that physically encapsulate antigen-encoding plasmid offer several potential benefits to genetic vaccine formulations, including protection of the encapsulated plasmid(21), and size based adjuvancy and targeting to phagocytic antigen presenting cells(22). Furthermore, unlike viral delivery, microparticle delivery systems possess the capacity to hold extremely large payloads, allowing for vaccines with multiple antigen expression constructs (multi-valent) and co-encapsulation of immuno-modulating cytokines. Despite these advantages, current microparticle systems prepared from PLGA exhibit extremely low levels of gene expression in antigen presenting cells. Although such low amounts of antigen expression may be sufficient to induce some immune responses, it is likely that increasing levels of gene expression will lead to a corresponding increase in vaccine potency. We hypothesized that the incorporation of a degradable, pH sensitive polymer in conventional PLGA microparticle formulations would increase gene delivery capacity by facilitating intracellular release of plasmid payload upon phagosomal acidification.

Incorporation of PBAE into the microsphere matrix did not alter the structure or loading of the particles significantly. It was possible to encapsulate relatively high

quantities of supercoiled plasmid in our formulations using standard double emulsion techniques, despite previous indications that this is difficult(23).

In the last chapter, we demonstrated that these PBAE formulations could generate and increase of up to 5 orders of magnitude in gene delivery and expression in an APC cell line when compared to PLGA alone. Although this increased expression is important, upregulation of co-stimulatory molecules on these cells during epitope presentation is also crucial to vaccine potency. Co-stimulatory molecules are particularly important as in their absence, antigen presentation by immature/inactivated dendritic cells may induce tolerance to that antigen (24, 25). It was thus notable that primary bone marrow-derived dendritic cells were strikingly stimulated by microparticle formulations to up-regulate expression of several co-stimulatory molecules (Fig 6.4.B). Interestingly, 25% PBAE microparticle formulations with no encapsulated DNA still activated dendritic cells to a greater extent than PLGA microparticles but not as fully as 25% PBAE microparticles with encapsulated plasmid, suggesting that the PBAE polymer microparticle on its own can activate dendritic cells. The mechanisms behind this effect on dendritic cells are not clear and warrant further investigation. One possible mechanism stems from the observation by Thiele et al that the addition of cationic polymer (poly-L-lysine) to the surface of polystyrene beads up-regulates CD83 on primary human dendritic cells(26). This introduces the possibility that the increasingly positive surface charge of the cationic PBAE containing formulations may be partially responsible for the observed effect.

To determine if antigen encoding plasmid DNA encapsulated within PBAE containing microparticles can generate $CD8^+$ T-cell response to a model antigen, we used

an antigen expression system based on a particular peptide-MHC complex in which the SIY octapeptide is associated with K^b, a class I MHC protein(27). Cho *et al.* showed that a fusion protein containing the SIY sequence can stimulate mice to produce polyclonal $CD8^+$ T-cells which react specifically to the SIY-K^b complex(11).

Accordingly, we compared the ability of the pCMV-SIY plasmid in various microparticle formulations and as naked DNA to stimulate SIY-K^b specific rejection of EL-4 (express SIY & K^{b+}(15)) tumor cells in B6 mice. Only in mice immunized with pCMV-SIY DNA in PBAE containing microparticles was growth of SIY-producing EL-4 cells reduced. This effect was antigen-specific since EL-4 cells not expressing SIY grew unhindered in the same mice where SIY^+ EL-4 cells were affected. In contrast, the same plasmid in PLGA microparticles or as unencapsulated naked plasmid, had no apparent effects on the SIY^+ EL-4 cells. In addition, preliminary experiments demonstrated naïve anti-SIY-K^b cells (2C T-cells(16)) adoptively transferred into B6 mice persisted and up-regulated a T-cell activation marker (CD69), following i.p. injection of the SIY peptide into 3 out of 4 mice, only if the mice had been previously vaccinated with microparticles containing PBAE (or with the naked pCMV-SIY DNA) but not with those made exclusively with PLGA (Figure 6.6.). The antigen-specific tumor regression observed was likely due to a polyclonal anti-SIY-K^b $CD8^+$ T-cell response, but $CD4^+$ T-cells that recognize class II MHC-peptide complexes derived from the fusion protein encoded by the plasmid could have contributed to the tumor regression. Nevertheless, the responses seen were antigen-specific, reinforcing and extending *in vitro* evidence that microspheres containing PBAE are far more effective than those made exclusively from PLGA in their transfection ability and effect on primary dendritic cells.

Due to their inherent adjuvancy, the PBAE containing microparticles may be widely applicable as a platform for delivery in circumstances where the antigen of interest is not immunogenic enough for plasmid DNA vaccination alone, as in the case of B-cell malignancies or in individuals with weakened or tolerized immune capacity(28). The presence of strong adjuvancy as a innate property of the delivery system also bypasses adverse effects from using cytokines or conventional adjuvants to augment the immune reaction(8). We are currently exploring combinations of PBAE microparticles with complimentary technologies such as plasmid encoded cytokine and immunogenic fusion constructs along with targeting moieties on the microparticle surface which may even further enhance vaccine potency. Finally, the intracellular delivery capacity of PBAE microparticles may have implications for delivery of other drugs to antigen presenting cells, such as in the case of lysosomal storage disorders, where targeted, effective delivery to macrophages could lead to enhancements in enzyme replacement therapy.

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7. High-Throughput Fabrication of Microparticles Containing Active Plasmid DNA

7.1 Introduction

The controlled release of proteins from biocompatible polymer matricies was first reported in 1976, and has since revolutionized the way therapeutic agents can be used in the clinic[1]. A popular and extremely attractive method for releasing these materials is through polymeric microparticles which entrap the drug to be administered. This technology has been utilized to encapsulate and release therapeutic proteins suitable for applications such as anti-cancer treatments (Lupron Depot), local delivery of anesthetics[2, 3], cytokine delivery[4], controlled release of steroids[5], sustained release of protein antigen[6], and targeted DNA delivery[7] to name a few. The particles offer protection for the encapsulated materials, which have the potential to be extremely sensitive to physiologic environments, and maintain the ability to release continuously or intermittently over periods of days to months[8]. Another advantage of this technology is the ability to non-invasively inject the particle delivery system through a needle, avoiding the surgical implantation required when using larger delivery platforms.

One common way to prepare polymeric microparticles is through a method called the double-emulsion/solvent-evaporation technique (for review see[9]). This method allows for practically any water soluble small molecule drug, protein, DNA, etc, to be loaded into particles made from polymers such as the extremely popular, poly α-hydroxy-acids (most notably the FDA approved, poly-lactic-co-glycolic acid, or PLGA). The relatively small amount of drug-bearing, aqueous phase is finely dispersed in the immiscible, organic solvent containing the polymer by vigorous agitation to form a primary emulsion. This emulsion is then transferred to another aqueous phase containing a suitable surfactant and agitation is repeated. The result is the formation of discrete solvent droplets (secondary emulsion) containing the original aqueous, drug-loaded primary emulsion. Evaporation of the volatile solvent by stirring, followed by freeze drying yields solid polymer particles with internal, drug loaded compartments. This process usually takes approximately 4-5 hours, and, due to the requirement of washing steps to remove detergent, on the order of 4-8 microparicle formulations can be conceivably prepared in one day.

Microparticles prepared in this manner are extremely versatile given that they can carry large payloads and encapsulate multiple agents. Also the size can be easily controlled by the concentration of the polymer solution, agitation speeds during fabrication, and amount of surfactant used in the outer aqueous phase. Finally, the particle surface can be coated with materials which can target or affect cells through many commonly known mechanisms. This flexibility of varying multiple parameters allow for combination therapies involving several agents, which may have synergistic effects. However, varying all of the available parameters to fully optimize a therapy can

be a daunting task. Further complicating this scenario is that some therapeutic molecules such as proteins[10] and plasmid DNA[11] are deactivated in the particle microenvironment, requiring the need for additional stabilization agents.

A relevant example of the number of parameters involved with optimization is in the case of microparticulate genetic vaccine delivery. In this scenario any number of plasmids expressing different antigenic epitopes can be encapsulated. Also, a number of cytokines have tremendous promise to alter immune cells and have been shown to promote vaccine effectiveness[12], and therefore should be considered. Similarly, it is feasible to think that certain known protein chemokines would attract immune cells to the particle and would be an attractive addition. Furthermore, molecules such as mannose and phosphatidylserine are involved in immune cell phagocytosis of particles and are prime candidates for microparticle surface coating for delivery to these cells. Other studies have shown that particle size plays a substantial role in the effectiveness of the vaccine formulation *in vivo* and may differ from system to system[13]. Finally, the polymer which is used in fabrication of the particles has been shown to drastically affect the delivery capacity of the particle[14] and blending two polymers together is sometimes desired. In this case, finding an optimal ratio is necessary[14]. The number of possible particle formulations would then follow by:

$$\mathbf{2^{(\#\ of\ cytokines)} \cdot 2^{(\#\ of\ chemokines)} \cdot 2^{(\#\ of\ surface\ labels)} \cdot (2^{(\#\ of\ plasmids)}) \cdot}$$

$$\mathbf{((\#\ of\ polymers) \cdot (\#\ of\ polymer\ ratios) + 1) \cdot (\#\ of\ particle\ sizes)}$$

$$= \quad \underline{\{\text{TOTAL \# OF FORMULATIONS}\}}$$

assuming that: a) all combinations of the first 4 terms are possible, and b) if more than one polymer is to be considered, that it would be evaluated in blends with one common polymer, such as PLGA[14] (# of polymer ratios does not include 100% of this common polymer to avoid repetition in the groups).

Therefore let us assume a minimalistic, but at least realistic, scenario in which we have a known, single-antigen system where *in vitro* and *in vivo* screening can be performed (i.e. the antigen is not being investigated). Also assume it was desired to investigate the effects of two different polymers on delivery of some already known dosage of 2 different cytokines without *a priori* knowledge of what particle size is optimal. A realistic evaluation of polymer ratios would be 100:0, 75:25, 50:50, 25:75, and 0:100 (polymer A : polymer B). Therefore:

(# of cytokines) = 2 (# of polymers) = 2

(# of polymer ratios) = 4 (# of surface labels) = 0

(# of particle sizes) = 2 {i.e. phagocytosis range, endocytosis range}

(# of chemokines) = 0 (# of plasmids) = 1

Using the above equation, the total number of particle formulations possible is 144. Experimental designs (factoral) may be feasible depending on what parameter is varied

and can bring this number down somewhat. However, the number of required combinations would still be extremely high and preparing all formulations in a reasonable timeframe would not be realistic.

Furthermore, we have recently synthesized a library of over 2000, structurally-diverse poly(β-amino ester)s, all of which may have potential to enhance genetic vaccine delivery and adjuvancy in a similar way as the one tested in our preliminary studies[15]. Clearly, to make progress in screening even a portion of this library, especially if it is desired to vary any other parameters, it would be necessary to develop rapid methods for synthesizing these formulations on a smaller scale. In this chapter, we describe for the first time, a high-throughput method for fabricating microparticles by the double emulsion procedure. We demonstrate that we can reproducibly produce particles with high surface integrity and controllable size distributions. Finally, and most importantly, we demonstrate that we can entrap a therapeutically relevant material in a biologically active form using this technique.

7.2. <u>Materials and Methods</u>

7.2.1. Materials

Poly(*d*,*l*-lactic-co-glycolic acid) polymer (PLGA, RG502H Resomer 50:50) was purchased from Boehringer Ingelheim (Ingelheim, Germany). Poly(β-amino ester)s (PBAE) were synthesized as previously reported ($M_n \approx$ 7-10 kD) [15, 16]. Plasmid DNA encoding firefly luciferase (pCMV-Luc) was obtained from Elim Biopharmaceuticals (Hayward, CA). Dextran conjugated tetramethyl rhodamine ($M_n \approx$ 70 kD) was purchased from Molecular Probes (Eugene, OR).

7.2.2. Cells

The P388D1 macrophage cell line was obtained from ATCC (Manassas, VA). Cells were cultured in RPMI 1640 media (Gibco Life Technologies; Carlsbad, CA) containing 10% FBS, 0.1 M HEPES, 1 mM Sodium Pyruvate, and 100 U/ml Penicillin/Streptomyocin.

7.2.3. High-throughput preparation of particles

Plasmid containing microparticles were prepared by the following modification of the double emulsion technique[9] to scale down and adapt to a high-throughput format. All steps described below were at 4°C to minimize structural defects of the particles due to variation in polymer glass transition temperature. Lyophilized plasmid DNA was dissolved in an aqueous solution (10 mg/mL) of sterile-filtered EDTA (1 mM) and D(+)-Lactose (300 mM). 12 µl of this solution was then added to 0.25 ml of CH_2Cl_2 solution with polymer at varying degrees of composition (50 mg/ml) in a deep, 96 well plate (Corning) with a staggered formation (Figure 7.1). To emulsify these immiscible phases, we utilized a 24 tip, probe sonicator attachment (Sonics and Materials Inc; Danbury, Conneticut) at a setting of 47 % amplitude for 10 seconds. The resulting emulsion was then immediately transferred to a solution of poly(vinyl alcohol) (120 µL into 1.5 ml, 1% PVA (w/w), 0.25M NaCl) in deep, round bottom 24 well plates (Corning) using a 96 tip fluid handling robot. This plate was then immediately sonicated at a setting of 37% amplitude for 20 seconds to form the final water-in-oil-in-water emulsion. This plate was then placed on a rotating plate and allowed to stir for 3 hours to allow for solvent evaporation. The plate was then transferred to a refrigerated centrifuge with plate attachments and rotated at 1200 rpm for 10 min. The supernatant was removed with a 6

well aspiration wand (V & P Scientific; San Diego, CA) and replaced with clean water. Particles were resuspended and the process repeated 3X to remove excess PVA surfactant. After the final wash, the particles were suspended in a minimal amount of water, frozen with liquid nitrogen, and allowed to lyophilize in a large vacuum chamber (Labconco; Kansas City, MS) at < 10 mTorr for 3 days. Products in the individual wells were white, fluffy powders. Microparticles and polymers were stored at -20°C in a desiccated chamber.

7.2.4. Characterization of particles

Microsphere size distributions were measured via volume displacement impedance using a Multisizer 3 using 30-200 μm orifice tubes (Beckman Coulter; Miami, FL). Zeta potentials were obtained using a ZetaPALS analyzer (Brookhaven Instruments; Holtsville, NY) with 10mM HEPES buffer at pH=7.4. Morphology of microsphere surfaces was imaged using scanning electron microscopy (SEM).

7.2.5. Reporter gene transfection

To determine if the encapsulation process yielded active plasmid DNA, we incubated microparticles with a P388D1 macrophage cell line as previously described[7]. Briefly, P388D1 macrophages were seeded at $5x10^4$ cells/well in fibronectin coated, white polystyrene 96 well plates and allowed to achieve 75% confluence. Media was then replaced with suspended of pCMV-Luc plasmid DNA containing microspheres in cell media using a 96 well fluid handling robot yielding 4 reps per microparticle sample (24 to a 96 well plate format). A titration of the soluble, lipid-based transfection agent, Lipofectamine 2000 (Invitrogen), was prepared with DNA as a positive control. After a 20 hr. incubation, the media was aspirated from the samples and cells were washed with

PBS. The cells were lysed by incubation for 10 minutes at room temperature with Glo Lysis Buffer (Promega, 100 µl, 1X). The wells were then analyzed for luciferase protein content using the Bright Glo Luciferase Assay System (Promega) and a Mithras plate reading luminometer (Berthold Technologies) with a 1 second read time.

7.3 Results and Discussion

With the recent synthesis of a library composed of over 2000 PBAEs, many new promising gene delivery polymers have emerged that can perform better than the best commercially available transfection reagents[15]. Also, these polymers, like the PBAEs initially studied, have the potential to exhibit pH sensitive solubility and are therefore promising agents for microparticulate formulations which are suitable to differentially release in the low pH environment of an endosome or lysosome. This property makes particles prepared from these materials extremely promising for the delivery of proteins to phagocytic cells such as in the case of enzyme replacement therapy where targeted, intracellular delivery to macrophages seems to be the most logical strategy. We have also shown in a previous chapter that anionic materials can be released with an adjustable delay depending upon how much cationic polymer is added to the formulation. Furthermore, some of these cationic PBAEs have further been investigated for the effects of polymer molecular weight[17] and drug binding and complexation effects[18] on delivery efficiency. To extend these types of studies to screen large numbers of polymers in a library such as the one mentioned above, however, would require an advance in the speed and efficiency in which microparticles are fabricated.

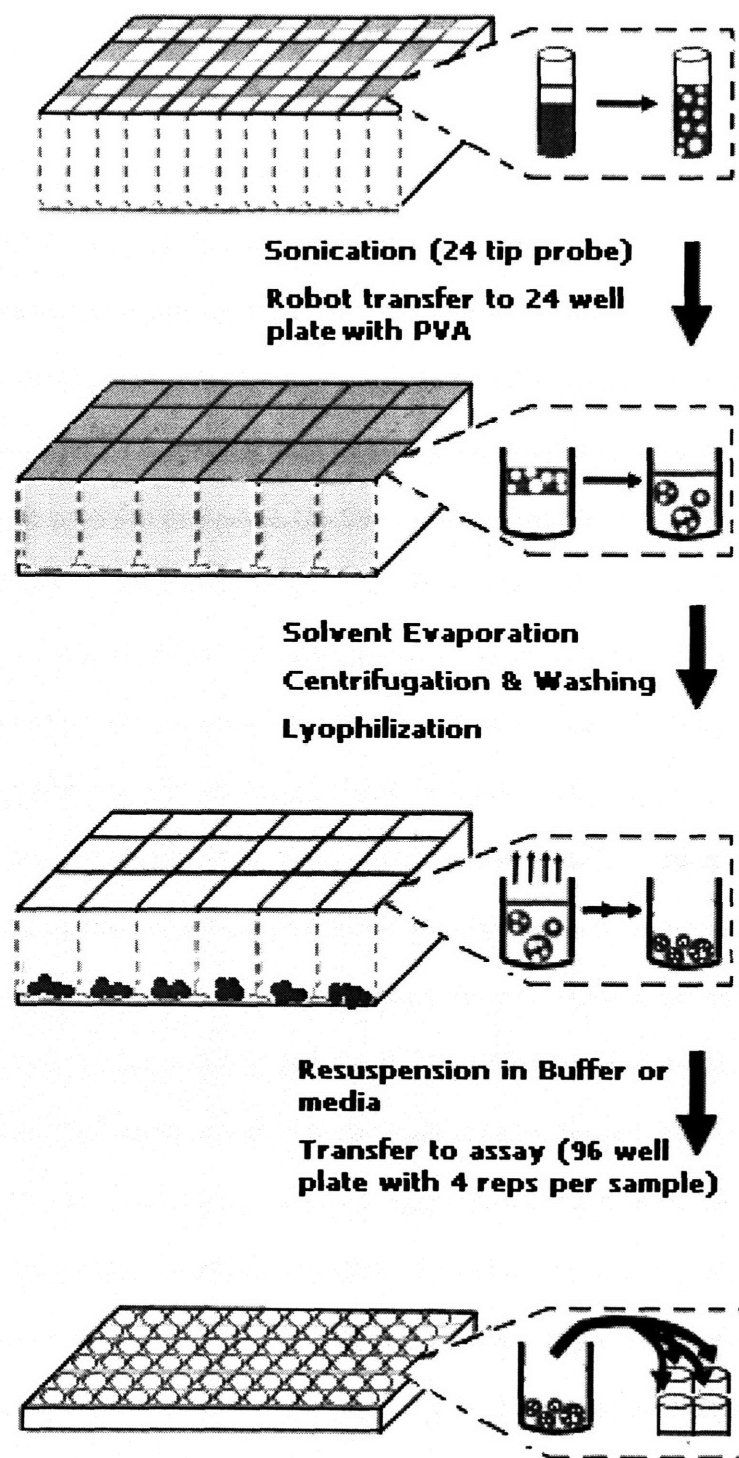


Figure 7.1. Schematic representation of the high-throughput double emulsion procedure.

7.3.1 High-throughput fabrication of particles

Figure 7.1 schematically represents a process intended to scale-down a standard double emulsion protocol and place it in a plate so that many particle formulations can be prepared at once. Due to differences between a standard double emulsion procedure and the proposed high-throughput method, there are several special circumstances worth noting. First, the transfer of the primary emulsion from the 96, deep-well plate to the 24, deep well plate with PVA solution must be performed as quickly as possible. In a standard double emulsion procedure, the time between these stages before the secondary emulsion is formed is close to 5 seconds. However, when transferring multiple primary emulsions, the fluid handling robot takes around 10 seconds, leaving little extra time before the droplets in this emulsion begin to grow in size. Secondly, the sonication was performed at intermediate intensities and only PVA was varied in order to alter particle size. Higher sonication rates would surely result in much smaller particles[19], however one needs to be cautious of the safety limitations of the probe in use which may limit the usage of this parameter to control particle size on its own. Thirdly, the solvent evaporation in our studies was performed at 4°C to avoid complications related to the glass transition temperatures which may vary substantially between polymers. It should be noted that solvent evaporation will take longer periods of time to come to completion at this lower temperature. Furthermore, since the double emulsion is in a plate, rather than in a beaker with a stir-bar, as is commonplace in a standard procedure, longer solvent evaporation times may be necessary (>3 hrs). Finally, since different particle formulations will settle differently, and some may aggregate at high centrifugation

speeds, care should be taken to use low rotor speeds and cautious supernatant aspiration during washing steps to avoid irreversibly damaging or losing product.

7.3.2. Characterization of particles

Rhodamine conjugated dextran sugar was encapsulated in particle formulations to demonstrate that a model material can be placed into polymer particles using our modified, high-throughput technique. Using fluorescence microscopy (Figure 7.2 A), particles seemed to encapsulate relatively high quantities of material and looked similar to particles prepared using standard double emulsion. This entrapment seemed to remain consistent throughout the plate, as determined by fluorescence microscopy of microparticles taken from several different wells (data not shown). Furthermore we were able to generate multiple 24 well plates with this same consistency in encapsulation. All formulations were prepared subsequently with plasmid DNA (pCMV-Luciferase). Particles prepared with this plasmid were examined using Scanning Electron Microscopy (SEM) after standard gold sputter coating. Results indicate that particles have spherical shapes and look similar to those from standard double emulsion techniques (Figure 7.2 B & C). These images also indicate that the particle has a relatively high integrity with minor flaws on the surface. These could be a result of not checking and balancing the osmolality of the internal and external aqueous phases, which has shown in previous chapters to affect drug entrapment and particle surface integrity drastically.

Sizes of particles were measured using a volume impedance principle on a Coulter Counter. This size seemed to be inversely dependant on the concentration of PVA used in the outer aqueous phase, as expected[9]. PVA concentrations of 5% yielded particles with mean diameters around 4 μm, while concentrations of 0.5% PVA resulted

in particles with a mean diameter below 1 μm (Fig 7.3 A-D). It is important that this parameter be easily adjustable given the many physical properties the particle size influences (e.g. cellular uptake, release, loading). The only limitation of the technique described herein is that particle size cannot be changed with respect to different wells in the same fabrication plate. This stems from the sonication amplitude output being constant in every tip of the 24 arm probe. This is demonstrated by sizing random wells on the periphery and the center and comparing mean diameters. In our study, there was no statistical difference between these two values in any case (Fig. 7.3 E & F).

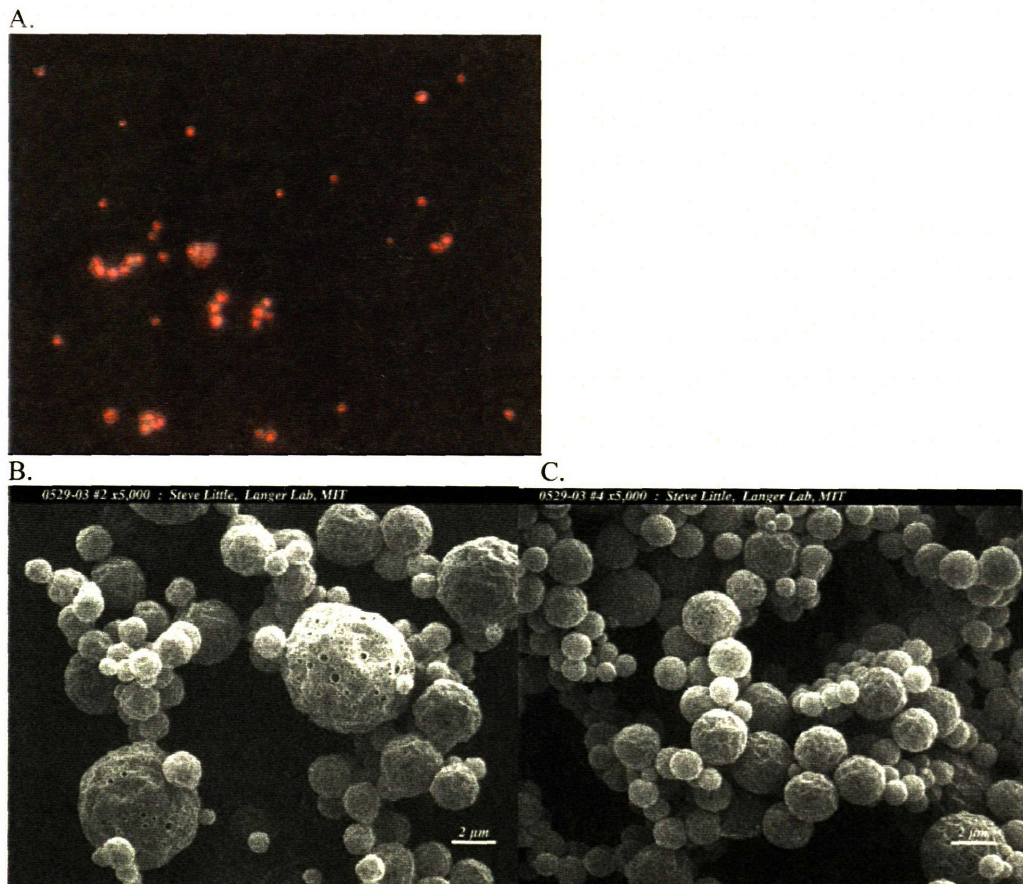
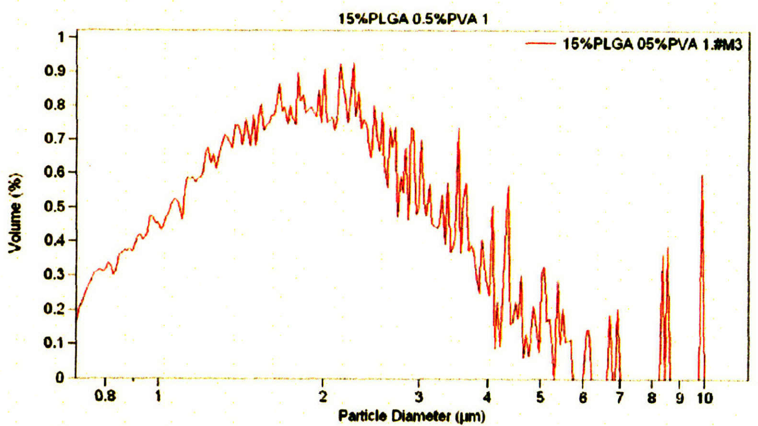
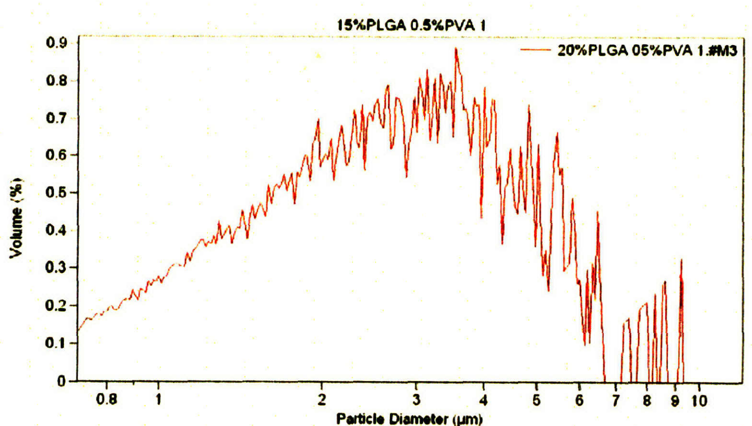


Figure 7.2. A. Fluorescent microscopy image of particles containing encapsulated rhodamine conjugated dextran sugar (red). B & C. Scanning Electron Micrographs (SEM) of particles prepared using the high-throughput double emulsion technique. Images are 5000X magnification. Bar in the bottom right hand corner indicates the length of a 2 μm reference.

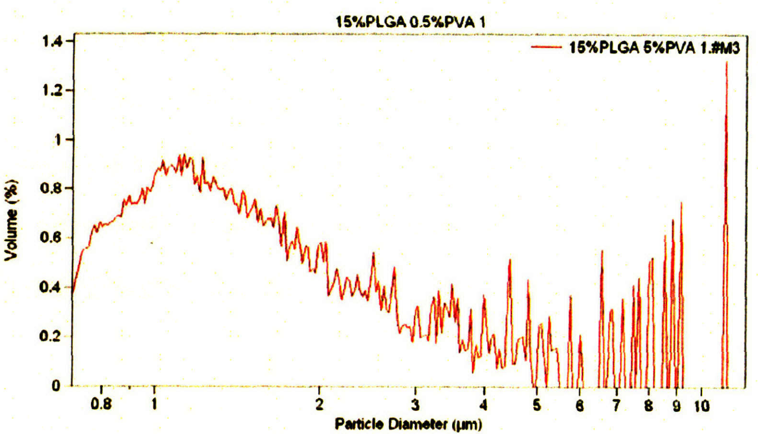
A.



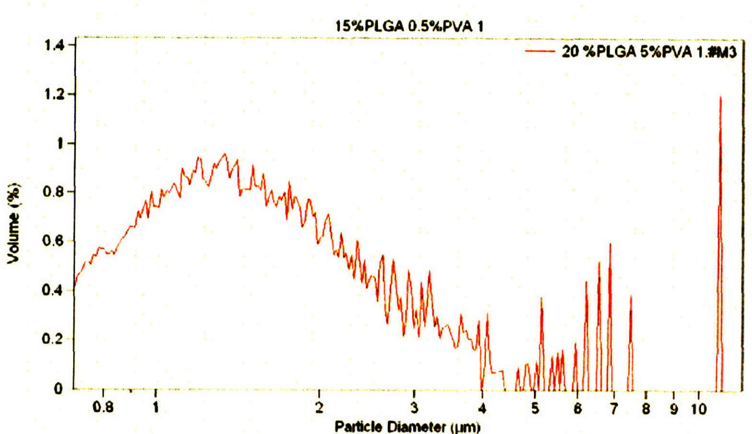
B.



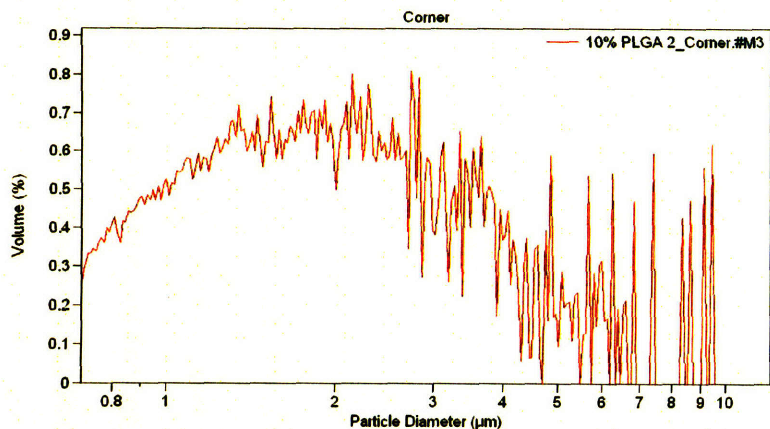
C.



D.



E.



F.

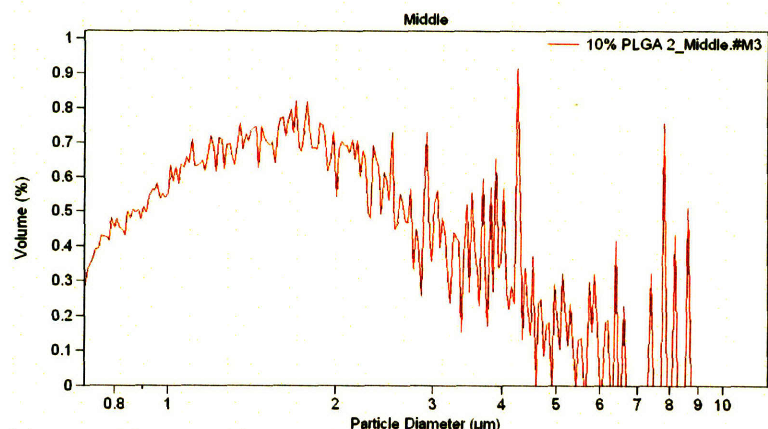


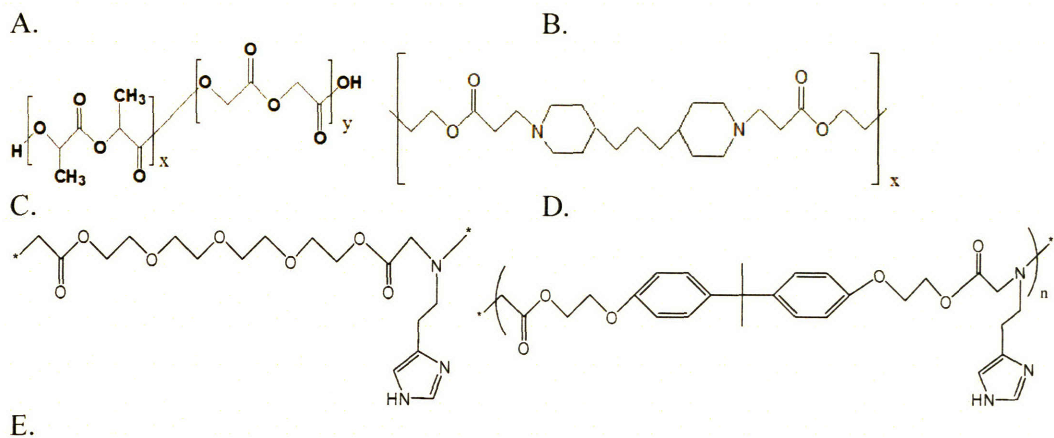
Figure 7.3. Volume impedance based size distributions of particles prepared using the high-throughput double emulsion technique. A-D. Varying PVA concentration in the outer aqueous phase results in particles with different sizes. A & B represent particles which were prepared with 0.5% PVA in the outer aqueous phase composed of 15% ($D_{ave} = 2.3 \pm 1.3$ µm) and 25% PBAE ($D_{ave} = 3.0 \pm 1.6$ µm), respectively. C &D represent particles which were prepared with 5% PVA in the outer aqueous phase composed of 15% ($D_{ave} = 0.9 \pm 0.4$ µm) and 25% PBAE ($D_{ave} = 0.9 \pm 0.7$ µm), respectively. E & F demonstrate that particles prepared in a random corner well ($D_{ave} = 2.2 \pm 1.4$ µm) are the same size as particles prepared in a random well in the center of the plate ($D_{ave} = 2.4 \pm 1.9$ µm).

7.3.3. Entrapment of active plasmid DNA

Therapeutic agents may not always be fully active after the encapsulation process. This can be due to many factors including: 1) sheer forces, 2) organic solvent phase interactions, 3) internal particle microclimate, and 4) drug-polymer interactions. Ando et.al. addressed this issue in the case of plasmid DNA encapsulation and suggested modifications to these processes to better suit this particular pro-drug[20]. Zhu et. al. addressed this issue from a protein standpoint using PLGA microparticles[10]. It is extremely important for any new fabrication technique to allow for encapsulation of a material in its biologically active state. As related to the new methods described here, different forces are present, such as vigorous sonication in place of a homogenization step and/or differences in turbulence between a 24, deep well vs. a 100 ml beaker. To evaluate the activity of encapsulated material, we used PLGA (Fig. 7.4 A) blended with a polymer (Poly-1, Fig. 7.4 B) which is known to exhibit transfection in a P388D1 macrophage cell line. Particles were prepared using different ratios of the two polymers (40% Poly-1 : 60% PLGA to 5% Poly-1 : 95% PLGA) and were resuspended in P388D1 cell culture media. These particles were added to the cells (similar to last stage of Figure 7.1) and incubated for 3 days before testing for luciferase expression using luciferin and ATP.

The results of this assay conform to the results obtained previously using Poly-1 as a delivery enhancer in a similar optimum polymer ratio range (Figure 7.4, blue bars, 4 repetitions). This data also confirms that active plasmid has been successfully encapsulated. It should be noted that only a 1 sec luminometer read time was used in these studies instead of the 10 read times used in previous chapters. The reason for this

change was to avoid going outside the linear range of the machine if one of the other polymers tested in this study proved to be as effective here as in the case of spontaneously formed polymer/DNA complexes[15].



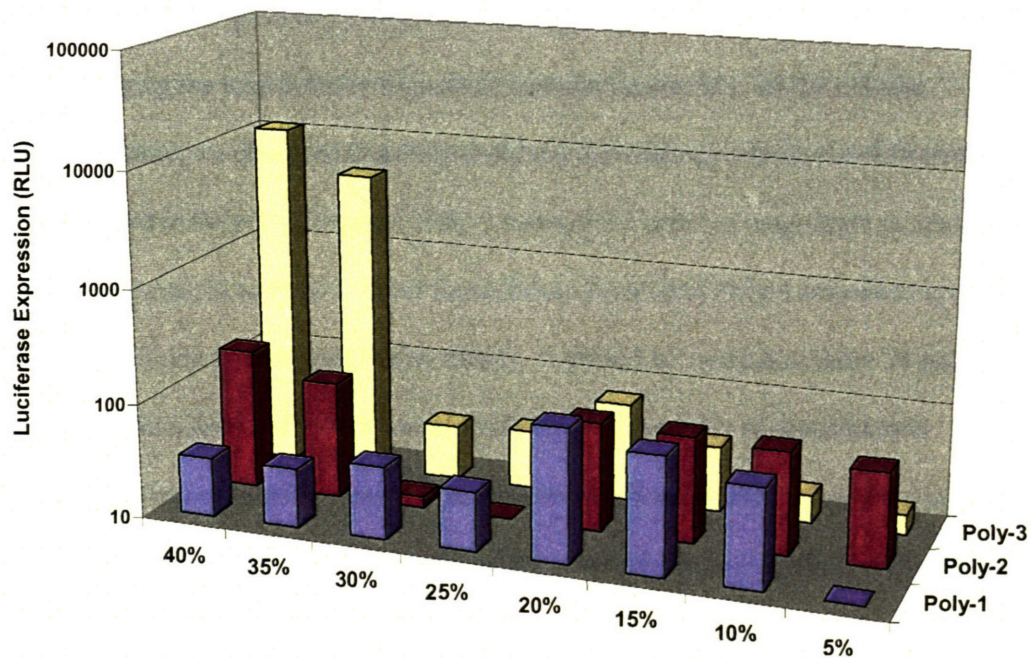


Figure 7.4. Structures of **A.** PLGA, **B.** Poly-1, **C.** Poly-2, **D.** Poly-3.

E. Transfection of P388D1 macrophages to demonstrate that active plasmid DNA can be incorporated into polymer microparcles prepared using the high-throughput double emulsion technique. Three distinct PBAEs (y-axis; Poly-1 (blue), Poly-2 (red), Poly-3 (yellow)) were

prepared in deep well plates in ratios varying from 5% PBAE/95% PLGA, to 40% PBAE/60% PLGA (x-axis). These particles were resuspended in cell media and added to cell culture wells containing P388D1 macrophages. Three days later, these cells were tested for luciferase activity asdescribed in the materials and methods section and displayed above in relative light units (RLU) on the z-axis.

7.3.4. Effects of varying polymer ratio of two new PBAEs in microparticle formulations

Two new PBAEs were chosen from the 2000+ library and incorporated into microparticle formulations using the high-throughput double emulsion procedure to serve as a pilot example for the usefulness of this technology. As previously discussed, Poly-1 was varied from 40% to 5% in 5% increments (8 total formulations compared to the 5 used in previous chapters with this polymer). In the same plate, Poly-2 and Poly-3 (Figure 7.4 C & D, respectively) were varied using the same ratios with respect to PLGA content (bringing the total number of particle formulations to 24). In the cellular transfection assay, we observed that Poly-2 did not demonstrate substantial differences when compared to Poly-1. However, Poly-3 boasted a 2 order of magnitude increase at 35 and 40% compared to Poly-1's best formulation (recall that Poly-1 transfects up to 5 orders of magnitude greater than PLGA alone). It should be noted that these 24 particle formulations were prepared in 4-5 hours, while the same number of formulations prepared by a standard double emulsion procedure would have taken 3 full days worth of work to produce. It will be extremely interesting to test the promising Poly-3, and other new polymers more extensively using this technology in the future.

The speed in which this technique allows for microparticles to be fabricated provides a valuable tool to study variations in particle formulations in many ways. Just one example of this is the enablement of rapid testing for release profiles. Particles could

conceivably be prepared using different ratios of polymers, molecular weights, and excipients containing drugs which currently can be detected in extremely low amounts using new technologies (proteins can be measured in the pico to nanogram range using ELISA; double stranded DNA such as plasmid can be measured in the picogram range using base-pair intercalating agents such as PicoGreen) by release in a 96 well plates. The plate can be centrifuged, supernatant removed/analyzed, and new buffer/media can replace and resuspend particles to collect released drug for the next time point. Furthermore, since the disclosed fabrication method now enables a researcher to prepare over 100 microparticle formulations in a day, the experiment involving genetic vaccines mentioned earlier which was infeasible with standard technologies, now becomes a reality. With respect to choosing the best reagents for final usage *in-vivo*, sadly, the appropriate understanding is now one step behind in the development of relevant assays which would best predict whether a particle formulation created on the bench-top will be useful to a patient at bedside.

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8. Conclusions

Synthetic, non-viral DNA delivery methods are extremely promising candidates to yield viable therapeutics in the field of genetic vaccines because of their safety and versatility. However, for these delivery systems to be implemented, new ways to increase their potency must be investigated. Given our current understanding of the deficiencies in current vaccine formulations, along with the growing understanding of the mechanisms of genetic vaccines and the cell types involved, it seems most logical to target delivery to dendritic cells (DCs). These cells are particularly difficult to transfect, and require better gene delivery systems than those currently implemented to target DCs. Furthermore, current state of the art delivery systems based on PLGA do not potently stimulate co-stimulatory upregulation on the surface of DCs, a necessary component to effective vaccine responses.

The recent synthesis of polymer libraries composed of degradable poly(β-amino ester)s has yielded many new gene delivery agents with similar structural properties when compared to PLGA. The goal of my thesis was to investigate these new materials which are better suited for the delivery of plasmid DNA to DCs. We hypothesized that these

materials would lead to a better delivery vehicle based on their physical properties, and therefore, a better genetic vaccine formulation.

As part of this approach, we incorporated a particularly promising PBAE with pH sensitive solubility profiles, blended with PLGA, into DNA microparticle formulations. We demonstrated that these formulations provide a more suitable environment for plasmid DNA, indicated by higher loadings, supercoiled content, and activity. These properties translate to an increase of up to 5 orders of magnitude in DNA delivery efficiency when compared to PLGA alone. Furthermore, these particles can be potent stimulators of antigen presenting cells *in vitro* as measured by several important co-stimulatory molecules. In addition, we have demonstrated that incorporating these new biomaterials into microparticulate genetic vaccines can lead to antigen-specific, immune-mediated rejection of a lethal tumor dosage *in vivo*, a significant advance over conventional formulations and proof that our original hypothesis is valid.

The potential of this class of polymers for use in microparticle formulations is tremendous. Therefore the screening of the remainder of the libraries for this application is certainly warranted. However, the sheer size of the libraries, along with the overall flexibility of adding multiple promising biological agents to particle formulations leads to an insurmountable obstacle: the preparation of all the necessary microparticle formulations using conventional techniques. Therefore, the second goal of this thesis was to investigate tools to aid in this daunting task. In this work, we report for the first time, a method for the high-throughput fabrication of microparticle formulations prepared by the double-emulsion technique. Using this method, it is possible to prepare over 100 particle formulations in a day compared to less than 10 using conventional methods. These

particles have similar physical properties to those prepared using the standard double emulsion technique, and it was demonstrated that encapsulated plasmid DNA was transcriptionally active. Furthermore, an initial trial using this new technology using only 2 of the polymers from the 2000+ PBAE library yielded a formulation which led to increases in gene delivery efficiency of over 2 orders of magnitude compared to the original PBAE tested for this application.

9. Future Work

The work performed in this thesis can be extended in two areas: 1) the development of new assays to better predict *in vivo* genetic vaccine potency, and 2) the screening of new polymer microparticle formulations using the technologies described herein. The former of these two is an extremely difficult task and strong arguments have been made that an assay like this may never be fully realized. However, steps toward this goal would involve using the extent of our current knowledge to create an assay which reflects the course of the genetic vaccine activation mechanism. The latter extension of this work can not only include the screening of new PBAEs from the described library, but also can be extended to the addition of co-encapsulated proteins and surface modifications intended to manipulate DC activation along with antigen uptake, processing, and presentation.

The development of a high-throughput assay to predict *in vivo* vaccine efficacy of microparticle formulations would most certainly involve primary dendritic cells, since these are the cells which most likely mediate this response. Also, it seems necessary to

incorporate one stage where lymphocytes are added to the treated DCs so that the effect of both antigen expression, and co-stimulatory up-regulation can be translated to some measurable change in the lymphocyte population. One possibility, which maintains the high-throughput plate format described in Chapter 7, would be: 1) to seed 96 well plates with primary dendritic cells isolated from muring bone marrow, 2) incubate these DCs with microparticle formulations prepared by the high-throughput method, 3) add T-cells specific for the antigen expressed by the plasmid encapsulated in the microparticles, and 4) measure T-cell activation and proliferation by tritiated thymidine incorporation, which should be sensitive enough to pick up minor changes in a small sample using current technologies. Eventually, both $CD8^+$ and $CD4^+$ cells could be tested in this assay using whole protein expression vectors which would express both MHC Class I and II associated antigens.

Once an assay like this is validated by favorable *in vivo* immune responses, the next obvious task would be to screen the polymer library using different blends of PLGA and PBAEs. However, assays such as the one discussed in the introduction to chapter 7 which would investigate cytokine co-encapsulation are also warranted. Cytokines such as GM-CSF, IFN-gamma, IL12, and IL-2 expression vectors are all promising candidates to increase vaccine potency. Similarly, chemokines such as RANTES and IP10 could attract DCs toward the particle administration site and T-cell cytokine expression vectors may attract T-lymphocytes to the transfected dendritic cell. Finally, modifications to the particle surface may significantly affect the level of uptake and how a DC views the phagocytosed material. Coatings such as bacterial lipopolysaccarides can react through DC surface toll-like-receptors to simulate pathogen phagocytosis. Alternatively,

phosphatidlyserine is one of the predominant molecules on the surface of apoptotic cells and has been shown to increase phagocytosis while maintaining the low level of surface co-stimulation seen in an immature DC. This may be a viable alternative to encapsulating materials to be delivered to DCs for the purpose of down-regulating the immune system, such as in the case of graft rejection and autoimmune disease.

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