Non-Viral Drug Delivery Systems for Immune Modulation

by

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Abstract:

Biodegradable polymer particles have diverse applications in drug delivery. The main objective of this thesis was to apply these delivery systems to modulating the immune system. We optimized particle formulations for the delivery of three novel immune modulating compounds, small inhibiting RNA, immunostimulatory RNA, and β-1,6-glucan.

Because microparticles formulated from PLGA and Poly(β-amino-ester) have been shown to target and transfect DNA in antigen presenting cells we studied their ability to knock down genes with siRNA. We discovered ways to improve particle morphology, encapsulation efficiency, and buffer the acidic microenvironment of degrading microparticles, all significant challenges with siRNA.

We next used fluorescent nanoparticles as imaging agents to study these siRNA delivery challenges. Cationic polymers were deposited on the surface of fluorescent core-shell silica nanoparticles electrostatically; the resulting particles were complexed with a nucleic acid and delivered to cells. We screened a library of 60 unique formulations to identify an optimal protocol for DNA transfection demonstrating efficiency equal to PEI. We screened a library of 30 unique formulations for siRNA delivery and demonstrated knockdown of 25%. Confocal imaging showed that polymer coating increased localization of the nanoparticles to the cell membrane, endosomes and nucleus. Polycation surface-modification seemed broadly extendable to a biodegradable polymer particle delivery system for siRNA.

Cationic lipids or lipidoids were promising polycations to apply to biodegradable particle surface-modification because they efficiently deliver siRNA. We screened 30 lipidoid formulations for optimal knockdown in P388-D1 macrophage cells, and isolated formulations that demonstrated up to 40% knockdown in P388-D1, 80% knockdown in primary macrophage, and 65% knockdown in mouse macrophage in vivo. We formulated microparticles from PLGA/lipidoid blends that demonstrated nearly 80% knockdown in P388-D1. This same formulation also induced sequence specific interferon response to immunostimulatory RNA in human peripheral blood mononuclear cells.

Finally we used PLGA microparticles to deliver a novel fungal cell wall component, β-1,6-glucan, to neutrophils. This approach induced neutrophil expression of reactive oxygen species in vitro. In a mouse model of blood stream Candida albicans infection 60% of mice survived lethal doses when treated with the particles.

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1. Thesis Introduction

It is not possible to define the exact moment when the field of immunology was born. As early as the 5th century Greek Plague, Thucydides observed that those who had previously recovered were immune. However it was the pioneering work of Louis Pasteur in "germ theory" that led to the first vaccine. Elie Metchnikoff's elucidation of immunology mechanisms and Paul Ehrlich's concept of autoimmune disease, chemotherapy and antibiotics brought immunology to become a scientific discipline around the turn of the 20th century [1]. These three researchers were among the first to understand that pathogens can exploit the immune system, and that this complex system can turn against the host. They were also the first to consider modulating the immune system to make it more effective against pathogens or less harmful toward the host.

Over the past century many discoveries have improved our understanding of the immune system in disease and many new medical approaches have been invented. Immune suppressing drugs have enabled organ transplantation. Disease progression can be slowed in autoimmunity with chemotherapy and immune suppressing drugs. Vaccines have eradicated small pox, polio and other diseases that once killed millions.

However, the true power of the immune system has not yet been harnessed. Autoimmunity and cancer are certainly far from being cured and still killing thousands each year.

There are several limitations to our ability to modulate the immune system and successfully treat disease. We do not have a completely clear picture of which targets will optimally direct immune response in a given situation. New cells, molecules, and pathways continue to be discovered which change our understanding of the immune system. As immunology advances in the genomic age, we will come closer to understanding which genetic, cellular and biological levels to adjust in order to harness the immune system. Moreover, in many cases we will have the biological tools to do it. Genes can be expressed or knocked down by delivering nucleic acids (either siRNA or plasmid DNA), cell surface receptors can be engaged with specific binding moieties, and soluble molecules have desired effects on specific cells. However, the immune system is composed of a complex network of cells that is interwoven with every other system in the mammalian organism. Targeting therapies to immune cells to direct their gene expression and phenotype is a very difficult task because these mobile cells reside in the tissues, migrate through the lymphatic system and circulate in the bloodstream.

To modulate the immune system individual cells, genes, molecules or signals must first be targeted and then regulated. There have been several approaches to targeting immune cells and these are outlined in the background section, but briefly they include antibody targeting, aptamer targeting, peptide targeting, natural ligand targeting and size exclusion.

There are also many approaches to regulating the immune cells which are outlined in the background section these include siRNA, plasmid DNA, small molecules, antibodies, polysaccharides, natural antigens, adjuvants.

There is no ideal combination of drug and delivery system to modulate the immune system. However, physical microparticles offer some distinct advantages: 1.) passively targeting antigen presenting cells based on size 2.) activation/ tolerization of dendritic cells 3.) delivering sustained payloads and 4.) interacting with phagosomal receptors. Because of these attributes they hold promise to solve some the delivery challenges related to immune modulation.

In the following chapter a brief background is presented, focusing on Immunology and the important cells, and molecules that control immune response. Then, a review of delivery/targeting approaches to particular immune cells follows and finally some approaches to controlling the immune cells are reviewed.

1.1 References

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2. Thesis Background

"The difference between destruction of the immune system and stimulation of the immune system is an interpretation."

-Deepak Chopra

2.1 The immune system

The mammalian immune system is a collection of mechanisms that protect the host organism from both pathogens and from harmful altered self cells (either tumor cells or infected cells) (reviewed in [1]). The cells of the immune system communicate with each other and with other systems to orchestrate or organized response to the pathogen with the best interest of the host in mind. The immune system has a mind of its own. The immune system uses a life over limb philosophy and it often kills self cells which are infected or damaged so that they will not harm the organism. This decision making ability, choosing which individual cells will live or die within the organism, is a large responsibility. Using its best intelligence the immune system collectively decides which cells are foreign or self, dangerous or harmless and then it acts. It also has the profound ability to "remember" which antigens it has considered harmful by instructing memory cells to be ready should the challenge reoccur.

Sometimes the immune system makes a "mistake". Either danger signals are inappropriately associated with a self antigen and an autoimmune disease results, or the immune system is not ready for a pathogen and is over come by the pathogens fast growth. If the pathogen is toxic it can cost the life of the host. Because pathogens can

evade immune surveillance and/or response we have been able to invent a number of immune modulating methods to treat the diseases caused by immune system malfunction.

To better understand which aspects of the immune system are appropriate to target therapeutically we will review the components of the immune system as well as some of the pathology of immune disease.

Immunologists have organized the immune system into organs, cells are molecules. Seems like a logical way to describe the components of the system here.

2.1.1 Organs

Bone Marrow – The bone marrow is the origin of all cells in the immune system. Stem cells resident in the bone marrow differentiate into immune cells precursors in a process called hematopoiesis. These immune cell precursors migrate out of the bone marrow and continue their differentiation in other organs. Most blood cells are produced in the bone marrow, this includes red blood cells and platelets as well as B cells, natural killer cells, granulocytes and immature thymocytes.

Thymus – The thymus is the location of t-cell maturation and selection. Immature t-cells migrate from the bone marrow to the thymus. During their differentiation process t-cells rearrange the genes determining the specificity of their t-cell receptor (TCR) to generate great diversity. In the thymus t-cells are screened by resident dendritic cells and selected for the binding properties of their t-cell receptor. Cells which could be harmful to self are removed and those which are non-specific to the extent they would bind antigen not

displayed in the context of self major histocompatibility complex (MHC) are also removed. This is how the immune system centrally determines the specificity of its response.

Lymph Nodes -- The lymph nodes are the target of immune cells, they collect draining lymph from tissue and therefore are the destination of activated antigen presenting cells (APC) which are normally resident in the tissues. These activated APC present their digested antigen in the context of MHC and come in contact with t-cells. The lymph nodes are an intersection of the draining APCs with t-cells from the blood stream. It is here in the lymph nodes that the immunological synapse forms between antigen presenting cells and t-cell and in fact where the immune response cascade begins.

Spleen -- The spleen is a reservoir for immune cells and a filter for the blood. It contains mostly T cells and B cells, but also macrophages, dendritic cells, natural killer cells and red blood cells. Many of the foreign antigens and cells which drain from the tissue through the lymph system ultimately reach the spleen. Much like the lymph nodes the spleen is the site of interactions between t-cells and antigen presenting cells. It is also the location where B cells become activated and begin producing antibody.

2.1.2 Cells

T-Cells – Immunologists generally divide T-cells into two types that are functionally different, t helper cells and cytotoxic t cells. T-helper cells are mainly decision-making cells which are selected in the thymus for appropriate specificity. These cells interact with antigen presenting cells in the lymph nodes and spleen and become activate if they receive the appropriate signals. The must receive an antigen specific signal through the interaction of MHC/peptide with their T-cell receptor (TCR) plus a combination of secondary signals that indicate danger. It is up to the antigen presenting cells to deliver these signals. When t-helper cells become activated they release cytokines which induce activation of other cells as well as local inflammation.

After selection in the thymus naïve t-cells migrate to the circulation where they pass though lymph nodes and spleen. Depending on which antigen specific and secondary signals they encounter they can differentiate to one of four lineages: Th1, Th2, Th17 or Treg. Th1 differentiation is typically associated with bacterial and viral infection and this type of immune response often results in an inflammatory response. Th1 differentiation is induced by expression of IL-12 by DC and INF-g by NK cells and t-bet is the major transcription factor leading to Th1 differentiation[2]. Th2 differentiation is often associated with parasitic infections such as helminthes and is induced by IL-4 through the key transcription factor GATA-3. Historically it was thought that a balance between Th1 and Th2 defined an appropriate immune response and that when the balance was inappropriately skewed in one direction of the other a disease state arose. It was believed that a lack of Th1 signaling could lead to overactive Th2 differentiation which or vice

versa. Fairly recently two new t-cell lineages were discovered, Treg and Th17. TGF-b starts the differentiation toward both Treg and Th17 cells, however the presence of IL-6, IL-23 and IL-21 change the direct the differentiation to Th17[3-5] which required the nuclear receptor RORgt[6] while IL-10 is related to the Foxp3 induced differentiation to the Treg[7]. All of these mechanisms are still under debate [8], but clearly Th17 and Treg differentiation have been brought into the spotlight recently and this is an example where the system itself may be teetering misadjusted and a small therapeutic influence could make the difference between a healthy and a disease state. In particular over-expression of Th17 signals seems to lead to a number of diseases[2].

Cytotoxic t-cells are activated by a similar mechanism to t-helper cells, but these cells migrate into tissue and circulation and find and induce cell death among altered self cells such as virally infected cells and tumor cells.

Natural Killer Cells -- Natural killer cells (NK) cells also function as effector cells that directly kill certain tumors and viral-infected cells. Unlike cytotoxic t-cells NK cells kill their targets without an antigen specific activation signal from antigen presenting cells in the lymphoid organs. However, NK become activated in the presence of the appropriate cytokines expressed by t helper cells, and once activated these NK cells can kill many dangerous invaders, especially those that lack MHC on their cell surface.

B Cells – Production of antibodies is the major function of B lymphocytes. Antibodies recognize and bind to one particular antigen, which can be any biological molecule.

Antibodies bind to antigens as a means to engulf, kill or remove that substance from the body. The non-binding end of the antibody is known as the Fc region and it is responsible for activating downstream immune effects at the site of antibody binding. This immune response can be induced though the complement system or through the activation of phagocytosis.

Granulocytes or Polymorphonuclear (PMN) Leukocytes -- PMNs include neutrophils, eosinophils and basophils these cells are part of the innate immune system and they recognize bacteria through surface molecules that bind polysaccharides, lipids, DNA, RNA or other molecules specific to bacteria or parasites. In some cases these cells will phagocytose bacteria and in other cases they will express reactive oxygen species which are toxic to bacteria as a method of eliminating them.

Macrophages – Macrophages survey the tissues and phagocytose bacteria, debris, dead cells and opsonized foreign material. Inside their phagosome macrophage degrade their payload by acidifying the phagosome and using proteolytic enzymes, RNAase and DNAase. Macrophage present their processed antigen to t-cells in the lymph nodes and spleen.

Dendritic Cells – Dendritic cells are professional antigen presenting cells. Their main purpose is the identify specific antigen and to determine how dangerous the antigen is based on local danger signals at the site. Dendritic cells are the main cells signaling to naïve t-cells which induce their differentiation to Th1, Th2, Th17 or Treg. Dendritic cells

are the target of all vaccination and therefore the target of modulation related to generating a more specific vaccine.

2.1.3 Diseases and Modulation

There are a number of diseases that are related to malfunction of the immune system. Although there are too many immune diseases and disorders to completely review, types of disorders and diseases we would potentially modulate are reviewed.

Since the thesis is focused on microparticle-based delivery, we will focus on the cells, which can be targeted and treated with these approaches. Perhaps the most interesting cells which can be targeted by microparticles are dendritic cells, these cells are on center stage of the immunological synapse. They are the gatekeepers of the adaptive immune system. They take up antigen and as described earlier they present antigen to t-cells along with a number of other signals which induce these cells to differentiate to either Th1,2,17 or Treg. There are a number of diseases were misregulation of these signals leads to disease. In addition, dendritic cells are the first point of processing for antigen or vaccine therefore the best opportunity for modulation of the vaccine induced immune response.

An additional cell type, which is capable of phagocytosis of microparticles, is neutrophil. Neutrophils are the first line of defense against bacteria, yeast and fungal infection. Although antigens phagocytosed by neutrophils are not presented to the adaptive immune system neutrophil are capable of initiating their own local immune response through the

release of reactive oxygen species. A common disorder involving neutrophils is neutropenia. In neutropenia there is a large and significant deficiency in the number and the activity of neutrophils, this is a common side effect of chemotherapy[9] and is also very common in diabetics, and often leads to infectious ulcers.

2.1.3.1 Autoimmune disease (unwanted immune response)

Unwanted immune response occurs in allergy, asthma, autoimmune disease and transplant rejection. Allergy is perhaps the most common unwanted immune response, with over 50 million sufferers in the US [10] the US market for allergy is predicted to exceed $10BN by 2010[10]. Autoimmune disease is broadly defined to include he following diseases: Rheumatoid Arthritis; Psoriasis; Multiple Sclerosis; Psoriatic Arthritis; Irritable Bowel Disease: Crohn's Disease; Ulcerative Colitis; Uveitis; Immune Thrombocytopenic Purpura; Myasthenia Gravis; Scleroderma; Sjoren's Syndrome; Systemic lupus Erythematous; Fibromyalgia. Together over 300 million people are treated globally for these diseases. The biologics market alone is predicted to top $17 BN for their treatment by 2010 [11].

Unwanted t-cell response is common to all these diseases , Th2 in the case of allergy[12] and Th1 or Th17 in the case of autoimmunity [13]. The classic model of t-cell selection (described earlier) describes how and why 90% of t-cells are deleted before reaching the periphery [1], but what happens when t-cells that are specific for self escape thymic deletion? Because, this happens often we are fortunate to have a mechanism that protects us against autoimmunity, peripheral tolerance[14-21]. In peripheral tolerance antigen

presenting cells take up self antigen process it and present it to t-cells, but since there is a lack of second signals that communicate danger, the t-cells do not become activated, instead they are induced into a state of tolerance. The mechanisms behind peripheral tolerance are very complicated, but involve Treg cells [22], cytokines[23], cell surface molecules [24], and neglect [25].

Sometimes one or more of the mechanisms of peripheral tolerance breaks down and autoimmune disease results [26]. In this situation the host harbors activated t-cells (or b-cells) that are specific to self. There have been a number of research studies artificially inducing peripheral tolerance in disease models. A number of studies have attempted to induce peripheral tolerance using monoclonal antibodies [27] or by dosing apoptotic cells [28] to dendritic cells. We believe that a microparticle system capable of targeting and modulating dendritic cells could be used to induce antigen specific peripheral tolerance by co-delivering an antigen. A number of microparticle systems have been developed which are capable of targeting dendritic cells for vaccination. We review these systems later in this chapter.

2.1.3.2 Immune deficiency

The number of individuals with compromised immune systems due to HIV/AIDS, immune suppression for organ transplantation and chemotherapy has dramatically increased in the past few decades[29]. Because the number of compromised individuals has grown a number of opportunistic infections have exploded in their incidence and prevalence [30]. One of the most profound increases has occurred in the area of fungal infections[31] and one of the most important immune challenges related to fungal

infection is neutropenia [32]. Because there are an ever increasing number of patients with neutropenia this is a very important area of study.

2.2 Modulation of the immune system and applications

There are countless mechanisms included in the human immune system. The areas where immune modulation may be the most important, particularly with particle-based approaches, are vaccination, autoimmune disease and immune deficiency. Each of these areas can leverage the strength of particle-based approaches with their targeting ability, the ability to interact with and activated cells and their ability to deliver payload in a controlled way. Here we review the areas in which particle based approaches to immune modulation have the most promise.

2.2.1 Better vaccination

When a vaccine activates the immune system there are numerous types of immune responses that can result. The antigen presenting cells which process the antigen can process it through the MHC class-1 or MHC class-2 system. The MHC-1 pathway is present in all mammalian cells. The MHC-1 molecule, once produced migrates to the endoplasmic reticulum where it ultimately associates with an assortment of peptides from proteins produced by the cell. Then, protected by a lipid bi-layer it migrates to the cells' surface were it presents the bound peptide antigen to any local cytotoxic t-cells.

Dendritic cells have a separate pathway, MHC-2. In the MHC-2 pathway protein antigens are phagocytosed by dendritic cells and processed. The resulting peptide antigens are further processed as the phagosome binds with lysosomes and becomes a "late" phagosome. Late phagosomes fuse with vacuoles containing MHC-2 molecules. These MHC-2 molecules are also present in the endoplasmic reticulum but cannot bind self-peptides since their binding site are occupied the invariant chain. This protein prevents the self-peptides produced by the dendritic cell itself from being presented as foreign antigen. After the phagocytosed antigen is processed and binds to MHC-2 in the late phagosome it then migrates to the cell surface where it can activate t-cells which the antigen presenting cell encounters in its migration to the lymph nodes and spleen.

Dendritic cells also use the MHC-1 pathway to present internally produced antigens to t-cells (for example, DC can be virally infected just as any other cell). When dendritic cells present antigen through the MHC-1 pathway they are much more likely to induce a cytotoxic response, by signally that they may be infected by a foreign antigen and also expressing danger signals.

Much research on genetic vaccines has focused on this concept of using DNA to transfect DC which will in turn translate antigen and therefore present it in the context of MHC-1, hopefully leading to a cytotoxic anti-tumor/anti-viral response[33]. In addition, dendritic cells have the ability to cross-present antigen a process where DC phagocytoses antigen, yet still presents it in the context of MHC-1[34-38]. It has been suggested that dendritic cells cross-present antigen via MHC-1 when they internalize it from self cells in the

presence of significant danger signals [39]. Therefore several reports have attempted to recreate this condition by delivering antigen in particles that mimic dead self cells[40-45]. In addition to using materials found in dead or dying cells such as their component lipids, particle based approaches could also be used to directly control gene expression that is involved in cross-presentation.

As previously mentioned an additional characteristic of any t-cell response is the t-cell's association toward a specific subset, Th1, Th2, Th17 or Treg. In particular for vaccination the goal is to generate Th1 or Th2 type responses. The th1/th2 polarization is particularly important in HIV infection [46]. Several attempts have been made to generate adjuvant that can direct the immune response in a particular direction Th1 [47-49] Th2[13, 50, 51] or both [52]. Recently it has been discovered that Th17 also plays a role in development of vaccine response [53]. Clearly adjutants that can tightly control the type of immune response which results from the vaccination are desirable.

2.2.2 Autoimmune diseases (and transplant tolerance)

There have been several attempts to treat autoimmune disease by suppressing the immune system [54] however it is challenging to use broad spectrum immune suppressing regiments because it increases the susceptibility to infection and disease. The ideal treatment for autoimmune disease will induce antigen specific tolerance while leaving the rest of the immune system intact to fight infection and disease. This has been attempted

in several cases in particular oral delivery[55-64] and aerosol delivery [65] of antigen has been studied as a possible mechanism for inducing tolerance.

A robust way to modulate antigen presenting cell signaling, in the context of antigen specific vaccination, could be very powerful in the treatment of autoimmune disease, allergy and for the rejection or organ transplants.

2.2.3 Immune deficiencies

The number of individuals with immune deficiencies has exploded in the recent past. Because so many individuals are now living with HIV/AIDS, organ transplantation, diabetes and other diseases immune deficiencies is a rapidly growing problem. A platform that would allow existing cells to recruit and trigger the proliferation of additional immune cells, especially in the case of neutropeania would be phenomenal.

2.3 Particles as a broad platform

There has been a large amount of research in the area of microparticle delivery [66-69]. Several methods exist for the production of microparticles to encapsulate drugs[70]. These include: emulsion/solvent evaporation [33, 40, 71-80], spray drying [73, 80-84], precipitation [85, 86] and a number of materials have been used in the formulation of these particles. Common materials used in the production of microparticles for delivery include PLGA[70, 87, 88], chitosan [89], Poly(ortho-esters) [76, 90] and blends of these polymers with functional polymers including PEI [91-94], Poly(beta-amino esters) [33, 77, 78]

Microparticles are useful as discussed earlier for a number of interesting applications in immunology and immune modulation. Because microparticles are large (typically in the range of 1-100um) they are in the same size range as cells, bacteria, yeast or fungi. As a platform this allows microparticles to interact with the immune system is a disguised intruder[95] and in fact are often used as adjuvant for traditional vaccines [96]. Since the goal of many immune modulation approaches is to induce an immune response that would be similar to the response to an actual infection microparticles have an intrinsic physical advantage and many reports have attempted to use microparticles to simulate bacterial or other pathogens [97, 98].

2.3.1 Passive Targeting of antigen presenting cells

Because of their size microparticles generally can not be taken up by non-phagocytic cells [33, 77, 99, 100]. This gives microparticles the ability to be targeted to the lungs [101] and the antigen presenting cells [33, 77, 99, 100]. The ability of microparticles to target antigen presenting cells is important because it allows the delivered payload to be selectively process and then trafficked to the lymph node and spleen where antigen presenting cells present it to active t-cells[102]. This approach has been successful in eliciting and antibody response [103, 104] and a cytotoxic t-cell response [105-108] in many disease models and in human trials.

2.3.2 Engaging surface receptors

Microparticles have an exquisite ability to deliver cell signals to cells by engaging cell surface receptors [109-111] [112]. The ability to bind with receptors both on the cells' surface and inside phagosomes allows microparticles to be designed to interact with the antigen presenting cells they are targeting in many diverse ways.

2.3.3 Controlled release

Another advantage of microparticle delivery is the ability to encapsulated a large payload and release this payload over an extended period, and/or release the payload in a triggered way. Several triggers can be used but pH[40, 83, 113] enzymatic degradation [114, 115] temperature [116], and redox potential [117] are commonly used.

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3. Microparticle encapsulation and delivery of siRNA

3.1 Introduction

The concept of DNA delivery or human gene therapy first arose in the 1960s[1, 2]. Since then many successful experimental results have validated the concept of genetic engineering and many studies have demonstrated successful transfection in both bacterial and mammalian cells [2]. Hundreds of clinical trials for gene therapy have been initiated [3]. However, as of Q1 2008 no gene therapy has been approved by the FDA in the United States. Recently one of the most intensely studied areas of gene therapy is DNA vaccines. Gene therapy is an especially attractive method of vaccinating dendritic cells to induce CD8+ cytotoxic immune responses because DCs producing antigen endogenously activate T-cells through the MHC-I pathway as compared to peptide vaccines which normally activate the MHC-II pathway[4].

Much work in the vaccine world has focused on using microparticles for the delivery of antigen, either DNA, or protein [5-9]. Microparticles are an attractive delivery vector because they can passively target antigen presenting cells (APC) based on size exclusion (non-phagocytic cells can not take up particles in the micron range)[10]. Additionally, microparticles have been used historically as an adjuvant since they have been shown to activate dendritic cells which helps induce a vaccine response[11].

Microparticles formulated from biodegradable polymers are capable of phagosomal escape and can be efficient transfection reagents. Little et al. have developed a

microparticle based DNA vaccine delivery system [12] which was shown to induce tumor regression in a mouse model. Because this system efficiently targets and delivers DNA to APC in vivo it was a logical starting point for siRNA targeting and delivery. To modulate antigen-specific immune response with gene knockdown several genes could be targeted. For example, knockdown could shift the balance of the immune response to a particular T-cell subset (Th1, Th2, Th17, Treg) or could extend the life of DC by knocking down Bak/Bax. Several examples are outlined in Table 3.1

Table 3.1 Example Gene targets for DC modulation

Gene	Agent	Action	Ref	
Nf-kb			[13]	
Bak/Bax	siRNA	Extend activation kinetics	[14-17]	
Th1/2/17/reg polarizing cytokines	siRNA	Direct immune response/treat autoimmunity	[18, 19]	
Costimulatory molecules	siRNA	Direct immune response/tolerize	[20-22]	

Because small inhibiting RNA has such broad potential for modulating any gene in dendritic cells, and because the poly-(beta amino-ester) microparticle system delivers nucleic acids to dendritic cells, a logical next step is to apply poly(β-amino-ester) to siRNA delivery. The ability to deliver siRNA together with other payloads to antigen presenting cells would be a potentially attractive approach to modulating the immune system.

3.2 Materials and methods

3.2.1 Materials

Poly(*d,l*-lactic-co-glycolic acid) polymer (PLGA, RG502H Resomer 50 : 50) was purchased from Boehringer Ingelheim (Ingelheim, Germany). Poly(vinyl alcohol) (Mw = 25 kD) was purchased from Polysciences Inc. (Warrington, PA).

3.2.2 Cell lines and siRNA

HeLa cells stably expressing green fluorescent protein were a kind gift of Carl Novina. These cells were prepared by transfecting HeLa cells with the vector pd1EGFP-N1 (Clontech) using Lipofectamine followed by selecting with G418 media and then single cell cloned [23]. P388-D1 cells were obtained from America Type Culture Collection ATCC and were transfected with vector pd1EGFP-N1 (Clontech) by electoporation, cultured in G418 media and then single cell cloned by flow cytometry. siRNA duplex specific for GFP was obtained from Dharmacon. siRNA sequence 5'- GGC TAC GTC CAG GAG CGC ACC -3' was used for all knockdown experiments [24]. Cholesterol modified siRNA was a kind gift from Alnylam (Cambridge, MA).

3.2.3 Flow cytometry

Cells were analyzed for GFP protein expression by flow cytometry fluorescent analysis. Cells were isolated from culture flasks by trypsinization (HeLa) or cell scraping (P388-D1). Cells were then stained with propidium iodine to stain dead cells. Cells with

propidium iodine in FACS buffer (2% FBS in PBS) were then analyzed using a Becton Dickenson FACScan Flow Cytometer. Cells were gaited for live cells and then histograms of FL1 (GPF) channel were reported.

3.2.4 Microparticle formulation

PLGA microparticles were prepared by modified double emulsion [25]. siRNA was suspended in a solution of (100 μL) of EDTA (1 mM) and D(+)-Lactose (300 mM). The resulting solution was emulsified with solution of PLGA and PBAE in in CH_2Cl_2 using a probe sonicator. This emulsion was added to a homogenized solution of poly(vinyl alcohol) (50 ml, 5% PVA (w/w), 5000 rpm) and NaCl (at varying concentrations of 0, 0.2, or 0.5 M). After 30 s, 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 h at room temperature and then 1 h at 4 °C. Microspheres were washed and centrifuged 3X to remove PVA prior to lyophilization. In some cases $Mg(OH)_2$ was added to microparticles to buffer the effects of acidic degradation products of PLGA. $Mg(OH)_2$ was encapsulated as a solid as $Mg(OH)_2$ has limited solubility in aqueous solution.

3.2.5 Encapsulation Studies

To examine the encapsulation efficiency particles (5mg) were first dissolved in CH_2Cl_2. siRNA was extracted by aqueous extraction into a solution of 1xTAE buffer for 2 hours at room temperature. Concentrations of siRNA in this aqueous solution were measured using RiboGreen (Invitrogen) and final encapsulations were calculated from this

information. Other samples of extracted siRNA were run on electrophoresis gels to determine the level in intact siRNA remaining.

3.2.6 Water Uptake and internal pH measurements

Internal pH was measured as previously described[26]. Briefly, microparticles were weighed in pre-weighed microcentrifuge tubes. Particles were incubated with 1 ml of 50 μM HEPES (pH = 7.4) for 24. Tubes were centrifuged, the supernatants were discarded, and the total weight of the microparticles and aqueous microenvironment was determined. Particles were then dissolved in acetonitrile (ACN) with 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.

3.2.7 Electron microscopy

To determine the surface morphology of the microparticles samples were dried on glass cover slips coated with gold and imaged using a Hitachi S-4800 FESEM.

3.3 Results

3.3.1 Cell based assays for siRNA knockdown

To generate data and screen potential particle delivery systems for RNA we wished to attain an immortal cell line that could give quick results and a comparison between several delivery technologies. At this stage we were interested in understanding and optimizing the delivery system as opposed to choosing a clinically relevant system. We chose Green Fluorescent Protein (GFP) from jelly-fish because it was easy to measure using flow cytometry and should not interact with endogenous gene expression.

To test delivery through both endocytosis and phagocytosis we used cell-based systems with positive controls for both professional antigen presenting cells and non- phagocytic cells. Because they were readily available and easy to work with we chose HeLa for the non-phagocytic cells and P388-D1 murine macrophage for the phagocytic lineage.

3.3.1.1 HeLa

Stable GFP+ HeLa cells were maintained in culture as described in the materials and methods section. GFP expression was measured by flow cytometry; cells were gated for live cells with propidium iodide. GFP intensity for non-treated and siRNA knock-down is shown in Figure 3.1. siRNA is delivered with Oligofectamine and described in the materials and methods section.

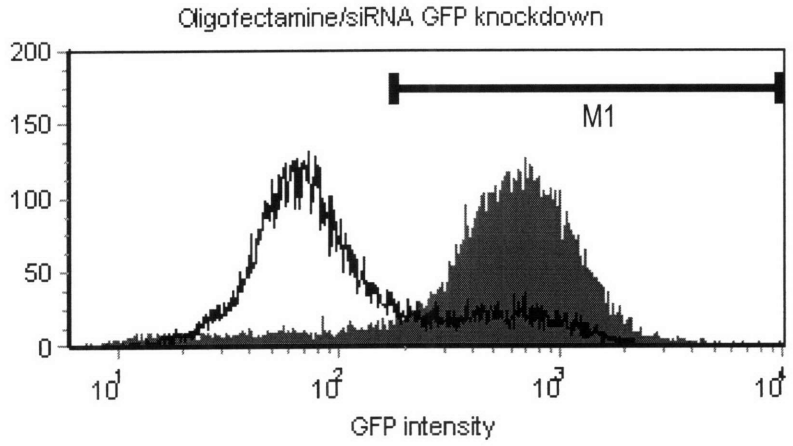

Figure 3.1 GPF HeLa cell line for knockdown assay

GFP+ HeLa cells express high levels of GFP as measured by flow cytometry (shaded histogram), when GFP siRNA is delivered with Oligofectamine knockdown (one order of magnitude) is observed in intensity of GFP fluorescence.

3.3.1.2 Macrophage cell line P388-D1

To determine if phagocytic delivery is efficient in a professional antigen presenting cell line we developed a stable clone of the immortal mouse macrophage cell line P388-D1. These cells behaved normally and grow similar to the non-GFP expressing cells, and expressed GFP at a level approximately one order of magnitude greater than the auto-fluorescence of the WT P388-D1 cells as shown in Figure 3.2.

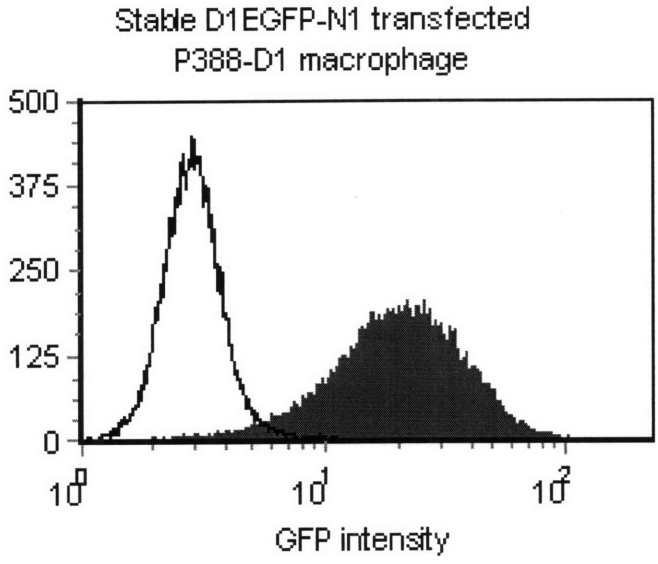

Figure 3.2 Stable GFP expressing P388D1 cell line

P388-D1 cells expressing GFP (solid purple histogram) and wild-type P388-D1 cells

3.3.2 Microparticle Encapsulation of siRNA

To determine the ability of PLGA/PBAE blend microparticles to deliver siRNA we developed an encapsulation technique minimizing conditions that could be harsh to RNA. We insured all surfaces and tools were RNAase free and minimized shear by using lower RPM in homogenization. Microparticles were fabricated as described in [12] with either 15% or 25% poly(beta amino-ester) (PBAE) and 85% or 15% PLGA; the PBAE structure is shown in Figure 3.3. These particular PBAE/PLGA blends were selected because they were previously shown to transfect P388-D1 with DNA for genetic vaccination [12] and because they were one of few PBAEs with physical properties

appropriate for making solid microparticles[27]. Osmotic pressure was balanced between the charged siRNA in the inner aqueous phase and the outer aqueous phase (which was adjusted to the appropriate ionic strength with NaCl) since PBAE acts as a membrane.

The formulation protocol described in the materials and methods section was designed to give the optimal conditions for preserving siRNA after encapsulation. To test this method and determine the biological activity of the siRNA after encapsulation we encapsulated siRNA, then dissolved the particles in organic solvent and extracted the siRNA into aqueous buffer. The extracted siRNA was delivered to the HeLa cell with the commercially available transfection reagent Oligofectamine. The results of these experiments are shown in Figure 3.4. Notice that the siRNA extracted from the newly developed microparticles maintains its activity as indicated by GFP knockdown equivalent to the non-encapsulated samples.

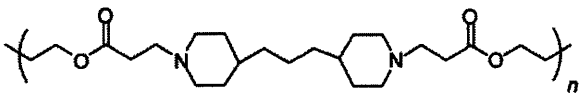

Figure 3.3 Poly Beta Amino Ester (Poly-c)

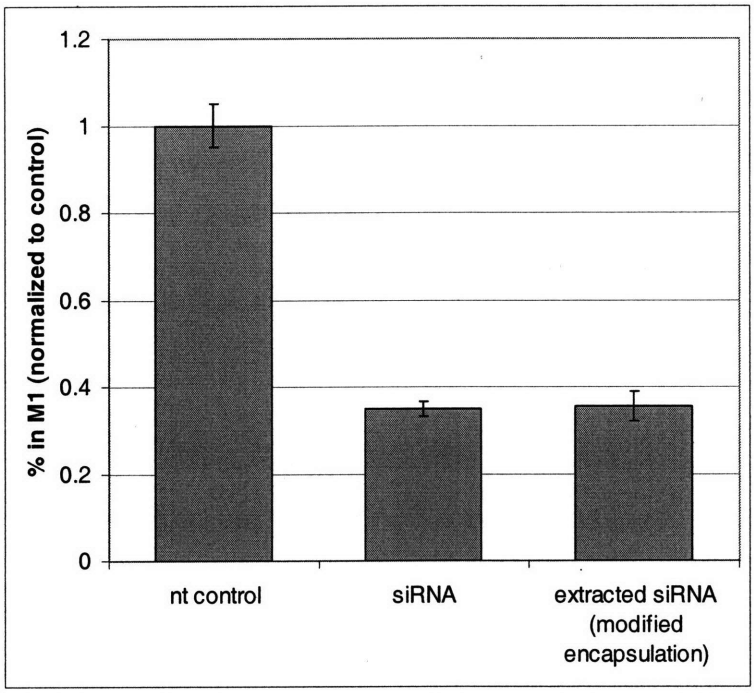

Figure 3.4 Bioactivity of encapsulated siRNA

GFP-siRNA was encapsulated in PLGA microparticles, and then extracted using solvent extraction. Extracted siRNA maintained its biological activity as compared to non-encapsulated siRNA. Results are reported as % in M1 gate. M1 gate is defined in Figure 3.1.

After validating the biological activity of the encapsulated/extracted siRNA we next tested the nano-particle's delivery of GFP-siRNA to HeLa cells and measured knock-down in GFP expression. The 15% and 25% PBAE particles were incubated with GFP+ HeLa cells. FACS analysis demonstrated GFP expression profiles as shown in Figure 3.5. Nanoparticles induced no measurable knockdown of GFP; in the same experiment knockdown was seen when siRNA was delivered with the commercially available transfection reagent Oligofectamine, see Figure 3.1.

We also delivered microparticles to phagocytic P388-D1 cells. This approach showed efficient DNA transfection previously [12], and therefore was a logical step for siRNA. Results in Figure 3.6 show no measurable knockdown with this system

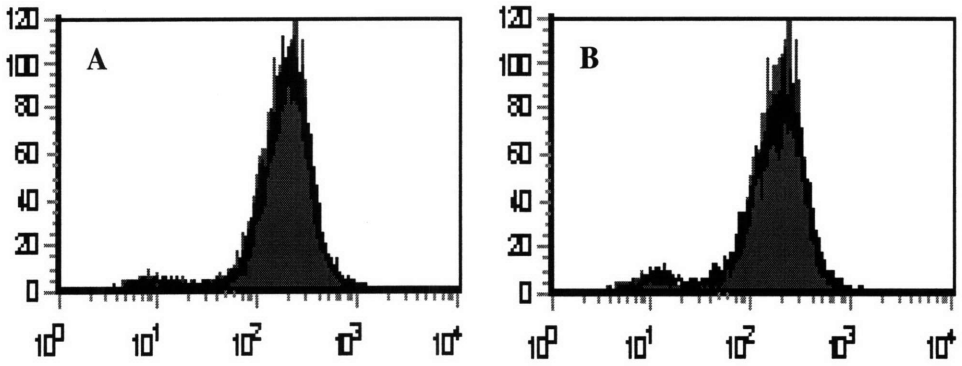

Figure 3.5 GFP knockdown with PBAE/PLGA nanoparticles in HeLa

Microparticles containing 15% (A) or 25% (B) PBAE are added to GFP-HeLa cells at 100ug/ml for 24 hours and then GFP fluorescence is analyzed by flow cytometry. Shaded purple histogram indicates non-treated samples, open black histogram indicates microparticles were added.

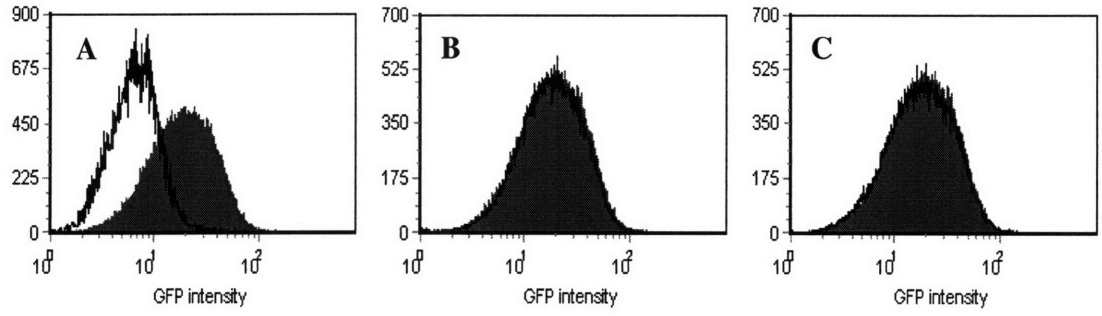

Figure 3.6 GFP knockdown with PBAE/PLGA microparticles in P388-D1

Stable GFP-P388-D1 cell line untreated (solid purple) and treated with GFP-siRNA delivered with oligofectamine black line (A). Microparticles containing 15% (B) or 25%

(C) PBAE are added to GFP-HeLa cells at 100ug/ml for 24 hours and then GFP fluorescence is analyzed by flow cytometry.

3.3.3 Modified Microparticle Systems for siRNA encapsulation

Because the PBAE/PLGA particle systems were efficient for DNA, but not for siRNA we hypothesized that there were additional challenges to siRNA delivery. We believed these additional challenges include:

1.) Acidic conditions inside degrading microparticles could degrade siRNA
2.) Encapsulation efficiency was lower with siRNA
3.) Endonucleases that attack RNA specifically in phagosomes

We tested these hypotheses experimentally. First, we examined the acidic nature of the degrading microparticle. As PLGA/PBAE microparticles are hydrated they quickly swell with water. This process is driven by the osmotic gradient generated by the colligative properties of species inside the particles. As water diffuses into the particles there are several steps in the degradation. If the degradation occurs in neutral media the process of PLGA degradation occurs through hydrolysis of the polymer backbone[28]. As hydrolysis occurs lactic acid and glycolic acid groups are released contributing to a more acidic environment. Because of acidic degradation products it has been shown that the internal pH inside degrading PLGA microparticles can be low [29]. The blending of PBAE into PLGA microparticles helps to buffer the acidic environment and in the case of

DNA delivery improves the stability of the payload after being expose to the degradation products[12].

To abrogate the effects of acidic degradation products we added magnesium hydroxide to the particles as a buffer. This approach has been used in the literature as a method for studying and improving the utility of PLGA particles for protein and DNA delivery [30-32]. Magnesium hydroxide is sparingly soluble in water but highly soluble in acid, so it is retained as a solid in the particles until pH effects cause its dissolution. To understand the microenvironment pH we measured the water uptake and pH as described in the materials and methods section. The data suggest, as expected, that the addition of $Mg(OH)_2$ leads to increased water uptake and a buffering effect as the microparticles degrade, see Figure 3.7 and Figure 3.8.

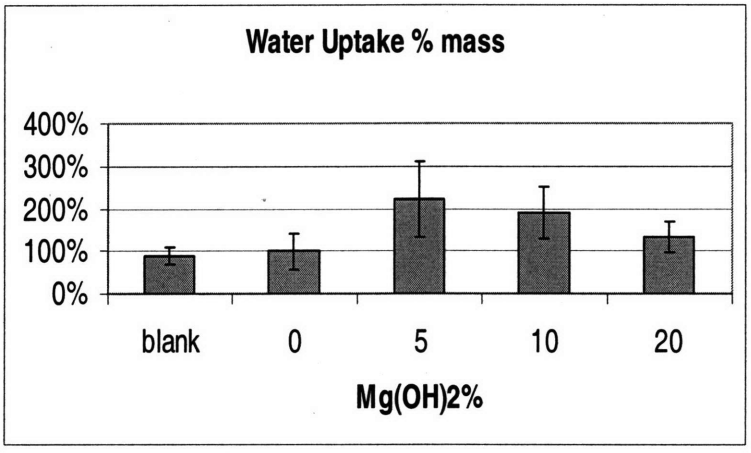

Figure 3.7 Water uptake in microparticles

Uptake of water by the microparticle microenvironment after 24 hours as measured in the materials and methods section. 0% $Mg(OH)_2$ refers to conditions where aqueous

solutions used in the encapsulations were saturated with $Mg(OH)_2$ but no solid was encapsulated.

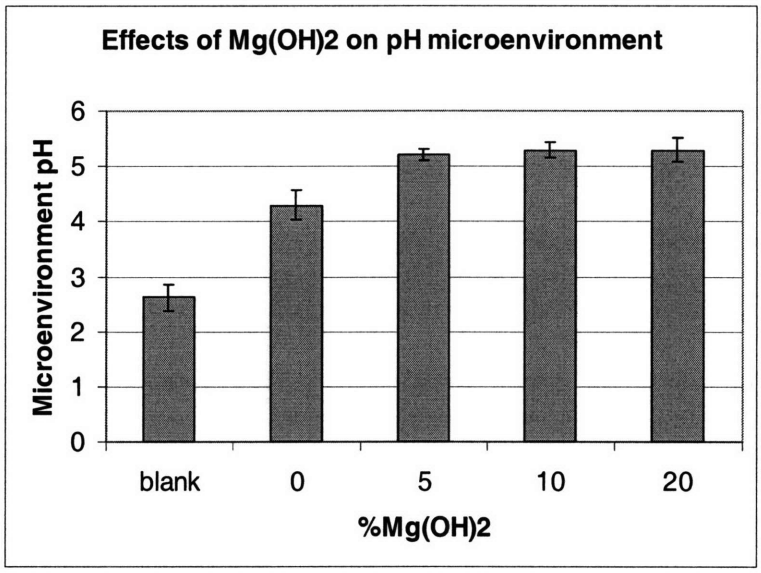

Figure 3.8 Internal pH measurements in microparticles

Microenvironment pH measured as described in the materials and methods section. 0% $Mg(OH)_2$ refers to conditions where aqueous solutions used in the encapsulations were saturated with $Mg(OH)_2$ but no solid was encapsulated.

The addition of magnesium hydroxide has a buffering effect on microenvironment pH. However, it is not completely clear how these measured pH values will affect the activity of the encapsulated siRNA. siRNA normally begins to degrade in serum within a mater of hours [33]. To examine the stability of siRNA in the particle microenvironment we incubated siRNA for various times in solutions with pH adjusted to several relevant levels. As shown in Figure 3.9 at pH of 4 or less biological activity is significantly impacted after 72 hours when measured in the cell based GFP-HeLa system.

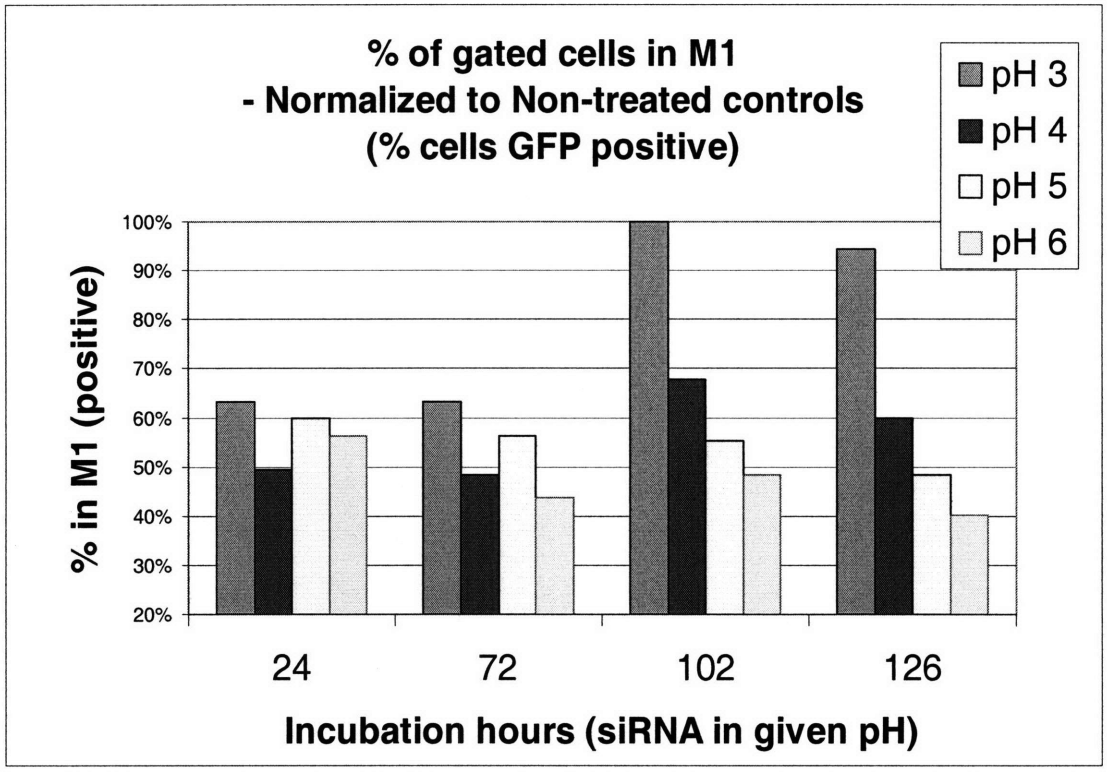

Figure 3.9 Effects of pH in siRNA activity

siRNA was incubated for various times in pH adjusted solutions. Results are reported as % in M1 gate. For reference M1 is defined in Figure 3.1.

To examine the effects of $Mg(OH)_2$ buffering on the activity of encapsulated siRNA we repeated the experiments in Figure 3.4 with an additional 24 hour incubation in aqueous media, and addition of 6% $Mg(OH)_2$. Results in Figure 3.10 indicate that the addition of 6% $Mg(OH)_2$ (by mass) generated an improvement in the activity of siRNA when encapsulated and extracted from PLGA microparticles. We used 6% $Mg(OH)_2$ as the

nominal standard because adding more did not seem to generate additional advantages in buffering the pH, but did cause additional water uptake.

These results suggest that improved acidic conditions inside the microenvironment might lead to a more effective delivery system. When tested in the cell based system the addition of $Mg(OH)_2$ did not improve the measured knockdown.

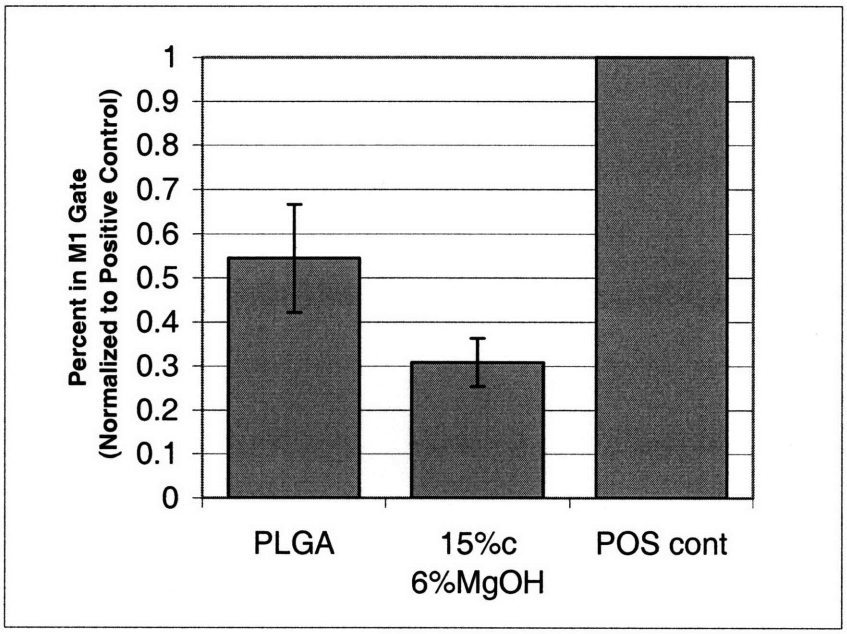

Figure 3.10 siRNA activity after encapsulation and aqueous incubation

GFP-siRNA was encapsulated in PLGA microparticles, with or without $Mg(OH)_2$ and then extracted using solvent extraction. Results are reported as % in M1 gate. M1 gate is defined in Figure 3.1.

Buffering of the microparticle microenvironment may lead to increased activity of the siRNA, but to better understand the mechanism of this improvement we examined the morphology of the $Mg(OH)_2$ buffered particles by scanning electron microscopy. As show in Figure 3.11 the morphology of the magnesium hydroxide buffered particles lacked the large craters seen in the non-buffered particles. This crater effect leads to higher particle porosity and it is likely that decreased encapsulation contributes to decreased activity of extracted siRNA. Perhaps with the porous morphology the siRNA in the non-buffered particles is able to escape during washing steps or during the initial phases of rehydration in buffer during the activity experiments.

When comparing the buffered particles with the non-buffered there is a striking similarity between the cratered appearance of the non-buffered particles and published data demonstrating mismatched osmotic gradients between internal and external aqueous phases[12].

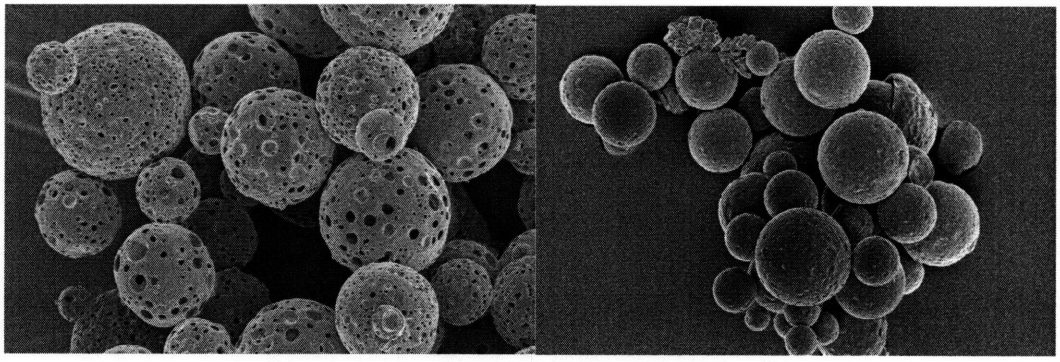

Figure 3.11 Effects of Mg(OH)2 on particle morphology

PLGA microparticles were fabricated without (a.) or with (b.) 6% magnesium hydroxide as a buffer to acidic microenvrionmental conditions.

We hypothesized that the non-buffered particles would not encapsulate siRNA as effectively because of their cratered morphology. To test this hypothesis we extracted siRNA and analyzed the concentration based on gel electrophoresis. Data in Figure 3.12 shows the amount of siRNA extracted from the 15% poly-c containing particles. Encapsulations of 15-60 pmol (~450ng) in 10mg of polymer microspheres are shown. During the formulation 0.3mg of siRNA was added to 200mg polymer giving 3% encapsulation efficiency (assume 30pmol). This result (3% encapsulation efficiency) is lower than the attainable encapsulation efficiency with DNA in the same system.

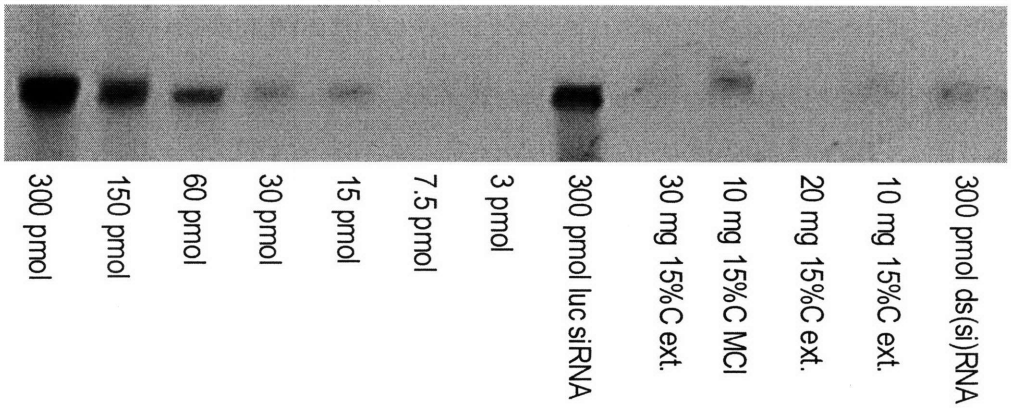

Figure 3.12 Encapsulation efficiency siRNA in PLGA microparticles

siRNA was encapsulated in the 15% poly-c/85%PLGA microparticles and extracted to be analyzed by gel electrophoresis.

The required quantity of siRNA needed for knockdown in our experimental system was determined by using the commercially optimized delivery vector Oligofectamine as an upper bound. We titrated the amount of siRNA added to each sample to determine how

little siRNA could be used to knock down GFP in the GFP-HeLa system. Results indicate that 45 pmol of siRNA is required for knockdown in a single well of a six-well plate. We can assume a maximum feasible microparticle concentration of 1mg/well and depending on functionality approximately 30% release is likely in day 1. Therefore, if we assume "transfectability" similar to Oligofectamine, encapsulation must increase by an order of magnitude from 3% to approximately 30%.

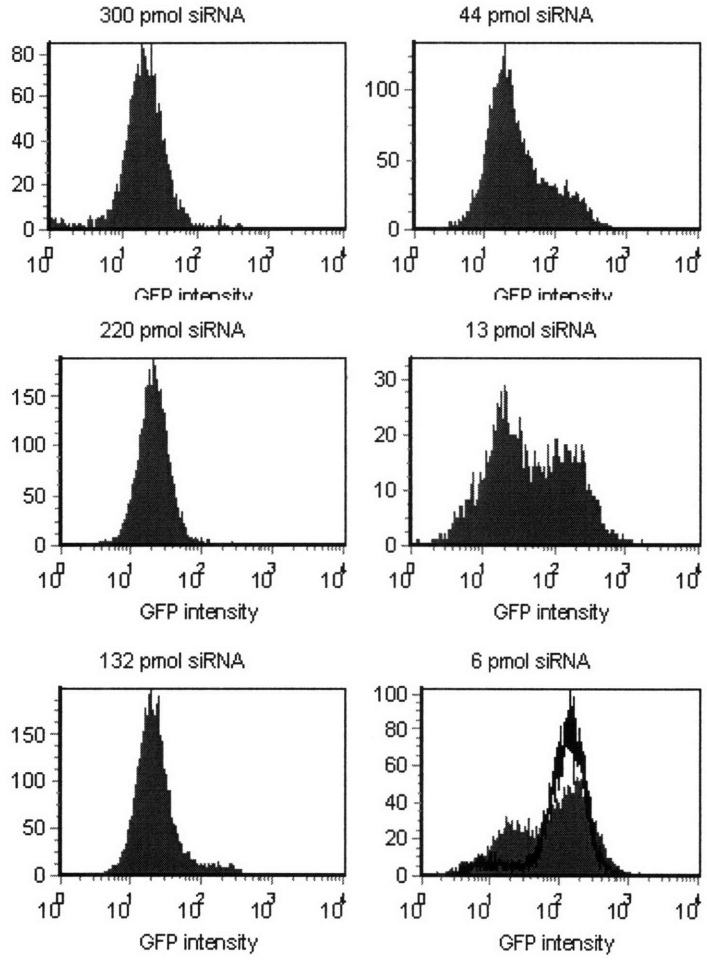

Figure 3.13 siRNA-Oligofectamine titration in HeLa

Various amounts of siRNA were used to determine the lower bound of concentration which would be effective in the GFP-HeLa model system.

Encapsulation efficiency needed to increase. Several literature sources have contributed techniques of generally increasing encapsulation efficiency by increasing polymer molecular weight [34] cryo-preparation [35] or additives [36]. None of these approaches demonstrated increases in encapsulation to the levels we needed.

Modification of siRNA with cholesterol improves circulating half life and transport across the plasma membrane [37]. Cholesterol, being a large lipophilic molecule increases lipophilicity of the siRNA and addition of cholesterol could potentially increase encapsulation, decrease endonuclease degradation by steric hindrance, and increase transport across membranes. We therefore obtained cholesterol modified siRNA from Alnylam [37] and tested the encapsulation efficiency using the standard extraction method and the RiboGreen method of quantification. As shown in Figure 3.14 we were able to improve the encapsulation efficiency from 3% to 20% with this method.

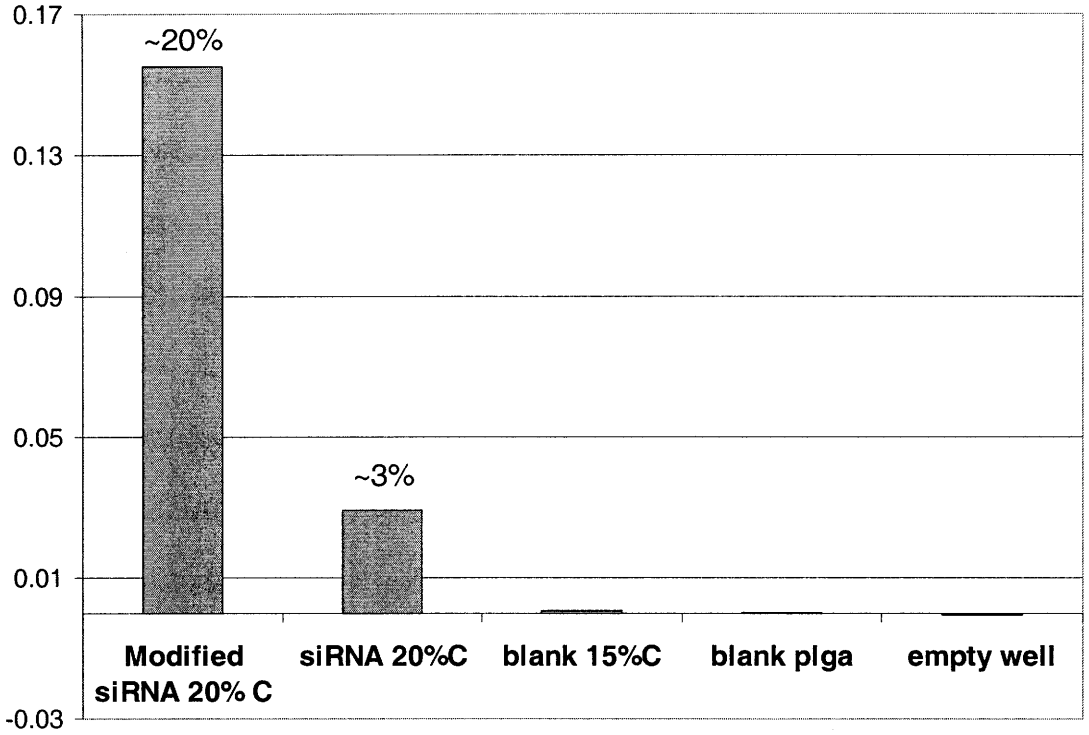

Figure 3.14 Improved encapsulation with cholesterol modified siRNA

Encapsulation efficiency of siRNA and cholesterol modified siRNA and microparticles without encapsulated siRNA. SiRNA was encapsulated and extracted from the indicated microparticle formulation.

3.4 Discussion

There have been numerous advances in nucleic acid delivery in the recent past, however the challenge of highly efficient, non-viral DNA and RNA delivery remains unsolved. Although viral delivery techniques tend to achieve efficient delivery, often they are plagued by unwanted sepsis like immune responses. Non-viral systems (polymer, lipid and particles) typically lack the threat of run-away immune reactions, they lack highly effective efficiency levels.

Another challenge faced by nucleic acid delivery systems is the high cost of DNA or RNA as a drug. This generates added pressure for delivery systems to be highly efficient at both targeting the appropriate cells and delivering the nucleic acid payload efficiently.

More specifically microparticle approaches have been described in the DNA vaccine arena and the adaption of these approaches to siRNA delivery could allow for effective and efficient modulation of the immune system, though targeted delivery to antigen presenting cells. Here we attempted the adaptation of the poly(β-amino-ester) system to siRNA. These preliminary studies demonstrated no measurable knock-down with siRNA using the PLGA/PBAE system. We identified several challenges of particular importance to particle based siRNA delivery.

Encapsulation efficiency using micro-emulsion techniques is a significant challenge. Because of its small molecular weight (100X smaller than plasmid DNA) siRNA may be more able to diffuse through condensing polymer during the microemulsion process and therefore escape to the external aqueous phase. We found encapsulation efficiencies 1/10 the typical encapsulation seen with plasmid DNA. One driving force to difficult encapsulation efficiency is the osmotic matching between inner and outer organic phases during the emulsion process. Since siRNA is 100 times smaller than DNA the molality will be 100 times greater for a solution with the same mass concentration. Future approaches to siRNA encapsulation, could involve production methods where multiphase encapsulation methods are not used. This is significantly challenging because siRNA is not soluble in solvents compatible with most biodegradable polymers viable for microparticle fabrication.

The second substantial hurdle is acid catalyzed degradation of siRNA inside acidic degrading microparticles. A potential solution is compensating for acidic degradation products during biodegradation by adding buffers. The addition of magnesium hydroxide buffered the acidic conditions in the degrading microparticles increasing the average microenvironment pH from 2 to 5. Based on our studies of pH effects on siRNA it should be relatively stable at pH 5 for several days.

The microparticle double emulsion production protocol requires a relatively large quantity of polymer (200 mg) and therefore siRNA (1mg) due to physical constraints and equipment size. Because of prohibitive costs this process is not easily adaptable to high

throughput screening. We did make attempts to miniaturize this process but even reducing batch size to 10% will still not allow screening of a full combinatorial poly(beta-amino-ester) library which contains over 3000 polymers[27]. In addition to prohibitive costs, most of the poly(b-amino-esters) are not suitable for particle production because their tackiness and viscosity at room temperature would compromise the physical structure of microparticles (especially during centrifugation for separation).

Future experiments should include high throughput methods for screening several variables including cationic material selection. Thousands of poly(β-amino-esters) and lipidoids have been studied for the delivery of siRNA using an cationic self-assembling techniques (liposomes or complexes). Small changes is monomer selection, end group modification or side chain modification can have profound effects on knockdown efficiency (for siRNA [38], for DNA see [27]). We demonstrate in Chapter 5 how screening lipidoid libraries lead to efficient microparticle-based siRNA knockdown using a non-emulsion techniques.

3.5 Conclusions

In this chapter we identified and studied many of the challenges to delivering siRNA with PBAE/PLGA microparticles.

We concluded that encapsulation efficiency was a major challenge. We also concluded that addition of a large lipophilic molecule, such as cholesterol to the siRNA increased encapsulation efficiency by 10X in PBAE/PLGA microparticles. We concluded that acidic degradation products from PLGA were a potential problem since the stability of siRNA was questionable in such conditions. The addition of magnesium hydroxide to microparticles buffers their pH and has significant positive effects on the particle morphology and encapsulation efficiency.

Encapsulation was increased to 30%, microenvironment was buffered to pH 5 and particle morphology was optimal yet these microparticles were inefficient in gene knockdown. Therefore, the next logical step is to study the cellular barriers which may be limiting the efficient escape from lysosomes and gene knockdown. High-resolution 3D confocal imaging could allow an understanding of particle localization during the delivery process. This is the focus of the following chapter (Chapter 4).

3.6 References

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4. Nanoparticle surface modification with cationic polymer: an approach to studying particle based siRNA delivery

4.1 Introduction

Fluorescent materials are useful for sensing, identifying and tracking intracellular structures[1-4] and local conditions such as pH[5] and redox potential[6]. Photobleaching severely limits the effective time scale for organic fluorophores, and although semiconductor quantum dots (Qdots) represent a bright, photostable substitute, expense, toxicity[7] and disposal issues make their use impractical in some cases. It has been suggested that core-shell fluorescent silica nanoparticles produced by the Stöber Synthesis provide safe, bright and more photostable alternatives with florescence intensities 20-30-fold greater than their unencapsulated (i.e., free) fluorophores. Ow et al. have recently demonstrated a novel method for generating core-shell fluorescent silica nanoparticles (C dots) with higher quantum efficacy and brightness than traditional dye-doped silica nanoparticles [8, 9]. Because C dots are optimized for brightness and stability [9] they hold promise as a new material for bioimaging applications.

For C dots to realize their full potential as intracellular imaging and detection agents, they must be delivered into the cell cytoplasm. C dots have physical properties that inhibit their free passage across the cell membrane. Cellular uptake typically confines unmodified silica particles to endosomes or phagosomes[5, 10], which limits their use as cytoplasmic and nuclear imaging agents. Similar to DNA delivery systems, delivering nanoparticles to the cytoplasm of live cells requires some active form of escape from

endosomes[11, 12], and this is currently one of the key hurdles of their use [12, 13]. Others have demonstrated the utility of silica particles as gene delivery agents when covalently surface modified with cationic groups[14-21]. Many DNA delivery technologies use endosomal delivery followed by endosomal escape. This approach uses the natural affinity of cationic particles to associate with the negatively charged cell membrane and be taken into endosomes. Many amine containing polymers have been shown to buffer endosomes and disrupt the osmotic gradient leading to escape of the payload outside endosomes.

A simple method of coating C dots with functional polymer could allow C dots to escape endosomes and ultimately accumulate in the cytoplasm. Such a system could enable C dots as an intracellular imaging agent, sensor of cellular activity or intracellular delivery tool.

4.2 Materials and methods

4.2.1 C dot preparation and coating

Core shell fluorescent nanoparticles with a nominal diameter of 60 nm were obtained from Hybrid Silica Technologies, Ithaca, New York. These particles were pelleted by centrifugation and resuspended in 25 mM sodium acetate buffer (pH 5.2). PEI-coated C dots were prepared by the dropwise addition of C dots to a stirring solution of PEI (25 kDa Sigma) in 25 mM sodium acetate buffer. The mixture was stirred for 10 minutes at

room temperature. Particles where then centrifuged and washed three times with fresh acetate buffer.

4.2.2 Thermal Gravimetric Analysis and Zeta Potential Analysis

TGA analysis were performed on a Perkin Elmer TGA7 Thermogravimetric Analyzer, under an oxygenated atmosphere, at a heating rate of 5°Cmin-1. Weight loss measured in the temperature range from 300 to 400 °C was used to calculate the percentage of PEI by mass. Previous experiments showed that PEI MW 25KDa degrades between 300 and 400 0C. Size and charge data were collected on a Brookhaven Instruments Corporation ZetaPALS analyzer.

4.2.3 Complexing particles with DNA

Plasmid DNA (CMV-luc) was obtained from Elim-Biopharmaceuticals (Hayward, CA). Particles prepared above were added in equal volume to a DNA solution (60 □g/ml in sodium acetate) and mixed vigorously. The solution was allowed to sit undisturbed for 5 minutes at room temperature after which time it was mixed 1.5:10 with fresh Optimem (Invitrogen).

4.2.4Transfection and viability assays

COS-7 and HeLa cells were maintained in Dulbecco's Modified Eagle's Medium (Invitrogen) containing 10% fetal bovine serum and Penicillin/Streptomycin. For transfections COS-7 cells were plated in 96-well plates and allowed to grow to 80%

confluence before transfections. Media was aspirated and 150 □l of particle/DNA solution in Optimem was added to each well. Particles were left on cells for 1 or 4 hours at which time Optimem was replaced with fresh growth media. Luciferase expression was analyzed three days later using Bright-Glo assay kits (Promega) following the manufacturer's instructions.

To demonstrate that particles are required for tranfection by this protocol some samples were centrifuged for 20 minutes at 8600 RCF (same conditions used for washing) to remove the C dot:PEI:DNA particles form solution and compared to identically prepared particles without centrifugation.

For viability assays, COS-7 Cells were treated with the same procedure as transfections and analyzed using the MTT Cell Proliferation Assay Kit (American Type Culture Collection) following the supplied instructions.

4.2.5 Microscopy and flow cytometry analysis

All confocal images were collected with a Carl Zeiss LSM510 Laser scanning confocal microscope. Cells were grown in chambered coverslips (gelatin-coated) and treated with particles in Optimem medium as for transfections. After 24 hours cells were treated with Lysosensor-Green (Invitrogen) according to the manufacturer's instructions, then fixed with 4% (v/v) paraformaldehyde, permeablized with 0.5% (v/v) Tween 80 (Sigma) and finally stained with TOPRO-3 nuclear stain (Molecular Probes). For flow cytometry analyses, HeLa cells after being exposed with C dot particles at several concentrations were dissociated with non-enzymatic cell dissociation solution (Sigma) for 10 min.

Single cells were aliquoted (2.5 × 105 cells per condition) and analyzed by a FACScan (BD Biosciences). Data analysis was carried out using CellQuest software. 3D video rendering (with rotation) is available in the supplementary information. For scanning electron microscopy (SEM) analysis, uncoated and PEI coated C dots (1:1 ratio PEI/C dots) were dried on glass cover slips coated with gold and imaged using a Hitachi S-4800 FESEM.

4.2.6 C dot preparation and coating

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4.3 Results

We chose polyethyleneimine (PEI) to coat the C dot particles because it has been documented as an effective DNA delivery tool that has a clear mechanism to achieve endosomal escape[22]. PEI and C dots were mixed in sodium acetate buffer and allowed to associate based on electrostatics. The ratio of PEI to C dots in the mixture was optimized to minimize aggregation and achieve a positive surface charge. PEI:C dot ratio, particle size, particle charge and percent PEI in final particle composition are summarized in Table 4.1 Sizing and Zeta potential dataTable 4.1

Table 4.1 Sizing and Zeta potential data

mg PEI/mg CDots[a]	Size (nm)[b]	Charge (mV)[c]	% PEI[d]
NT[e]	66.5 ± 1.5	-2.84 ± 7.13	--
0	114 ±1.4	-25.68 ± 3.64	--
0.001	99.0 ± 4.7	-27.34 ± 2.48	2.7%
0.002	>1000	-30.94 ± 2.94	2.0%
0.004	>1000	-18.71 ± 2.18	2.7%
0.01	>1000	-10.71 ± 2.46	3.0%
0.1	218 ± 7.1	47.90 ± 3.58	5.5%
1	117 ± 1.1	31.22 ± 2.68	5.5%

[a] PEI:particle mass ratio used for coating
[b] Effective particle diameter following coating measured by dynamic light scattering
[c] Zeta potential of coated particles measured by phase analysis light scattering
[d] Percent mass of PEI in coated particles measured by TGA
[e] NT Particles were measured without centrifugation or resuspension

Because the silica shell surface is negatively charged, the particles can be electrostatically coated with cationic polymers in a self-assembly process. As the PEI:C dot ratio was increased, coated particle charge approached neutrality and eventually became positive. Aggregation was evident in samples where an intermediate amount of PEI was added and charge was nearly neutral. Aggregation is commonly observed in solution when similar

amounts of oppositely charged materials self-assemble [9]. Because particles were less aggregated and had a desirable surface charge when coated at a 1:1 PEI:C dot mass ratio, we chose this condition for the remainder of the study. Scanning electron micrographs of coated and uncoated C dots are shown in Figure 4.1. No significant changes in particle morphology were observed.

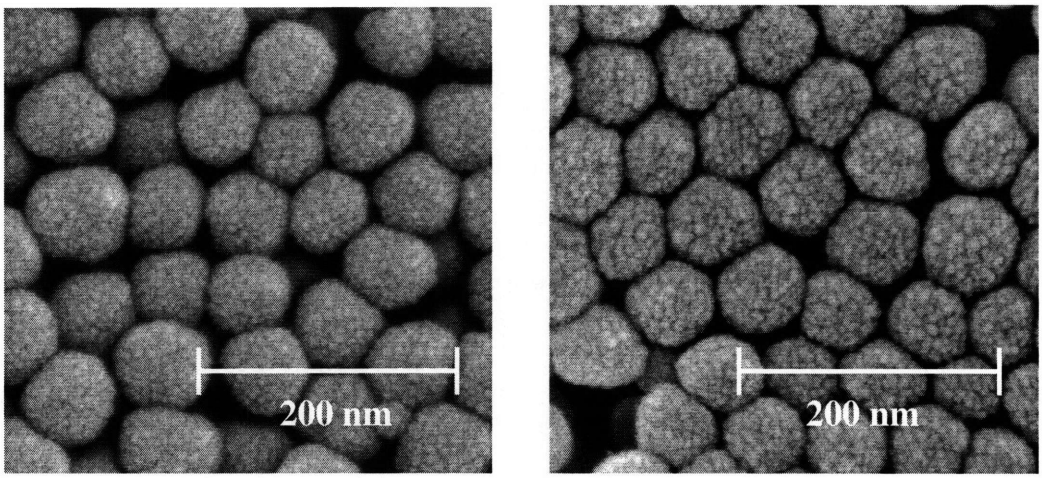

Figure 4.1 SEM micrographs of coated and uncoated C dots

No significant changes in particle morphology were observed after coating C dots with PEI.

Toxicity is a common characteristic of materials typically used for intracellular delivery because of their ability to alter or disrupt cell membranes[23]. Since our goal is to develop particles to be used with cells, we quantified the effects of PEI-coated C dots on the viability of COS-7 cells in vitro. Figure 4.2 compares viability of cells treated with PEI, C dots and PEI-coated C dots over a range of concentrations that are typically used with cells.

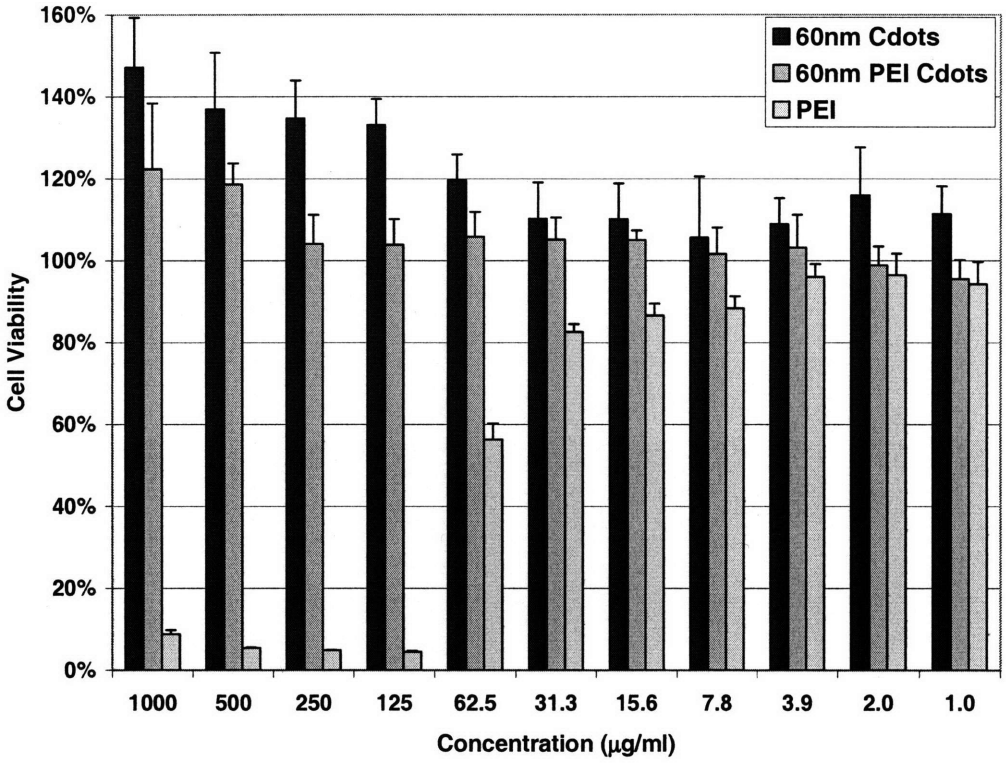

Figure 4.2 Cell viability in the presence of PEI-coated C dots

C dots, PEI-coated C dots, and free PEI were incubated with COS-7 cells and viability was analyzed after 24 hours using the MTT assay. Mean cell viability is shown as a function of the particle or polymer concentration (± SD, n = 4). For comparison, PEI-coated C dots are composed of 5.5% PEI by mass.

PEI alone affects viability of COS-7 in concentrations above 50 ug/ml, however C dots, both coated with PEI and uncoated, do not negatively affect cell viability over the full range of concentrations measured (1-1000ug/ml) and that would reasonably be used for

labeling or intracellular detection. The increased measured viability seen with increasing concentrations of particles seems to be caused by particles activating cells.

Cellular association and uptake of rhodamine (TRITC) dye-based C dots [24], with and without a PEI coating, were determined using flow cytometry (Figure 4.3). Median fluorescence levels in the FL2 histograms indicate that two-fold more coated particles were taken up by HeLa cells at 100 ug/ml compared to uncoated particles. A ten-fold increase in fluorescence was observed between coated and uncoated particles when delivered at 500 ug/ml. This increase in cellular association is most likely the result of non-specific electrostatic interactions between the positively charged PEI-coated C dots and the negatively charged cell surface[18]. Cellular uptake and endosomal escape are also possible with these particles, since they have an appropriate size and surface functionalization.

Confocal microscopy was used to verify the location of the particles relative to the cells. Coated and uncoated C dots were added to cells at 50 ug/ml. Figure 4.3a depicts confocal images of cells and C dots; green regions represent endosomes (LysoSensor Green), the nucleus is stained in blue (TOPRO-3) and red indicates C dot TRITC fluorescence.

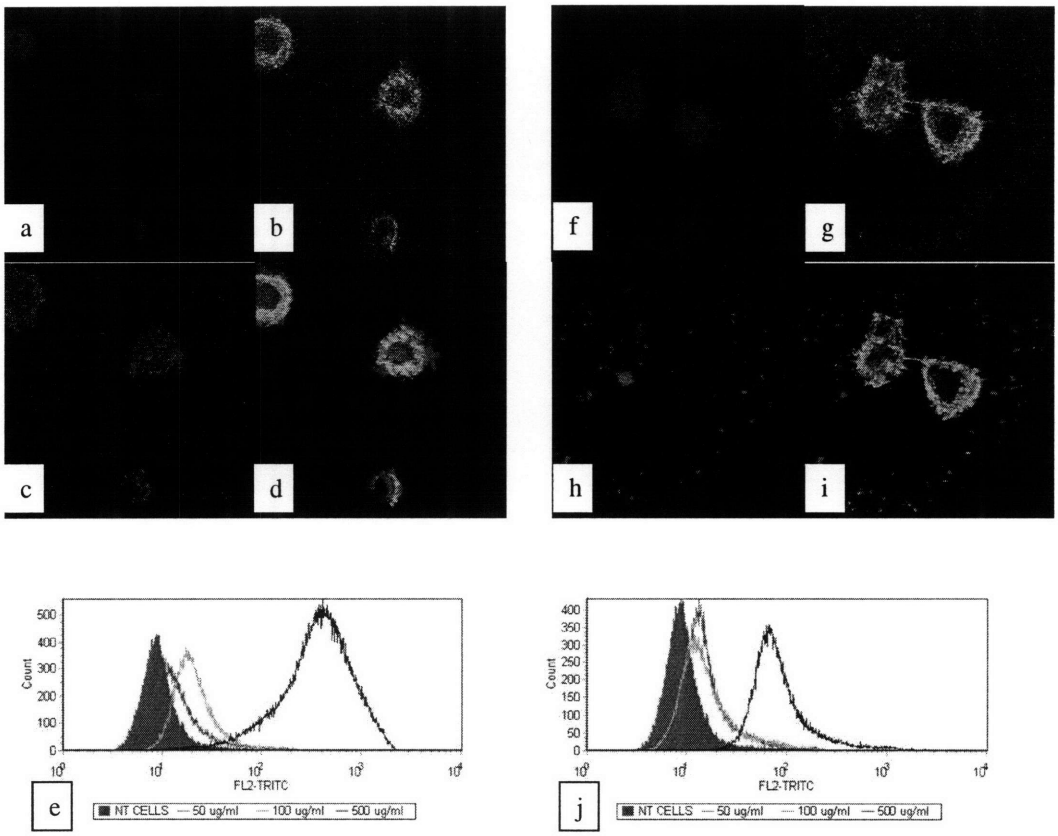

Figure 4.3 Analysis of Intracellular Delivery

Images taken at 100× magnification with laser scanning confocal microscope. Blue TOPRO-3 stains the nucleus, green Lysosensor-Green indicates endosomes and C dots are displayed in red. Single color images are single z-slice and merged images are 3D renderings of z-stack. PEI coated C dots (a-d.) can be seen co-localized with endosomes as a yellow color and distributed throughout both the cytoplasm and nucleus of the cell. Uncoated C dots (f-i.) are aggregated and less distributed throughout the cell, with no nuclear localization apparent. Quantitative Uptake Assay by FACS. PEI-coated (e.) and uncoated (j.) C dots were incubated on HeLa cells for four hours at 50, 100 and 500 ug/ml. After 24 hrs cells were harvested and analyzed with flow cytometry. Cell counts versus the TRITC fluorescence (FL2) are shown for condition.

Yellow regions represent the co-localization of C dots and endosomes. Figure 4.3a shows the uptake of coated C dots into endosomes (red+green or yellow) and association with the nucleus (red+blue or violet). Particles are well distributed throughout the cell and appear to be adjacent to the nucleus. In contrast, uncoated C dots show much less association with and distribution throughout the cells (Figure 4.3b). By comparison, the PEI coating results in much more C dot abundance in the endosomes (compare yellow regions of Figures 4.3a and 4.3b), as would be expected because of PEI's properties. In addition, PEI-coated C dots appear to be co-localized with the nucleus as evidenced by the red and blue (or purple) regions. Although the cytoplasm is not specifically stained, three-dimensional imaging suggests that coated particles appearing "between" endosomes and the nucleus are resident in the cytoplasm or other cellular components of cells.

These results are consistent with the interactions of cells with PEI[25, 26] silica particles [14-21, 27-30] and the proton sponge hypothesis of endosomal escape. Others have used covalently modified silica particles as gene delivery agents, however transfection often requires the addition of chloroquine[9] or is less effective than with soluble polyethyleneimine (PEI) [27].

Particles without the polymeric coating seem to be distributed in much larger aggregates that are not as effectively endocytosed (Figure 4.3). This may be due to the effects of salt and protein in the media which induce aggregation by reducing charge repulsions between particles. We noticed that generally particle aggregation is less pronounced for the coated particles than uncoated particles. This difference may be attributed to the high

charge density of the PEI coating which can effectively limit particle-particle surface interactions.

The high cytoplasmic accumulation and nuclear association of PEI-coated C dots suggests that these materials could be useful as gene transfection agents. In fact, PEI alone is well known for it's ability to transfect plasmid DNA into cells in the form of PEI/DNA complexes. In combination with C dots, the cellular viability is significantly improved and it now becomes possible to image the PEI-DNA transfection. Furthermore, the particle surface is amenable to chemical modification to potentially enhance delivery, either by direct functionalization or coating with other polycationic materials.

To examine the versatility of C dots as a transfection platform, we coated C dots with PEI and other polycations that are typically used for DNA delivery: PEI MW 1,800, PEI MW 25,000, PEI MW 70,000, Poly-L-Lysine low molecular weight, Poly-L-Lysine high molecular weight and Eudragit EPO. These hybrid particles were then complexed with plasmid DNA encoding firefly luciferase and delivered to cells. The transfection was carried out using a high-throughput, 96-well plate based protocol to simultaneously screen a number of surface coatings and complexation conditions. This approach allowed us to identify coated particles with transfection efficiencies similar to that seen with PEI/DNA complexes (Figure 4.4). Perhaps not surprising, the transfection ability of polymer-coated C dots closely correlated with the free polymer. And since PEI is the superior reagent, it also proved to be the most effective coating for enabling C dot-mediated transfections.

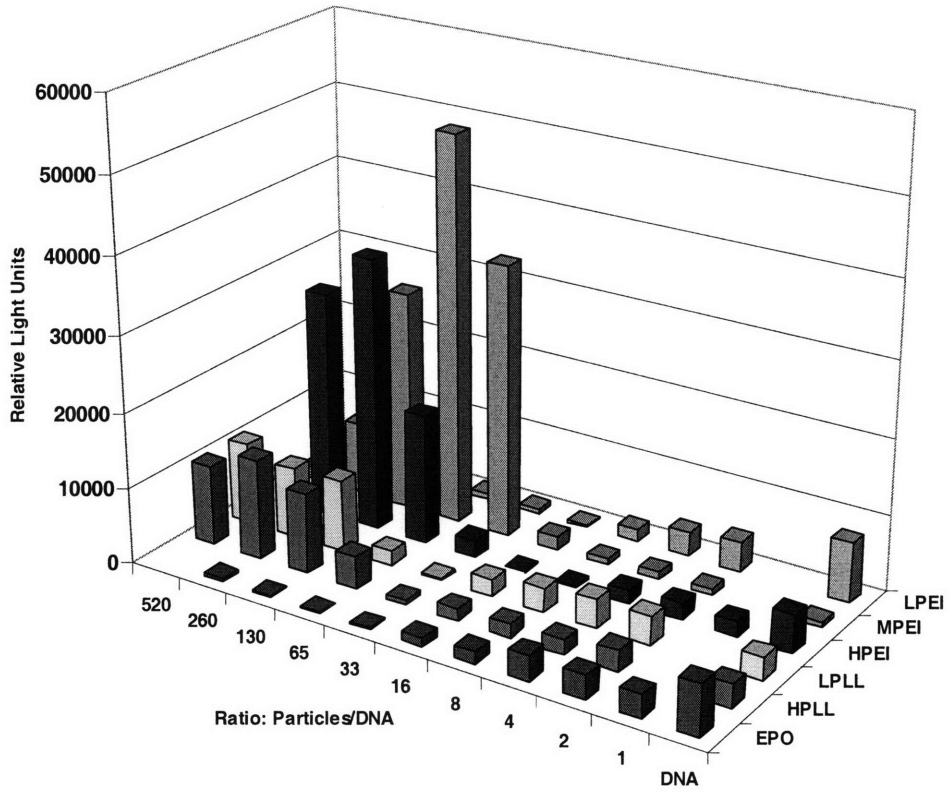

Figure 4.4 Transfection as measured by Luciferase luminescence

C dots coated with: PEI MW 1,800 (LPEI), PEI MW 25,000 (MPEI), PEI MW 70,000 (HPEI), Poly-L-Lysine low molecular weight (LPLL), Poly-L-Lysine high molecular weight (HPLL) and Eudragit EPO (EPO) were complexed with DNA at the mass ratios indicated. DNA row indicates naked DNA with no polymer or particles. DNA/PEI complexes (no particles) transfect at 58,000 RLU, the amount of PEI used in that transfection corresponds to a ratio of 82 on this chart. Mean relative light units of luciferase expression are shown as a function of both the polymer coating and particle:DNA ratio (n = 4).

To demonstrate that transfection is not a result of PEI separating from the C-dots to form PEI:DNA complexes we showed that removing the C dots from the transfection solution (by centrifugation) eliminated transfection but the same centrifugation did not affect PEI:DNA transfection (Figure 4.5).

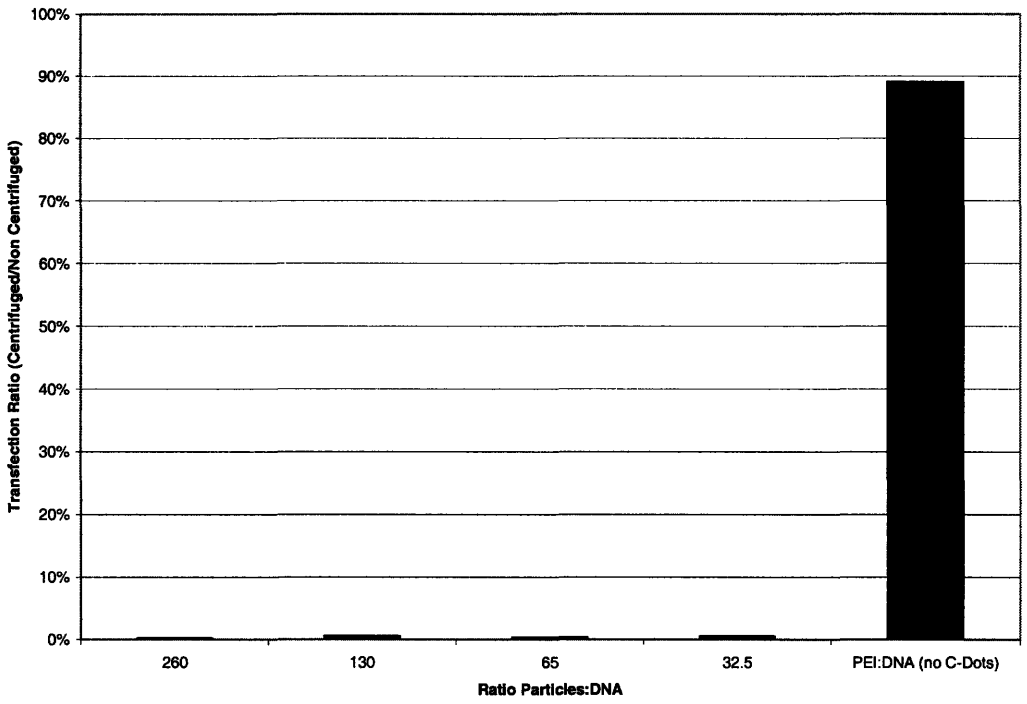

Figure 4.5 C dots are required for cell transfection

C dots coated with PEI MW 25,000 (MPEI), were complexed with luciferase DNA at the mass ratios indicated on the x-axis. C dot:PEI:DNA complexes or PEI:DNA complexes (no C dots) were added to COS-7 cells. Before being added to cells solutions were centrifuged to remove the C dots from solution while the solution with PEI:DNA complexes (no C dots present) was incubated at RT for 20 minutes (referred to as "non-treated" in the figure). The vertical axis represents the ratio of Relative Light Units measured with centrifugation to non-treated.

These results suggest that C dots electrostatically coated with PEI are effective gene delivery vectors and could be used as a simpler and brighter alternative to covalently modified dye doped silica particles [19] for the intracellular delivery and imaging of drugs and macromolecules.

As discussed at the end of the previous chapter a cationic nanoparticle approach to siRNA delivery seems attractive. Moreover, the approach developed here is easily adaptable to high-throughput screening of cationic polymers for surface modification. Next, c-dots were coated with a number of cationic polymer of varying molecular weight and composition and then complexed with siRNA. These potential isRNA delivery vectors were screened for knockdown in the GFP-HeLa cell line described in Chapter 3. Results are shown in Figure 4.6. These results represent the first solid particle based siRNA delivery we have demonstrated. High throughput screening identified from a large set of experiments the combination of variables leading to the desired outcome. As a general approach the concept of complexing siRNA to the surface of solid particles has demonstrated its usefulness and will be studied further.

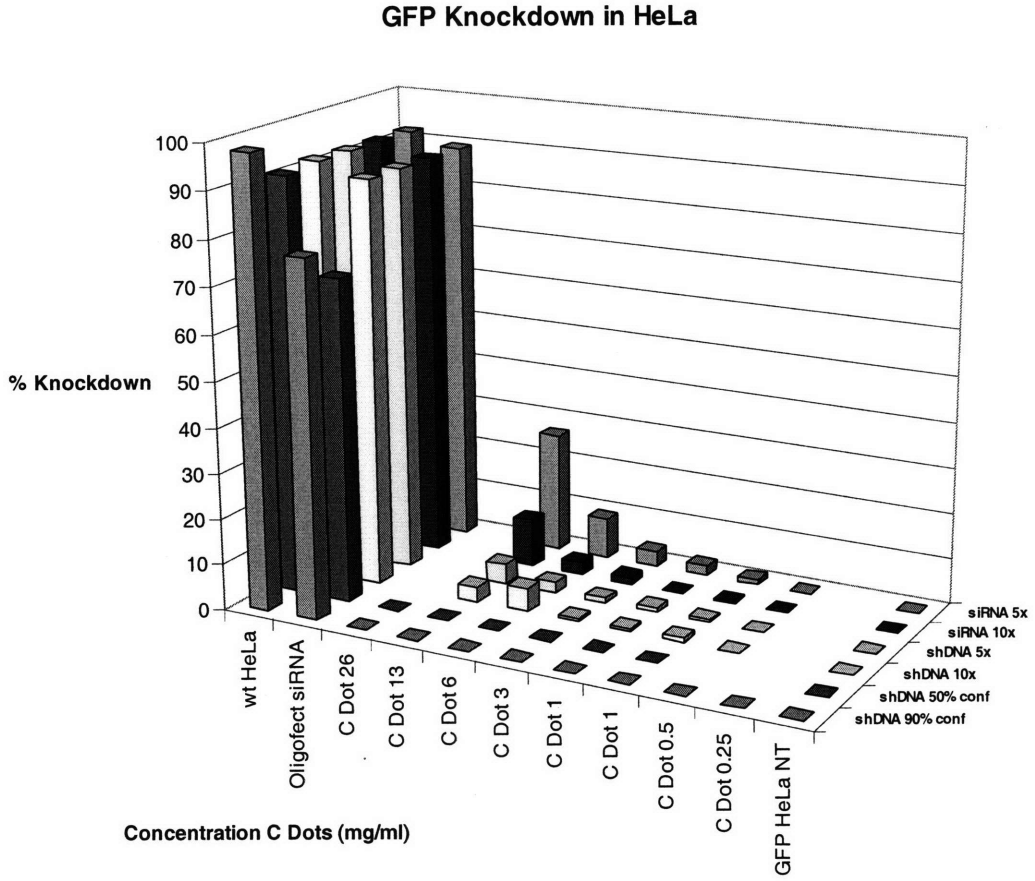

Figure 4.6 c-dot polymer siRNA complexes, knockdown in GFP-HeLa

C dots coated with PEI MW 25,000 (MPEI) and complexed with DNA or siRNA at the mass ratios indicated. Particles were then incubated with GFP+ HeLa cells which were analyzed by flow cytometry after 24 hours for GFP knockdown. To more easily compare small differences results are presented as (1-% in M1) indicating percent knockdown.

4.4 Conclusions

We have developed a method for coating and delivering silica nanoparticles to mammalian cells. Coating the particles with a polycation improved the cellular association and uptake levels. In addition, confocal microscopy confirmed that a PEI coating was both necessary and sufficient to enable endosomal escape and nuclear association of C dots. We used DNA delivery as a functional assay to show that these materials have the potential use as drug delivery systems. We believe that C dot internalization and distribution throughout the cell may be an important advance, allowing core-shell silica nanoparticles to be used for intracellular imaging, sensing and delivery. Finally, demonstrated effective siRNA knockdown using solid particles for the first time. The concept of complexing siRNA to the surface of functional cationic particles may be generalizable and will be studied further.

4.5 References

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5. Lipidoid nanoparticles and Lipidoid/PLGA microparticles for gene knockdown in antigen presenting cells

5.1 Introduction

Nanoparticles with appropriate surface functionality efficiently deliver DNA and siRNA to cells in vitro. In chapter 4, we demonstrated that 60nm silica nanoparticles functionalized with cationic polymer localize to the cell membrane, endosomes and the nucleus. These particles appear to efficiently deliver surface associated DNA or siRNA through endocytosis and endosomal escape. Although c-dots are non-photobleaching fluorescent imaging agents that enable deep stack confocal microscopy their non-degradable silica structure makes them less ideal for drug delivery. It would be particularly valuable to identify a biodegradable delivery vehicle with functionality to efficiently deliver surface associated DNA or siRNA to antigen presenting cells. This delivery system could be a nanoparticle or microparticle formulation.

5.1.1 Nanoparticles

We have confirmed that self assembling (commercially available) cationic liposomes efficiently deliver siRNA for gene knockdown and Akinc et al. have a developed a library of highly diverse lipid-like functional compounds termed "lipidoids" [1] for RNA delivery. The combinatorial nature of lipidoid synthesis allows for large libraries to be generated and screened in parallel high-throughput. Akinc et al have performed initial screens and identified several candidate lipidoids as effective siRNA delivery agents. Screening this library to identify lipidoid formulations optimal for siRNA delivery to

antigen presenting cells could identify chemical functionalities promising for endosomal and phagosomal escape.

5.1.2 Microparticles

Given earlier work with microparticles we know that the encapsulation of siRNA in micro-emulsion formulated particles was a limiting challenge (Chapter 3). We also demonstrated that functional surface modification of nanoparticles lead to efficient sub cellular localization and delivery (Chapter 4). Therefore, a potential approach to microparticle siRNA delivery could be surface fictionalization followed by surface association with siRNA and delivery. Because the process of phagosomal uptake and escape is conceptually similar to endocytosis, there may be similarities in optimal functional groups as shown before [2-5]. It is possible that the chemical functionalities isolated from screening would also be great candidates for phagosomal delivery[6].

5.2 Materials and methods

5.2.1Lipidoids

Lipidoids were synthesized as described [1] and were a kind gift from Michael Goldberg. Lipidoids were synthesized by addition of acrylamides or acrylates to amines. Amines were purchased from Sigma-Aldrich (St. Louis, MO, USA) and TCI America (Portland, OR, USA). Acrylates were purchased from Sigma-Aldrich (St. Louis, MO, USA), Dajac Monomer-Polymer (Feasterville, PA, USA), Hampford Research (Stratford, CT, USA), Scientific Polymer (Ontario, NY, USA), and TCI America (Portland, OR, USA). Acrylamides were synthesized by the drop-wise addition of acryloyl chloride to the appropriate 1-aminoalkane (see Supplementary Information for details). The ester portion of the lipidoid library was synthesized at a ratio of 2:1 acrylate:amine, with no solvent, unless otherwise specified. The amide portion of the lipidoid library was synthesized at the maximal ratio of acylamide:amine for each amine (e.g. 6 acrylamide:amine for amine monomer 98). All library reactions were carried out in 5 mL Teflon-lined glass screw-top vials. 200 mg of amine was added to the corresponding amount of acrylate or acrylamide. The mixture was stirred at 90°C for 1 or 7 days for acrylate or acrylamide monomers, respectively. After cooling, the lipid mixtures were used without purification unless otherwise specified.

5.2.2 P388-D1 macrophage cell line and primary murine macrophage

P388-D1 cells were obtained from American Type Culture Collection (ATCC, Manassas, VA) and cultured as suggested by ATCC. Cells were grown in monolayers in T-75 flasks

in media of high glucose supplemented RPMI 1640 plus fetal bovine serum plus antibiotics. When they became confluent cells were removed from their monolayer using a cell scraper (VWR) and plated in opaque 96 well plats at 15,000 cells per well. Primary peritoneal macropages were obtained from mice 9 weeks (or older). Mice were sacrificed and sunk in a 5% water solution of Dettol (Chloroxylenol) for 1 min, at RT. Then 5 ml of ice-cold IDDM-FCS (5%, decomplimented) was injected I.P. followed by gentle massage of the anterior and lateral walls of the abdomen. Dissection of the tissues and opening the peritoneum cavity followed. Peritoneum washes were collected and placed on ice. Then washes are filtered thorough a nylon filter into the 15 ml Falcon tube placed on ice. Cells were then centrifuged and resuspended and placed on ice. They were then plated at 15,000 cells per well in 96 well plates.

5.2.3 GAPDH Assay

KDAlert assay was purchased from Ambion Inc. (Austin, TX) and used according to the manufacturers directions. Briefly, at the time of analysis cells were lysed using KDAlert lysis buffer and 10ul of lysate was pipetted into a new 96 well plate. 90 ml of assay solution was prepared and added to the 96 well plate which was then read on a plate reader for flourescense. Data reported in kinetic data, time points were taken every two minutes for 10 minutes. The time point at t=6 minutes was used as the end point and the time point at t=2 minutes was used as t_0. Data reported is x, defined as.

$$x = \frac{(t_e - t_0)_{GAPDHsiRNA}}{(t_e - t_0)_{GFPisRNA}}$$

Where the numerator refers to siRNA specific to GAPDH and the denominator refers to the same measurement and calculations for control siRNA, in this case a sequence specific for green fluorescent protein.

Optimal balance factor is calculated according to the manufacturer's directions.

5.2.4 Flow cytometry

To analyze the expression of both green fluorescent protein and CD45 we used flow cytometry. GFP+ primary macrophages were isolated from GFP+ mice as described above.

5.2.5 Mouse experiments

C57Bl/6J mice (Jackson Labs) were injected intraperitoneally with 1 ml of 4% Brewers Thioglycollate medium (Difco) 3 days prior to injecting 10 mg/kg of CD45 siRNA, or GFP siRNA i.p (4 mice per group). Peritoneal lavage was collected 4 days later and stained with fluorophore conjugated antibodies to CD11b, Gr1 and CD45 (BD Biosciences). Flow cytometry samples were ran on the LSRII flowcytometer (BD Bioscience) and FlowJo software (Treestar) was used to identify the $CD11b^{high}Gr1^{low}$ macrophage population and quantify CD45 expression.

5.2.6 Microparticle formulations

PLGA microparticles were prepared by modified double emulsion [7]. In this protocol the inner aqueous phase was eliminated and to instead allow a single emulsion process. PLGA and lipid was dissolved in methylene chloride. Solution was added to a homogenized solution of poly(vinyl alcohol) (50 ml, 5% PVA (w/w), 5000 rpm) or Poly(ethylene alt maleic anhydride) (PMEA) or a mixture of PVA and PMEA at the designated ratio. After 30 s, 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 h at room temperature. Microspheres were washed and centrifuged 3X to remove PVA prior to lyophilization.

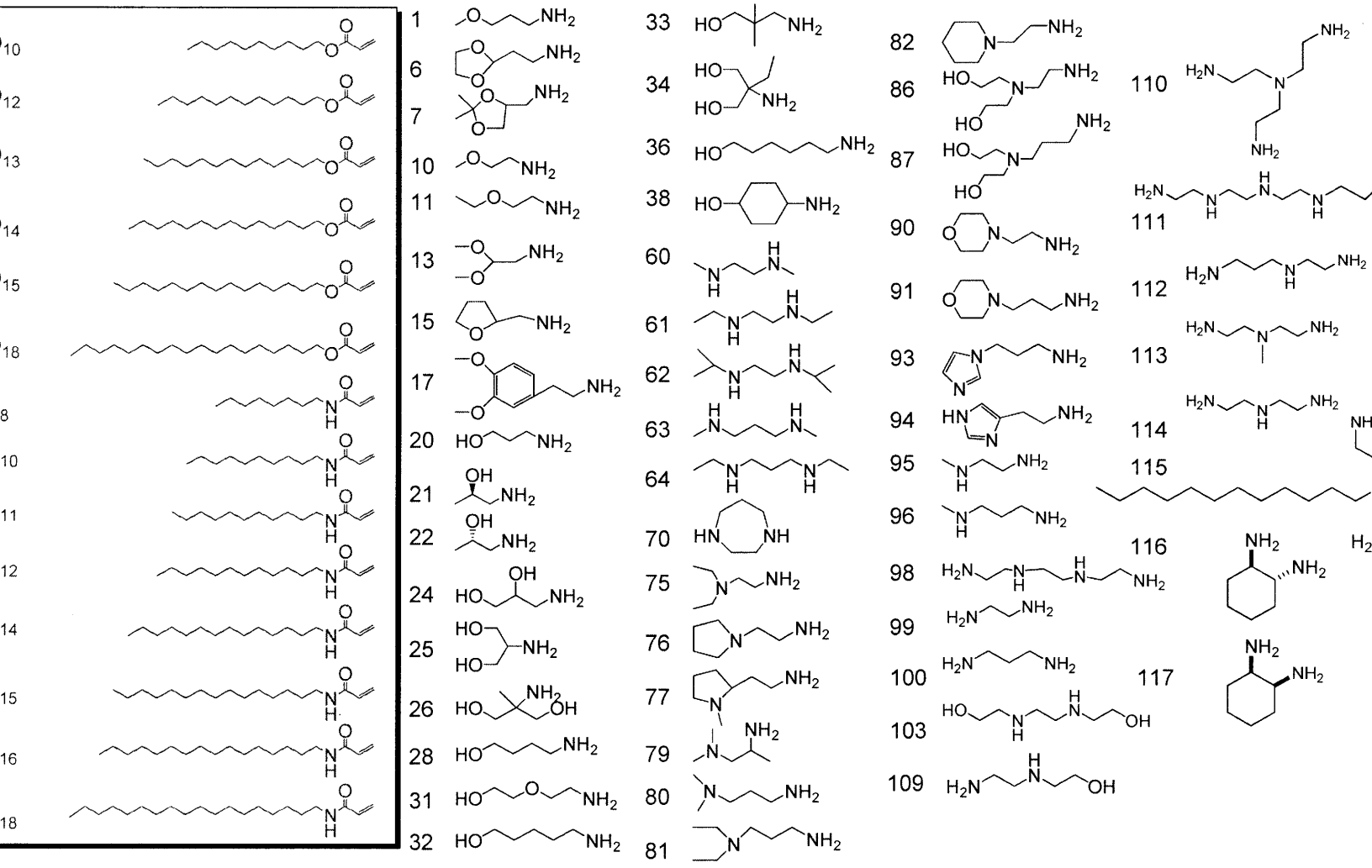
5.3 Results

5.3.1 Lipidoid nanoparticle knockdown in antigen presenting cells

In collaboration with Akin et al. we have developed a highly diverse lipidoid library [1]. These lipidoids are synthesized by a simple addition of alkyl-acrylates or alkyl-acrylamides to primary or secondary amines as shown in Figure 5.2. To generate a large diversity of polymers the library monomers come from the set shown in Figure 5.1. This library demonstrates great diversity and the simplicity of the chemistry allows rapid parallel synthesis [1].

The nomenclature used to describe the lipidoids in the library is outlined here: alkyl-acrylamides are termed as follows NA=N_8, NB=N_{10}, NC=N_{11}, ND=N_{12}, NE=N_{14}, NF=N_{15}, NG=N_{16}, and NH=N_{18} primary or secondary amines are referred to by number and a number following a hyphen indicates the number of tails. Therefore NC101-5 indicates monomers N_{11} and 101 with five tails.

By screening a dual reporter HeLa cell line for knockdown an efficient subset of lipidoids has been identified. The most efficient siRNA delivery was demonstrated by: NA98-5, ND98-5, NA110-6, NA110-5, NA11-7+6, NA112-5, NA114-4+3, NA115-4+3, NC116-3, and NC100-3. We used this sub-library in our screening experiments.



re 5.1 Lipidoid monomer structures

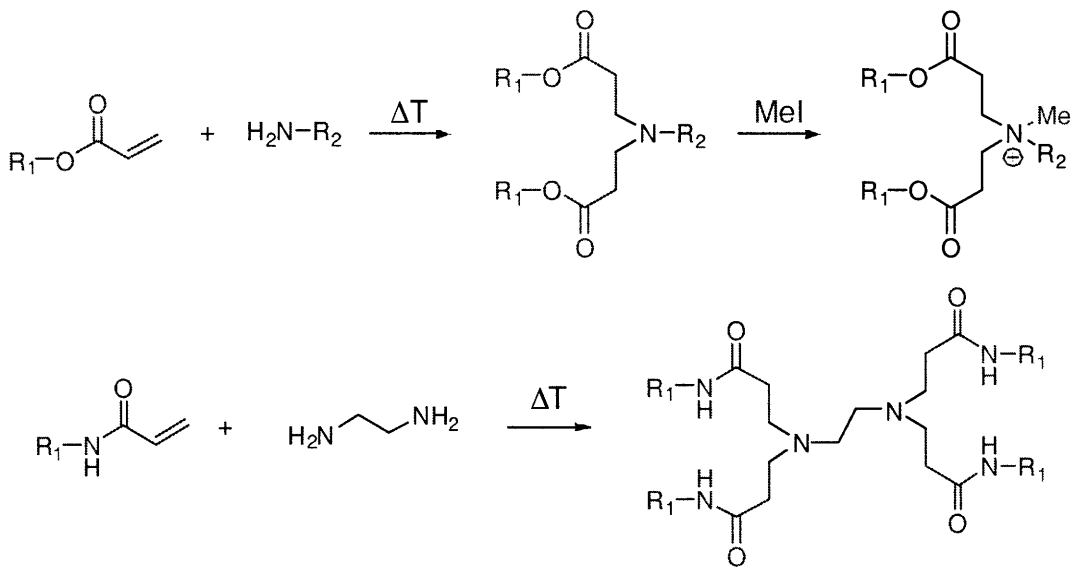


Figure 5.2 Lipidoid Chemistry

We used the P388-D1 cell and GAPDH siRNA to screen the lipidoid sub-library for gene knockdown. The transfection procedure is outlined in the materials and methods section. Cells were plated in 96 well plates and lipidoid complexes were delivered in various lipid to DNA ratios. Results are shown in Figure 5.3. It seems that in general higher lipid to DNA ratios result in more efficient knockdown. We report remaining expression in Figure 5.3 which is defined:

$$\text{remaining expression} = \frac{\left(flourescence_{t2} - flourescence_{t0}\right)_{GAPDH}}{\left(flourescence_{t2} - flourescence_{t0}\right)_{GFP}} = \frac{\Delta_{fluor}}{\Delta_{fluor,neg}}$$

Where t2 is four minutes later then t0. This variable defines the dimensionless knockdown in GAPDH expression induced by GAPDH siRNA relative to the non-specific knockdown induced by mismatched (GFP) siRNA.

The results were similar to the results found in the duel HeLa cells with the same siRNA delivery vectors[1].

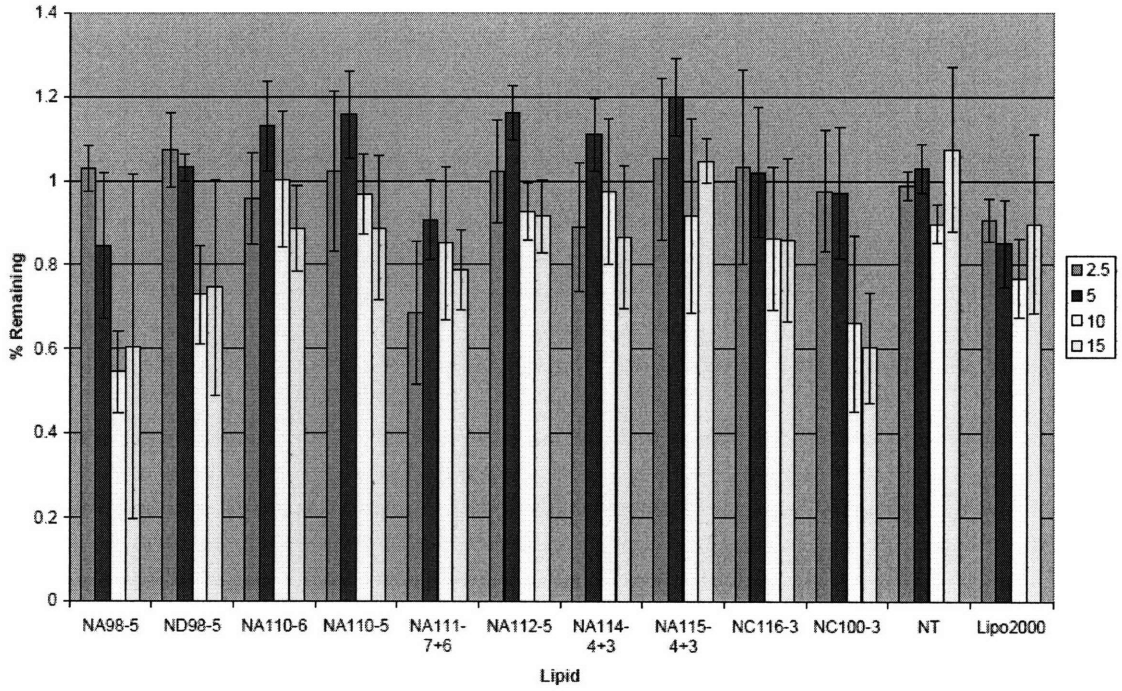


Figure 5.3 GAPDH knockdown with siRNA delivered using lipidoids

The indicated lipidoid structures were complexed with siRNA at the indicated lipidoid:siRNA ratio and added to culture with P388-D1 cells. After 48 hours cells were lysed and GAPDH concentrations were determined with the KDalert assay.

To further understand these results remaining expression was normalized to total protein expression in each well. This normalized data is shown in Figure 5.4, and the conclusions were the same. The NA115-4+3 lipidoid seems to interfere with the protein assay so one may disregard the high GAPDH expression indicated in this data series.

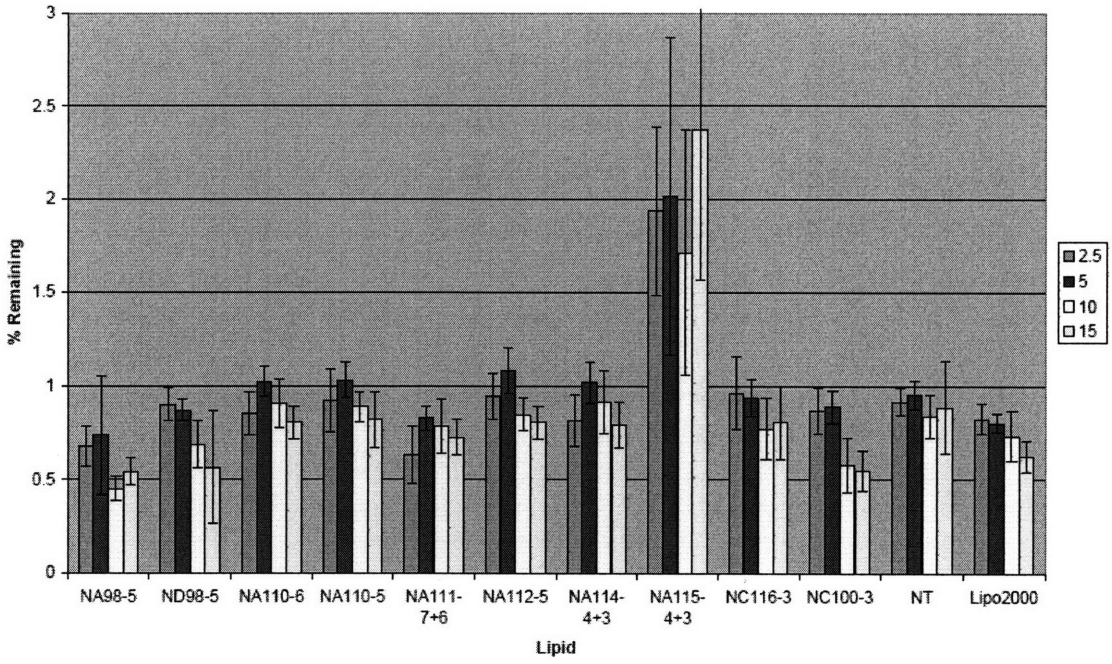


Figure 5.4 GAPDH knockdown normalized to total protein

The indicated lipidoid structures were complexed with siRNA at the indicated lipidoid:siRNA ratio and added to culture with P388-D1 cells. After 48 hours cells were lysed and GAPDH concentrations were determined with the KDalert assay. Results are normalized to total protein as determined by BCA assay.

An ideal transfection reagent demonstrates a balance of high transfection efficiency with low toxicity. To compare formulations we report the optimal balance factor as defined

$$OBF = \Delta_{fluor,neg} \times (1 - \text{remaining expression})$$

The optimal balance factor is reported in Figure 5.5. This calculation is suggested by the KDalert protocol. Notice various lipidoids and their efficiency when considering both cell viability and knockdown together.

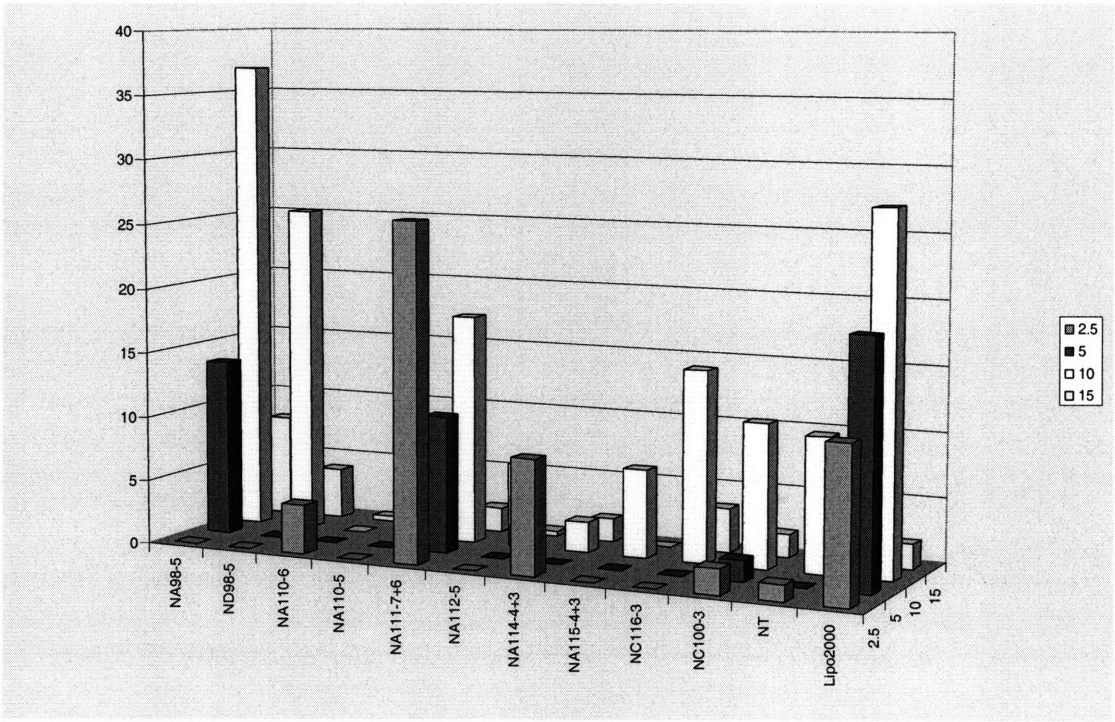


Figure 5.5 Optimal Balance Factor GAPDH knockdown Lipidoid/siRNA

The indicated lipidoid structures were complexed with siRNA at the indicated lipidoid:siRNA ratio and added to culture with P388-D1 cells. After 48 hours cells were lysed and GAPDH concentrations were determined with the KDalert assay. Optimal balance factor is reported.

Because ND98-5 seems to offer such a good combination of knockdown efficiency and tolerability to cells (and large quantities were available), we chose it at the optimal lipidoid for these experiments. Since the knockdown efficiency was so great in our initial P388-D1 model, we believed that the next experiment should be to deliver the siRNA-lipidoid complexes to primary macrophages.

We isolated peritoneal macrophages from mice as described in the materials and methods section and delivered the appropriate siRNA sequence to them in vitro. The results of these experiments are shown in Figure 5.6. This experiment shows that the ND98-5 is effective at knocking down both CD45 and GFP in a sequence specific way.

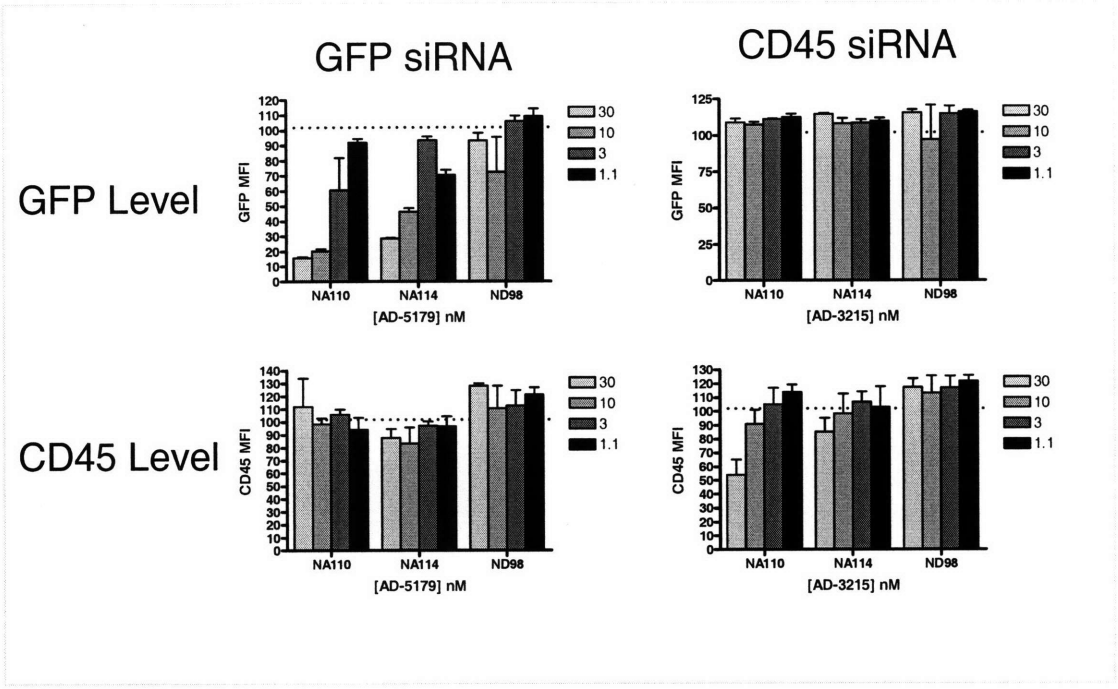


Figure 5.6 Primary macrophage knockdown with ND98

Primary murine macrophages were isolated from the peritoneal cavity of mice and cultured for 6 days. On day six the indicated siRNA and lipid were added at 1.1, 3. 10 or 30 to 1 ratio (lipid to siRNA). Cells were isolated from GFP positive mice and CD45 was analyzed with a fluorescently labeled monoclonal antibody by flow cytometry.

5.3.3 In Vivo knockdown with siRNA lipidoid nanoparticle-complexes

Because we observed efficient knockdown in primary cells in culture we chose a thioglycollate induced model of peritoneal inflammation to study siRNA delivered with ND98-5 in vivo. We injected mice I.P. with thioglycollate, an inducer of local inflammation and CD45 expression in macrophage. Three days later injected CD45 siRNA formulated with ND98-5 and 4 days after administering siRNA peritoneal

macrophages were harvested and analyzed for CD45 expression by flow cytometry. ND98-5 delivered siRNA for CD45 induced a 65% decrease in the expression of CD45 as shown below.

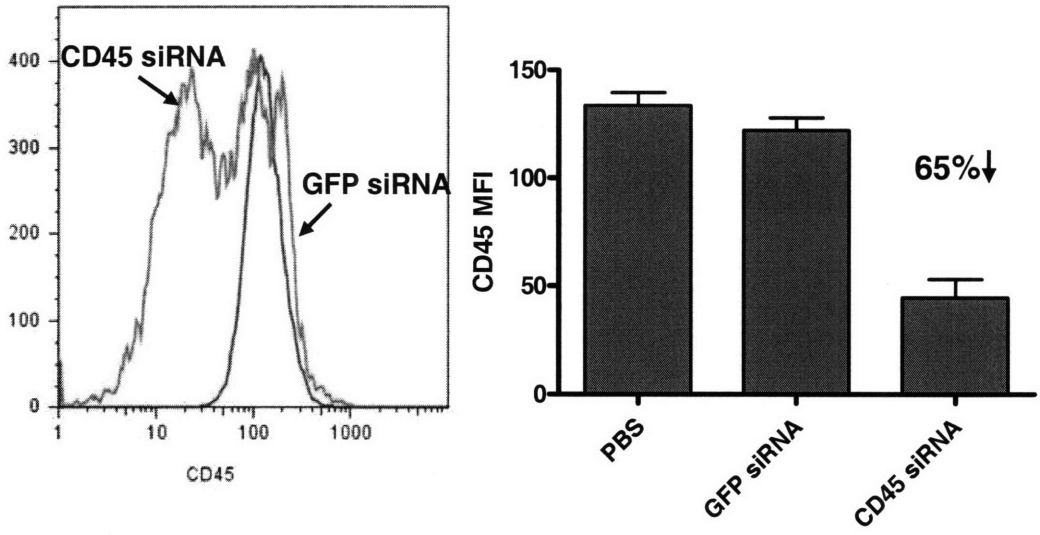


Figure 5.7 In vivo knockdown with ND98

Inhibition of CD45 protein in thioglycollate elicited mouse peritoneal macrophages. Mice (n = 4) were injected with thioglycollate (i.p.) 3 days prior to treatment with 10 mg/kg of lipodoid-formulated siCD45, or siGFP administered via i.p. injection. 4 days post administration CD45 expression on macrophages was analyzed by flow cytometry. Macrophage cells were gated (see Methods) and median fluorescence intensity of the CD45 staining is plotted.

5.3.4 Lipidoid/PLGA microparticle siRNA Knockdown in vitro

Chemical functionalities present in NA98-5, ND98-5 and NA111-7+6 demonstrated optimal balance factors equal or greater than Lipofectamine 2000, Figure 5.5. Because we were interested in developing a microparticle system for siRNA delivery we wanted to incorporate these functionalities to the microparticle surface. The possibility of encapsulating siRNA and lipidoid inside microparticles was ruled out because encapsulation efficiency is so low (Chapter 3), and because the surface association of siRNA with particles was efficient (Chapter 4).

To simulate the surface functionality of polymer coated c-dots (chapter 4) and lipidoid nanoparticles we developed a protocol that generated particles with lipid on the surface; this was accomplished by blending lipidoid with PLGA during a single emulsion process described in the materials and methods section.

The particles resulting from this approach were complexed with siRNA for GAPDH and incubated with P388-D1 cells. The results are shown below in Figure 5.8. We report remaining expression as before.

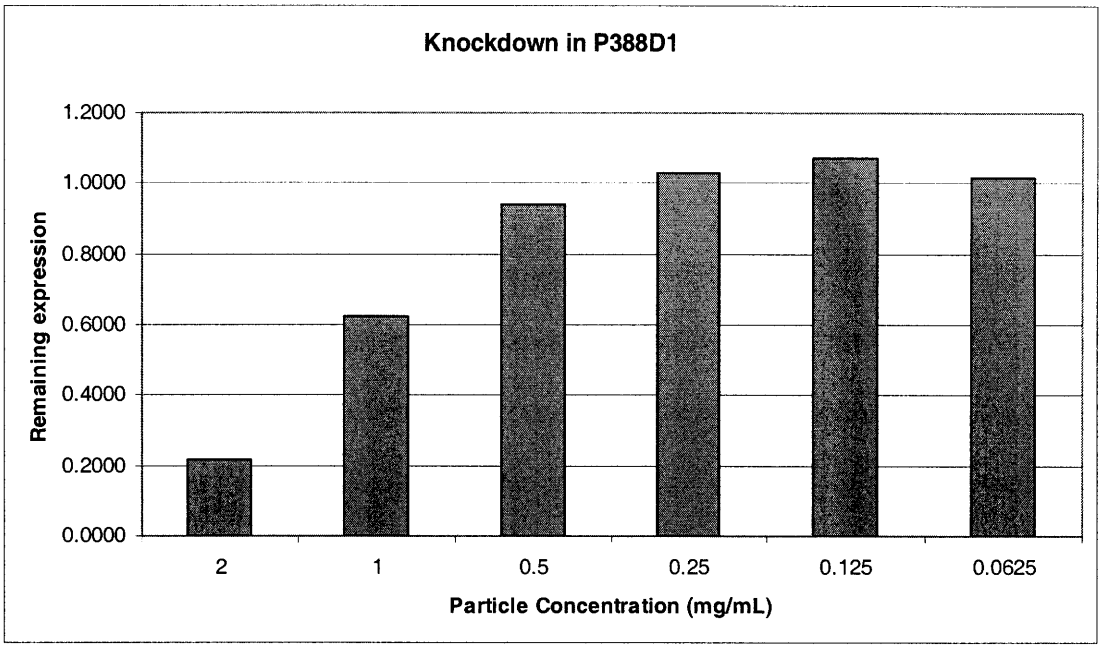


Figure 5.8 Microparticle-Lipidoid delivery system

Microparticles (20%ND98-5, 80%PLGA) were complexed with GAPDH siRNA and added to culture with P388-D1 cells. After 48 hours cells were lysed and GAPDH concentrations were determined with the KDalert assay.

This experiment was completed with microparticles formulated from both PLGA/ND98-5 and PLGA/ND95. The ND95 used on this experiment was in a less pure form than ND98 so it is an excellent control for toxcicity. Optimal Balance Factor for these two particles formulations is shown for both phagocytic P388-D1 cells and non-phagocytic COS-7 cells in Figure 5.9. Notice how the OBF for ND98 trends up as the concentration increases, while the OBF for ND95 trends down as the concentration increases. This can be explained by the additional toxicity demonstrated by ND95.

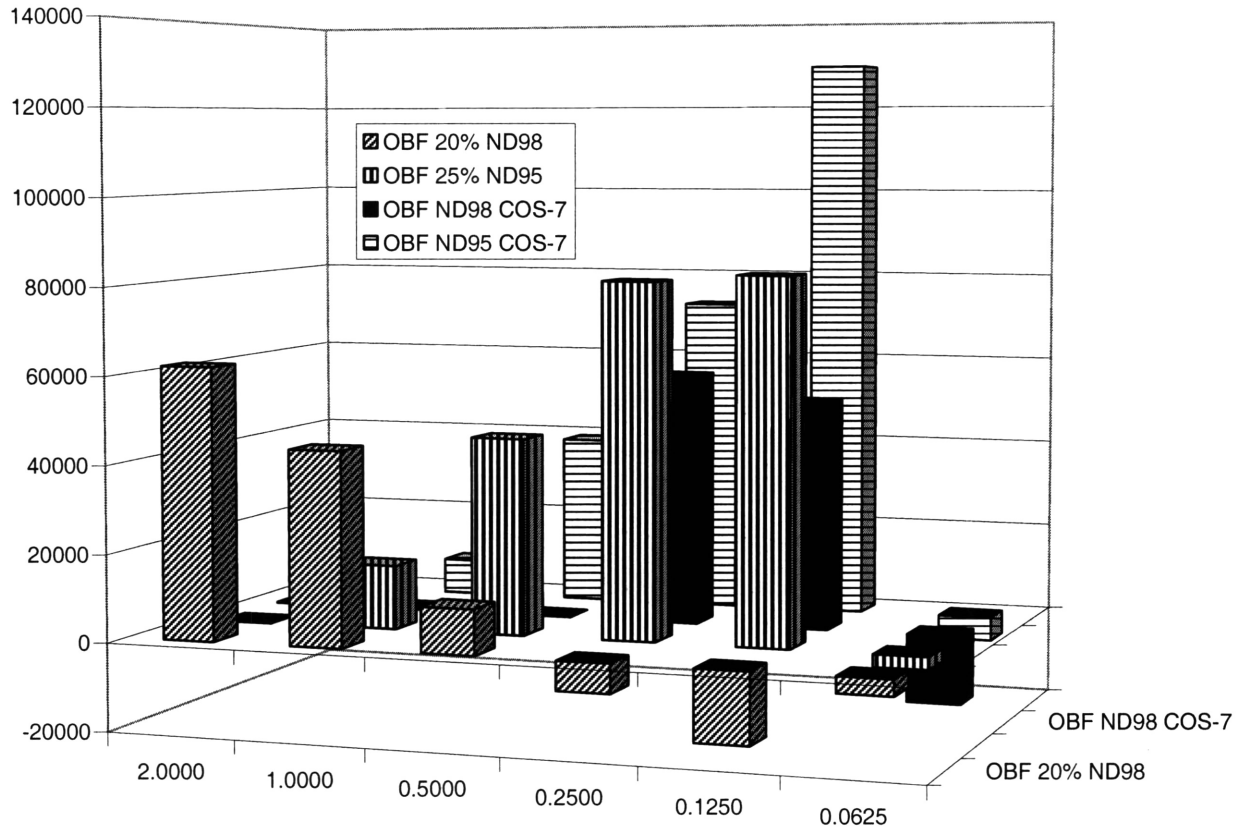


Figure 5.9 Optimal Balance Factor of microparticle knockdown

Microparticles containing the indicated percentage of selected lipidoids my mass (the balance PLGA) were complexed with siRNA and incubated with P388-D1 or COS-7 cells as noted in the figure.

We also tested the knockdown of microparticles in primary peritoneal macrophage see results in Figure 5.10. There was no statistically significant knockdown and error bars indicate a large standard deviation; this could be explained by the stability of the primary

macrophage being less than the cell line. It does appear that the toxicity of the microparticles is higher in the primary cells as compared to P388-D1.

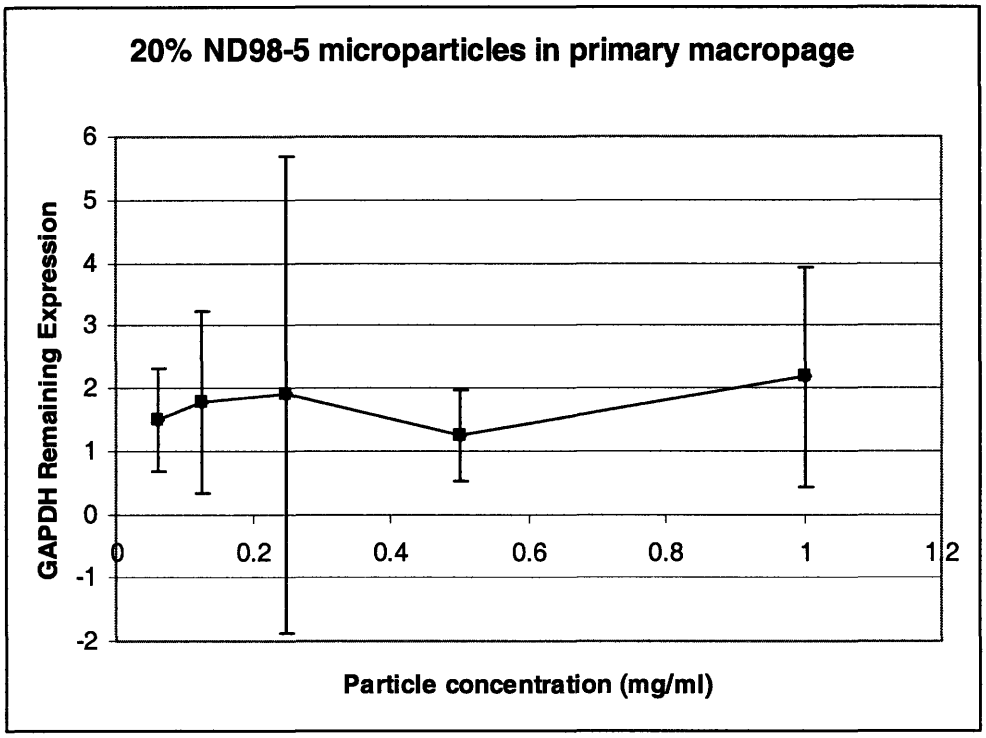


Figure 5.10 20% ND98-5 knockdown in primary macrophage

Microparticles formulated from 20% ND98-5 and 80% PLGA were complexed with siRNA specific for GAPDH and incubated with the cells at the indicated concentrations. 24 hours after transfection the cells were analyzed with the KDalert assay.

To determine if toxicity shown in Figure 5.10 was due to the microparticles or if the lipidoid and/or the siRNA were separating from microparticles and affecting the cells independently we performed an additional experiment. Microparticles were complexed with siRNA and then pelleted by centrifugation. The supernatant was collected and added to the cells. The results are shown in Figure 5.11. Notice that the remaining

expression of GAPDH is still greater than 1 and therefore appears that there is no knockdown observed.

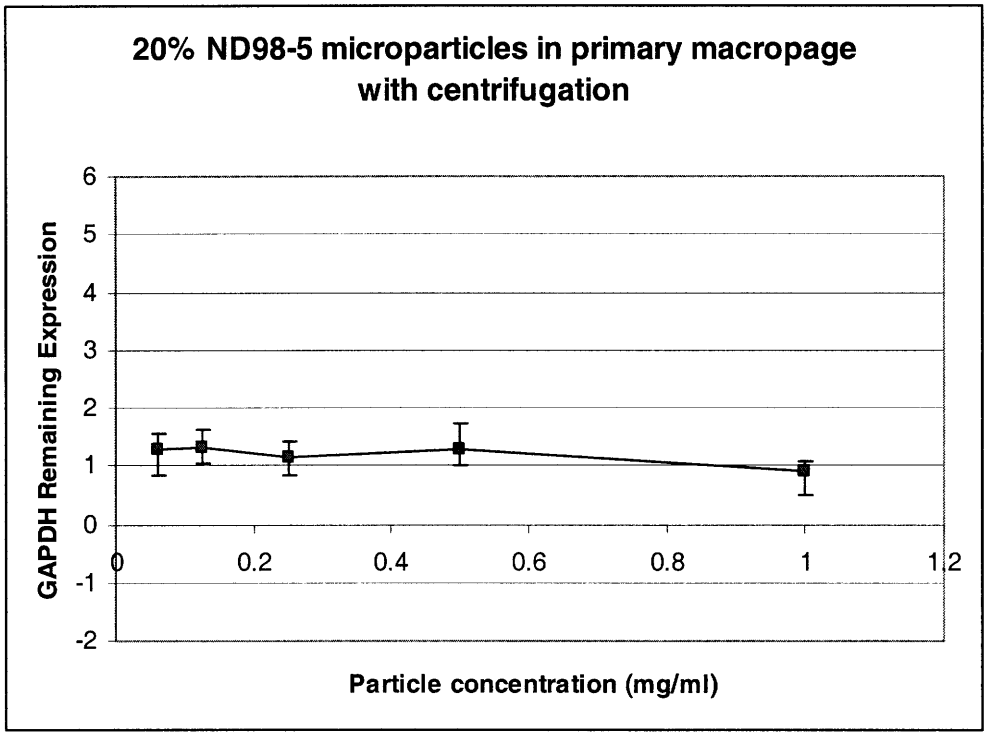


Figure 5.11 Knockdown in primary macrophage, supernate from ND98-5 containing microparticles

Microparticles containing 20% ND98-5 were complexed with siRNA and then pelleted by centrifugation, the supernatant was collected and added to cells. The cells were analyzed by KDAlert assasy 24 hours later.

The decreased variation in the supernatant samples could indicate that the particles were causing toxicity.

5.4 Discussion

The lipidoid nanoparticle system has demonstrated knockdown of 40% in P388-D1, 80% in primary macrophage and 65% in mouse macrophage in vivo while demonstrating favorable lack of toxicity as measured by GAPDH expression. This system could have broad application in the immune system as there are a number of applications, which are outlined in chapter 3.

This project has validated the high throughput screening approach as a method for identifying chemical and physical functionalities for drug delivery. The chemical structures of the most efficient lipidoids, NA98-5, ND98-5 and NA111-7+6, have some general chemical characteristics. They all contain primary and secondary amines, an attribute shared with PEI (chapter 4). This observation is in agreement with many hypotheses of endosomal buffering and escape[8, 9].

The microparticle based delivery of siRNA has remained a substantial challenge [10]. Recently there have been several advances in the delivery of siRNA using nanoparticles, even solid nanoparticles and other materials [11-13] but this has not been extended to microparticles.

Herein we report a method of using microparticles to deliver siRNA to antigen presenting cells and measured 80% knockdown. This approach could potentially be used to

passively target antigen presenting cells in vivo, and could therefore potentially be used to modulate the response to a genetic or protein vaccine, or otherwise modulate the immune system in vivo. In its current state, this system requires a large amount of siRNA and polymer to achieve knockdown. In comparison to the lipidoid nanoparticles formulations the microparticle system requires 100 times more siRNA to demonstrate similar knockdown. The best formulation with microparticles is 2mg/mL particles, 6.5 ug/mL siRNA while the best formulation with lipidoidisomes 16.5 ng/mL siRNA for the same transfection.

Because of requirements for so much siRNA and a seemingly impractical amount of polymer microparticles the microparticle system seems less practical than the lipidoid nanoparticles system.

5.5 Conclusions

We have demonstrated that the lipidoid siRNA delivery system developed by Akinc et al. can be effectively used to knock-down gene expression in phagocytic cell lines, primary cells and in vivo. This approach may be adapted to a therapeutic clinical system someday.

We also formulated lipidoids with PLGA in microparticles for targeted (passive) delivery of siRNA to antigen presenting cells. We showed 80% knockdown in the P388-D1 cell line and no measurable knockdown in primary mouse macrophage. Despite their ability to passively target antigen presenting cells Lipidoid/PLGA microparticles require 100X more siRNA to demonstrate similar knockdown when compared to lipidoid nanoparticles.

5.6 References

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6. isRNA and TLR activation of immune system

6.1 Introduction

Microparticles in the size range of 3-5um are phagocytosed, especially if their surface is cationic [1-4]. This chapter will focus on modulating the immune system by engaging receptors on the cell surface and inside phagosomes. Specifically this chapter will focus on the delivery of agents that will activate toll like receptors in antigen presenting cells to modulate their immune response.

Also, Chapter 7 will discuss the delivery of β-1,6-glucan to neutrophils for their activation. Both of these applications share similar characteristics; the goal is to deliver a biological agent, on the surface of microparticles to activate a receptor, either on the surface of cells or inside phagosomes.

Toll like receptors (TLRs) are pattern recognition receptors (PRR) which recognize molecules generally shared by pathogens but distinguishable from native host molecules. This class of molecules is called pathogen-associated molecular patterns (PAMPs). Toll like receptors recognize bacterial DNA [5], double stranded RNA [6], single stranded RNA [7, 8] and other (non-nucleic acid) PAMPs [9-12].

Many groups have studied the therapeutic potential of agonists for the toll like receptors [13-17] and in particular TLR9 agonists have been intensely studied as potential therapeutics. Some have attempted to use mono-clonal antibodies to activate TLR[18],

while others have used CpG motifs [14] or isRNA[19, 20]. CpG motifs target TLR9 activation while, isRNA targets primarily TLR3, TLR7 and TLR8 to induce a type I interferon response[20]. In both DNA and RNA approaches sequence, length and extent of hybridization determine how well the PAMP is recognized by the TLR and how well the approach will activate the immune response.

Although several labs and companies have studied the effects of CpG motifs and isRNA sequences on TRL activation there has been little publication in the academic literature on technologies optimized for the delivery of these compounds to the cell surface or to phagosomes where they can engage TLR and induce an immune response[21]. The isRNA mechanism has often been considered a side effect or obstacle to siRNA delivery[22].

It is possible that a delivery system optimized to passively target antigen presenting cells and deliver known isRNA sequences to the phagosomes could be valuable for generating immune modulation.

6.2 Materials and methods

6.2.1 Surface coating of microparticles with antibody

PLGA microparticles were synthesized as described earlier. After lypholization microparticles were resuspended in buffer containing monoclonal antibody for the given TLR and allowed to incubate overnight at 4^0 C. This was an adaptation of a published protocol[23].

6.2.2 Isolation of natural killer cells

B6 mice age 6-12 weeks were sacrificed and spleens were removed. Spleens were then pulverized and ground against a screen using the plunger using the plunger from a 5 ml syringe. Screen were then rinsed into a petri dish and splenocytes were then passed through a cell strainer to remove any large clumps of cells. The isolated splenocytes were then purified to naturally killer cell populations using the NK Cell Isolation Kit from Miltenyi Biotec. Untouched NK cells are isolated from splenocytes by depletion of non-NK cells. B cells, T cells, dendritic cells, macrophages, granulocytes, and erythroid cells are indirectly magnetically labeled using antibodies against CD19, CD4, CD8a, CD5, Gr-1, and Ter-119. These magnetically labeled cells are removed using a magnetic sorting column and the remaining cells, highly enriched in NK cells are collected for use in experiments.

6.2.3 Isolation of bone marrow derived dendritic cells

B6 mice age 6-12 weeks were sacrificed and femurs and tibias were removed. Using C10 media marrow was flushed from the bones. After being collected red blood cells were lysed from the bone marrow using RBC lysis buffer. Cells were then plated in petri dishes using C10 media supplemented to rGM-CSF. After 6 days of culture cells were harvested and prepared for experiments.

6.2.4 Microparticle formulation

PLGA microparticles were prepared by modified double emulsion. In this protocol the inner aqueous phase was eliminated and to instead allow a single emulsion process. PLGA and lipid was dissolved in methylene chloride. Solution was added to a homogenized solution of poly(vinyl alcohol) (50 ml, 5% PVA (w/w), 5000 rpm) or Poly(ethylene alt maleic anhydride) (PMEA) or a mixture of PVA and PMEA at the designated ratio. After 30 s, 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 h at room temperature. Microspheres were washed and centrifuged 3X to remove PVA prior to lyophilization.

6.2.5 Cell based type-1 interferon assay

This assay was developed by David Nguyen in the Langer Lab and will soon be published. Briefly a cell line was developed which fluoresces in the presence of type-1 interferon. Nguyen demonstrated that there is a linear range of detection for this assay over several orders of magnitude when cells were analyzed by flow cytometry. We added supernatants from PBMCs incubated with microparticles to these cells and analyzed them to determine the concentration of type-1 interferon.

6.3 Results

6.3.1TLR antibody modified PLGA microparticles activate NK/DC

Several recent reports demonstrate that natural killer cell cross-talk with dendritic cells is an important interaction that leads to natural modulation of the immune system [24, 25]. We believed this could be an exciting approach to controlling immunity.

We studied the activation of NK cells using antibody for CD27 and NK1.1, two surface markers known to activate NK cells [26]. Following the protocol developed by Takeda et al. we demonstrated that culture of primary murine NK cells culture in monolayers on surfaces modified with anti-CD27 and anti-NK1.1 demonstrated increased proliferation as show in Figure 6.1. This result was expected given the results described by Takeda et al. [26].

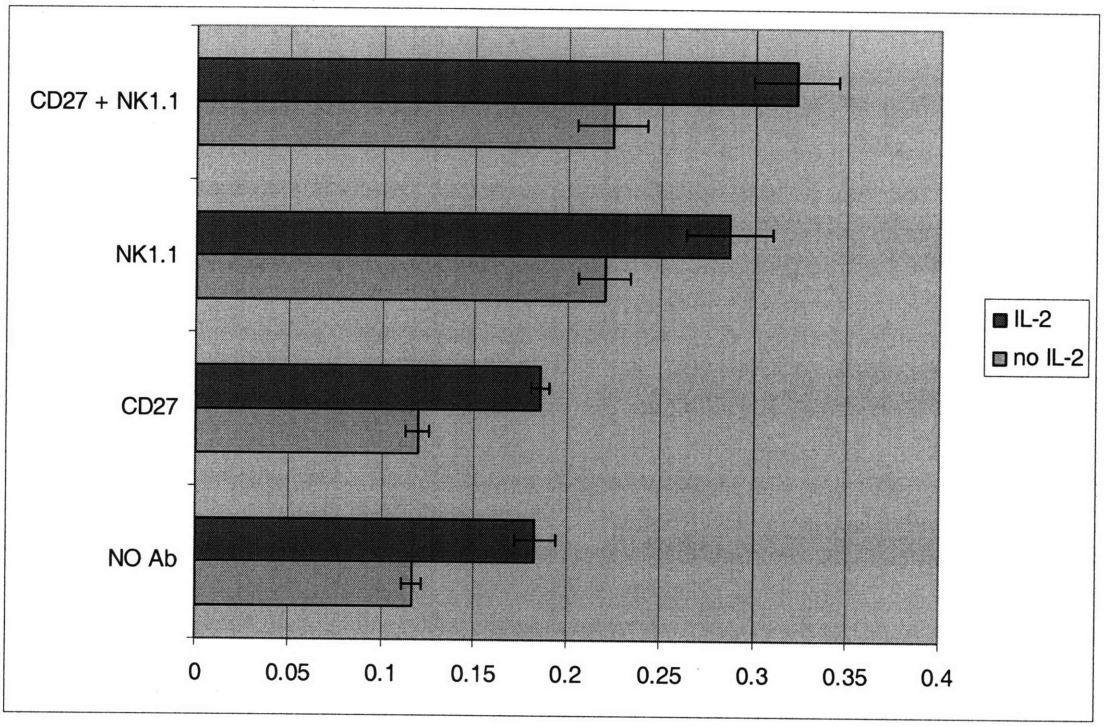

Figure 6.1 Proliferation of murine NK cells activated anti-CD27 and anti-NK1.1

Primary NK cells were isolated from spleens of mice and cultured in monolayers with the indicated antibodies adsorbed to their surface. After 24 hours metabolism was measured by MTT assay.

Next we examined the response of NK cells to microparticles coated with the same antibodies. We coated microparticles with the appropriate antibodies as described in the materials and methods sections and added these coated microparticles to NK cells in culture.

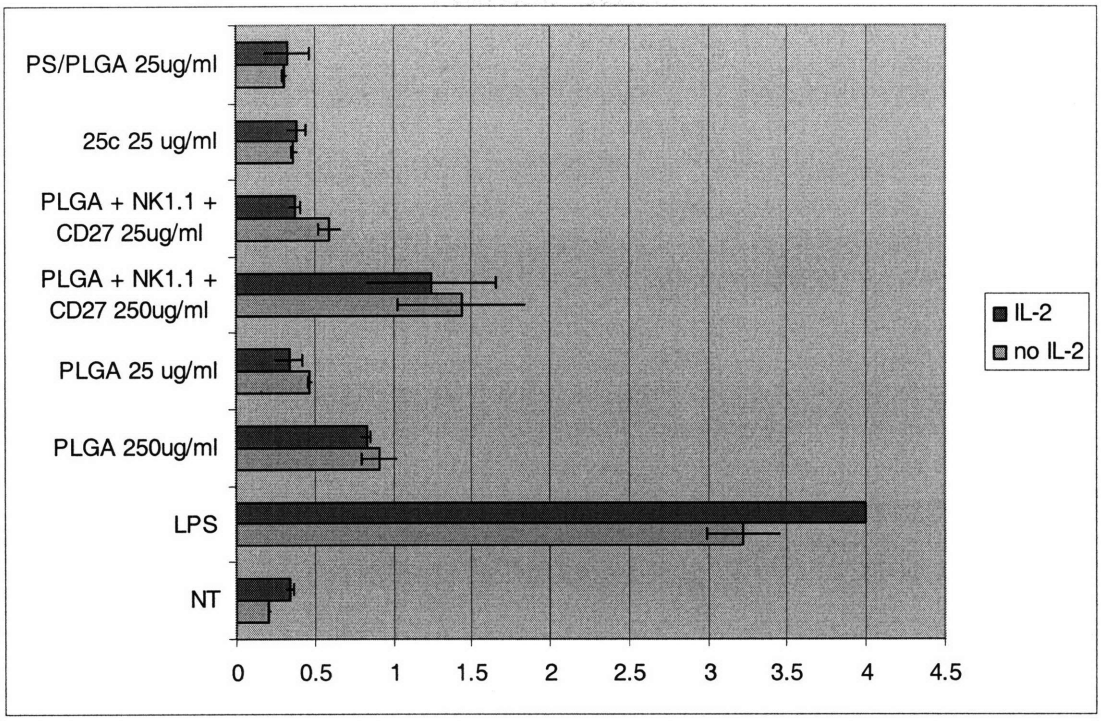

Figure 6.2 NK cells activated by CD27 and NK1.1 on microparticles

NK cells were isolated from mice and cultured. Microparticles were added to NK cells in culture and proliferation was measured by MTT assay after 48 hours.

Results shown in Figure 6.2 indicate that microparticles coated with NK1.1 + CD27 induced metabolism among the natural killer cells. This result indicated that these particles could potentially be used to activate DC. To further study the potential of NK cells to activate dendritic cells we measured the concentrations of cytokines commonly associated with NK cell activation and subsequent cross-talk leading to immune response[25, 26]. We examined expression of INF-g as this is an autocrine indicator of NK cell activation.

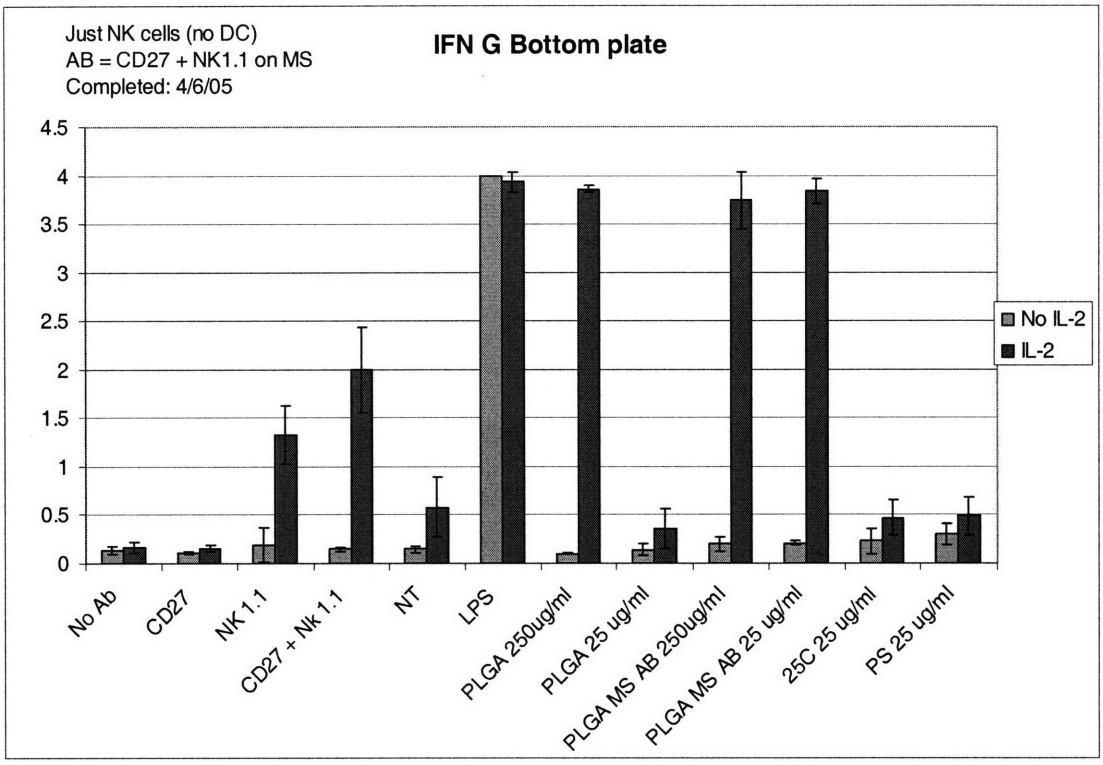

Figure 6.3 INF-g expression by NK cells incubated with microparticles

NK cells were isolated from mice and cultured. Microparticles were added to NK cells in culture and IFN-g expression was measured by ELSA after 48 hours.

The expression of IFN-g is shown in Figure 6.3. It appears that incubation with particles leads to significant IL-2 dependent expression of IFN-g.

In the following experiments, we tested the hypothesis that a particle system could activate interferon response in dendritic cells in vitro. We chose to modify the surface of microparticles with antibodies by absorption as described in the materials and methods section.

We first isolated bone marrow derived dendritic cells (BMDC) from mice as outlined in the materials and methods section. These cells were incubated with microparticles coated with antibodies for toll like receptors, TLR4, TLR6, TLR7 and TLR9 which are expressed in all subsets of murine DC [25]. Although TLR4 is reported to be expressed in murine DC by several reports there is some conflicting data [27, 28].

Results of the experiment are shown in Figure 6.4. All anti-TLR antibody coated microparticles induced DC to activate natural killer cells leading to increased proliferation when co-cultured. TRL9, TLR4 and TLR6 were the most efficient in educating DC to induce natural killer cell proliferation in vitro. This indicates that particles capable of agonizing toll like receptors may be effective at inducing a natural killer response in vivo or perhaps other immune response mediated dendritic cell cross talk.

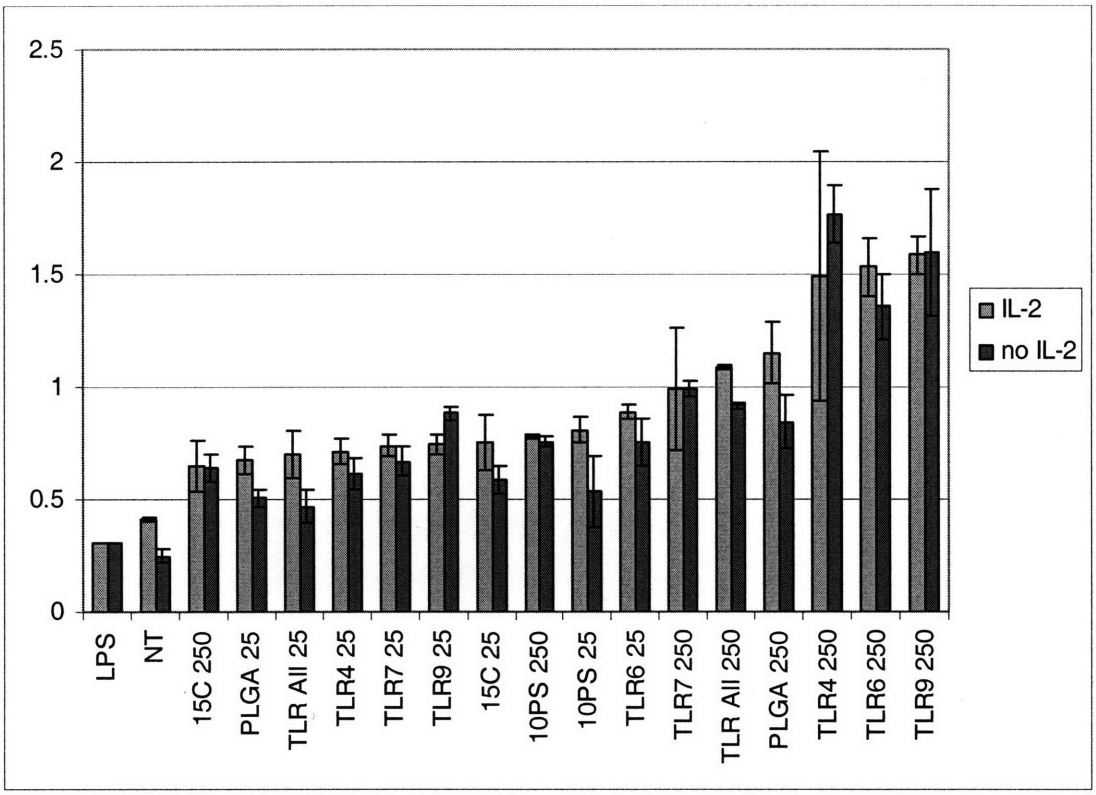

Figure 6.4 BMDC activate NK cells after activation by TLR angonist microparticles

BMDC were harvested and differentiated, microparticles with the indicated antibodies absorbed to their surface were added to the culture before co-culturing with natural killer cells. After 48 hours of co-culture proliferation was determined by MTT assay.

Finally we also measured the expression of INF-g by the natural killer cells during co-culture with the microparticle activated BMDC. The results of the second co-culture are shown in Figure 6.5.

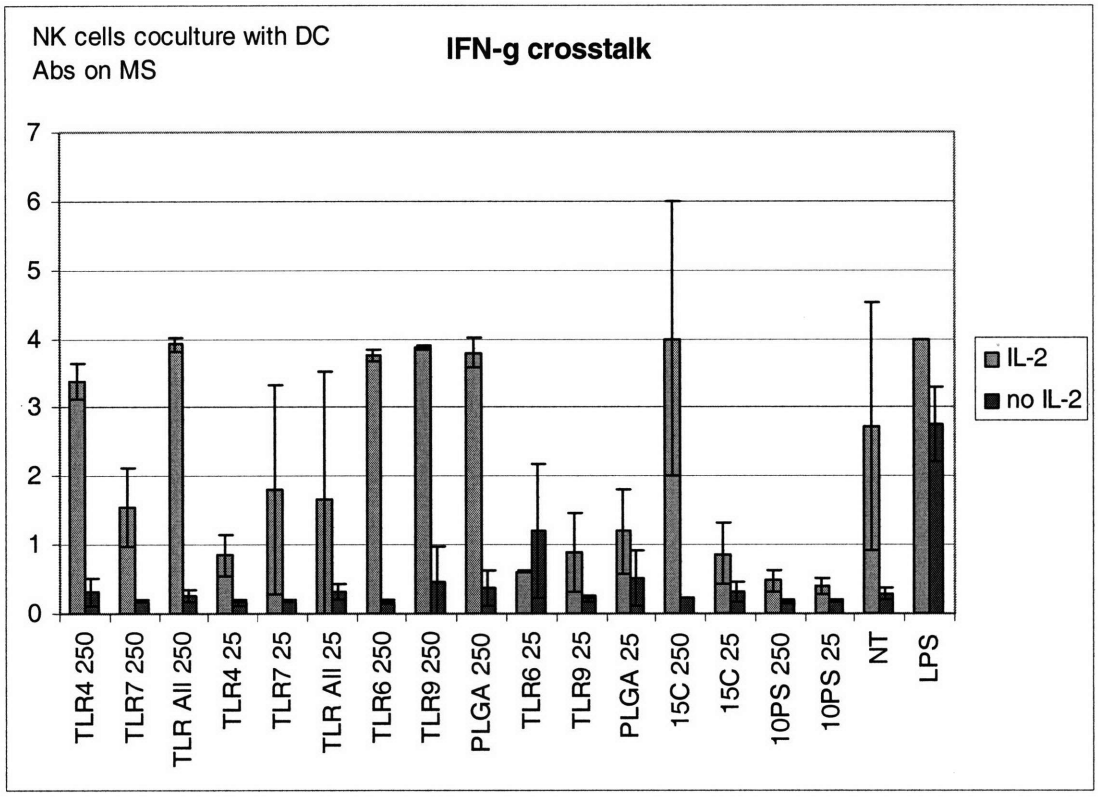

Figure 6.5 Activation of NK cells when co-culturesd with BMDC

BMDC were harvested and differentiated, microparticles with the indicated antibodies absorbed to their surface were added to the culture before co-culturing with natural killer cells. After 48 hours of co-culture IFN-g was measured by ELISA.

Natural killer cells are again activated by DCs previously pulsed with TLR agonizing microparticles in an IL-2 dependent manner. This result is not surprising given the previous results that the same dendritic cells induce proliferation of the natural killer cells.

6.3.2 isRNA delivery with lipidoid/PLGA microparticles

After completing the proof of concept data with the antibody system we revisited the idea of using the siRNA microparticle delivery system described in the previous chapter for the delivery of isRNA to activating TLR. Because isRNA is structurally similar to siRNA we were hopeful that the delivery system we developed for siRNA would also be applicable to isRNA. To test this hypothesis we used microparticles containing ND98-5 or ND95 as described in the previous chapter and complexed them with RNA sequences: GFP siRNA, beta Gal siRNA, Coley 1362 or Coley 1263. Coley 1362 is a known isRNA sequence, the others are control sequences. After complexing the microparticles with RNA they were incubated with human peripheral blood mononuclear cells (PBMC) and then analyzed by cell-based type 1 interferon assay. The results are shown in Figure 6.6 and Figure 6.7.

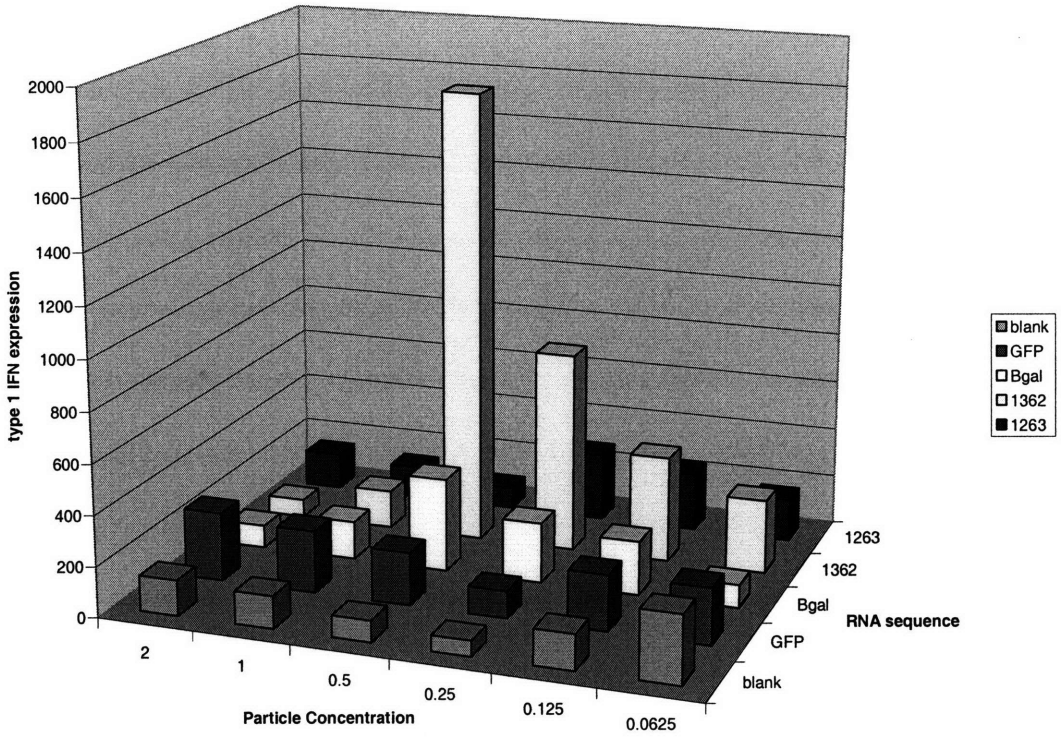

Figure 6.6 20% ND98-5 particles deliver isRNA

Microparticles formulated with 20% ND98 and 80% PLGA complexed with the indicated RNA were incubated with PBMC and supernatant was then analyzed for type-1 IFN

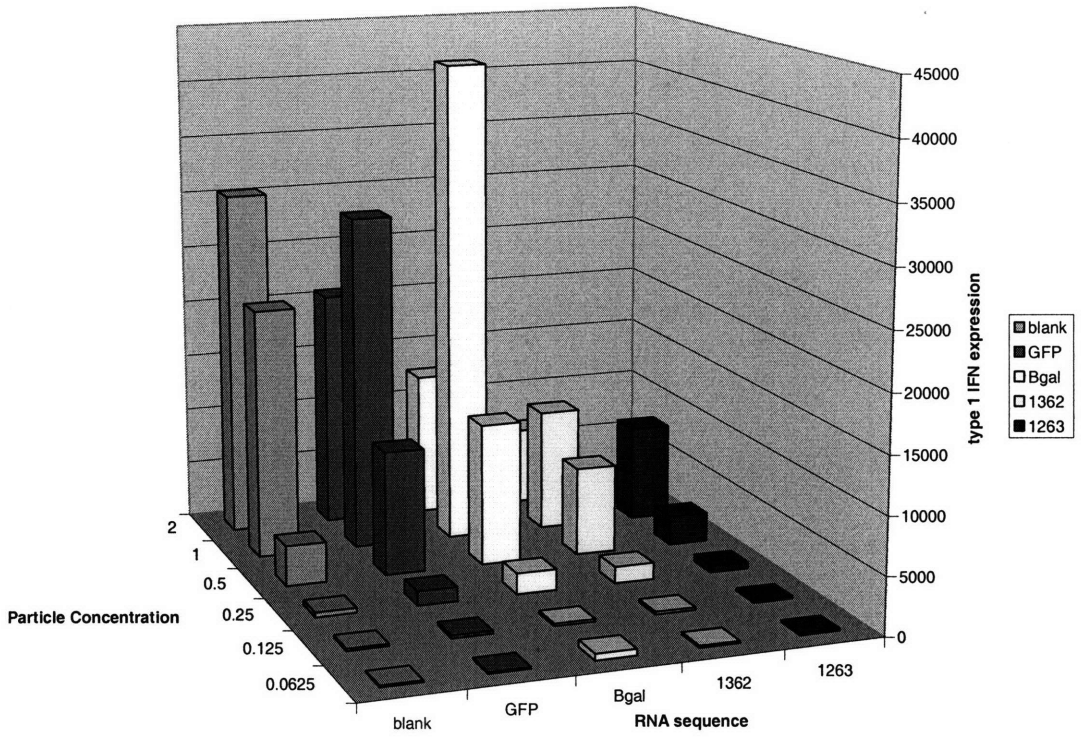

Figure 6.7 25% ND95 microparticles deliver isRNA

Microparticles formulated with 25% ND95 and 75% PLGA complexed with the indicated RNA were incubated with PBMC and supernatant was then analyzed for type-1 IFN

The results indicate that microparticles (~3 um) containing the purer ND98-5 appear to activate PBMC in a sequence specific manner, however it appears that the activation of PBMC by ND95 containing particles is non-specific and at a much higher level, indicating that the material itself may likely be activation the cells.

6.4 Discussion

The focus of this chapter was to explore the possibility of using lipidoid/PLGA microparticles to deliver RNA or other payloads to cells to trigger activation or otherwise modulate the immune response. As an initial proof of concept we first considered the delivery of agonistic antibodies on the surface of PLGA microparticles to activate natural killer cells or to activate dendritic cells through TLR. Later in the chapter we used lipidoid/PLGA microparticles to deliver known isRNA sequences which we obtained from Coley Pharmaceuticals.

These Lipidoid/PLGA microparticle approaches could prove to be interesting alternative options for delivering isRNA therapy. isRNA is an area of intense study and there has been little effort to develop or optimize delivery systems for isRNA. In fact isRNA was considered an undesired side effect of siRNA until only very recently.

Perhaps these proof of principle experiments will continue to generate interest around the use of microparticle approaches to activating the immune system using isRNA. The combination of microparticles with immune activation agents such as isRNA is a logical choice because microparticles themselves are often used as an adjuvant in vaccinations[29-31].

6.5 Conclusions

We have demonstrated that antibodies can be adsorbed on the surface of PLGA microparticles and subsequently used to activate cells as agonists. We have also shown that TLR4, TLR6, TLR7 and TLR9 antibodies can be used in this manner (adsorbed on microparticles) to educate dendritic cells to activate natural killer cells. We also showed that the lipidoid/PLGA delivery system could be used with isRNA; in the case of ND98-5 coated microparticles sequence specific isRNA was demonstrated.

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7. Biodegradable mircroparticles encapsulating β-1,6-glucan treat systemic fungal infection in vivo

7.1 Introduction

Once thought to be of little relevance, fungal diseases have exploded as a clinically significant problem over the past several decades[1, 2]. With the growing number of immune compromised transplant recipients, cancer patients and individuals infected with HIV the incidence and prevalence of human infection caused by fungi has dramatically increased [3-5]. Mortality rates remain over 50% in most studies and have been reported as high as 95% in bone marrow transplant recipients[6].

Unlike bacteria eukaryotic fungi share many biological mechanisms with humans making it more difficult to find therapeutics without toxicity[7]. There is no current clinical vaccine for the prevention or treatment of fungal infection by activating the immune system[6]. However some research progress has been made in modulating the immune system to prevent fungal infection[4] and ultimately the manipulation of the immune system may be a promising approach to treating fungal infections[6].

Yeast whole glucan particle (WGP) has been demonstrated to induce proinflammatory cytokine conditions [8] by activating macrophage and has been suggested and used as an immune stimulatory compound[9, 10] to activate neutrophils and treat tumors in combination with monoclonal antibody[10]. However, Rubin-Bejerano et al. recently

demonstrated that a single component of fungal cell wall, β-1,6-glucan, is the primary component which induces neutrophil phagocytosis and expression of reactive oxygen species (ROS). Moreover, when absorbed on the surface of a microparticle, β–1,6-glucan has been shown to strongly bind complement C3 [11].

Previous *in vitro* experiments by Rubin-Bejerano have focused on the use of non-degradable microparticles which are not a realistic option therapeutically[11]. The development of a of biodegradable microparticle system expressing β–1,6-glucan on its surface could open a new mechanism of treating and preventing microbial infection especially in patients with compromised immune systems or mild? neutropenia.

7.2 Materials and methods

7.2.1 Polymers

Poly(*d,l*-lactic-co-glycolic acid) polymer (PLGA, RG502H Resomer 50 : 50) was purchased from Boehringer Ingelheim (Ingelheim, Germany). Poly(vinyl alcohol) (Mw = 25 kD) was purchased from Polysciences Inc. (Warrington, PA). β–1,3-glucan (Laminarin from Laminaria digitata) was purchased from Sigma, B–1,6-glucan (pustulan from Umbilicaria papullosa was from Calbiochem).

7.2.2 Preparation of microparticles

PLGA microparticles were prepared by modified double emulsion[12]. Lyophilized β–1,6-glucan was dissolved in Dimethyl Sulfoxide (DMSO) at room temperature (50mg/mL) and diluted with an equal volume of deionized water. This mixture was added to a solution of PLGA in Dichloromethane under sonication with a probe sonicator. The resulting suspension was added to a solution of 1% polyvinyl alcohol in water and homonogized to generate polymer microparticles. Evaporation of Dichloromethane proceeded during 3 hours of stirring at atmospheric pressure and room temperature. The resulting particles were washed 3X with deionozed water and lyophilized to give a dry powder.

7.2.3 Scanning Electron Microscopy

For scanning electron microscopy (SEM) analysis, particles were dried on glass cover slips coated with gold and imaged using a Hitachi S-4800 FESEM.

7.2.4 Microparticle Characterization

To determine release kinetics microparticles were suspended in PBS pH 7.2 (PBS) and incubated at 37^0 C with mixing. Particles were isolated by centrifugation at the specified time point and the supernatant was collected and analyzed for sugar content by the phenol-sulfuric acid method[13]. Particles were resuspended and returned to 37^0 C with mixing.

7.2.5 Preparation of human neutrophils

Neutrophils were isolated from fresh human blood [14] collected from healthy volunteers in accordance with a protocol approved by the MIT Committee on Use of Humans as Experimental Subjects.

7.2.6 Reactive oxygen species assay

ROS production was assayed using DHR123 (Molecular Probes) as described before [14].

7.2.7 Surface exposure of β-1,6-glucan

Particles were suspended in PBS and allowed to incubate at 37^0 C for the indicated time (zero or three days). They were then incubated in 2% bovine serum albumin to block

non-specific antibody binding. Exposed β–1,6-glucan was detected by FACS following indirect immunofluorescence with a polyclonal anti-β-1,6-glucan antibody.

7.2.8 Mouse survival experiments

$5x10^6$ cells of the pathogenic fungus *Candida albicans* (starin CAF2-1) were injected into the tail vein of 8 weeks old C57BL6 mice. When indicated, the mice were also injected with β-1,6-glucan containing microparticles.

7.3 Results

7.3.1 Solubility of β–1,6-glucan in double emulsion solvent systems

Because the activation of neutrophils by β–1,6-glucan is mediated through particle surface binding of complement C3 [11] the ideal particles will display the β-glucan on their surface as they degrade. Several challenges exist in attempting to generate polymer microparticles which encapsulate and consistently display β–1,6-glucan on their surface. The structure of linear β–1,6-glucan is shown in Figure 7.1.

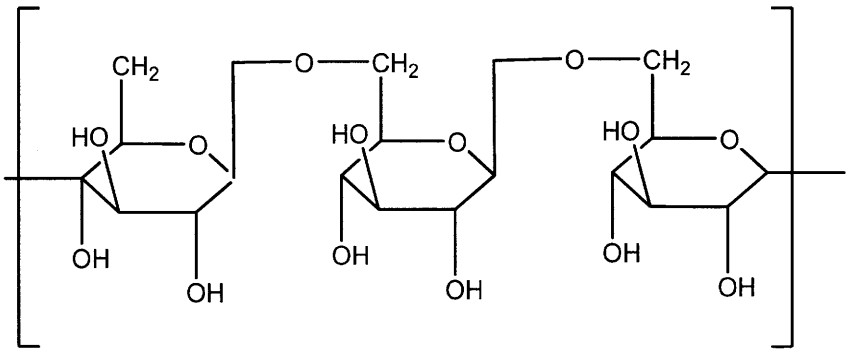

Figure 7.1 Linear β-1,6-glucan

Typical molecular weight 20,000 Da

This polysaccharide demonstrates limited solubility in water (less than 10 mg/ml at room temperature) and in methylene chloride (less than 10 mg/ml at room temperature). To co-encapsulate β–1,6-glucan with a biodegradable poly lactic co glycolic acid (PLGA) polymer using a standard single solvent emulsion technique requires the identification of

a solvent which is immiscible with water and able to solubilize both PLGA and the glucan[12, 15, 16]. Such a solvent was not identified. Dimethyl Sulfoxide (DMSO) was the only solvent found capable of dissolving concentrations high enough to expect reasonable encapsulation (data not shown).

Thus, we developed a nanoprecipitation-suspension encapsulation procedure as described in the materials and methods section. In this protocol b-glucan in DMSO is added to PLGA in methylene chloride solution creating a glucan nanoprecipitate suspension. This nanoprecipitation was then added to an aqueous solution and homonogized and after solvent evaporation and lyophalization generates polymer particles on the order of 1 um as shown in Figure 7.2.

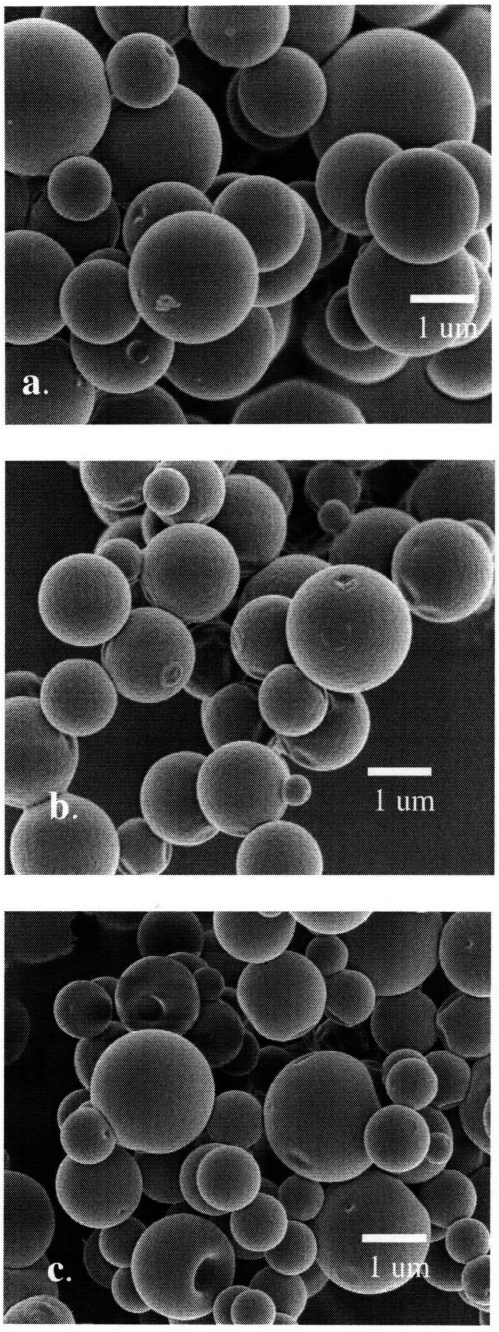

Figure 7.2 Polymer Microparticles

SEM micrographs of PLGA microparticles: (a) blank or (b) encapsulating β–1,6-glucan (c) encapsulating β–1,3-glucan

7.3.2 Encapsulation and release kinetics

To determine the b-glucan encapsulation efficiency and release kinetics particles were allowed to degrade in aqueous solution and sugar content was analyzed by the phenol sulfuric acid method as described in the materials and methods section. Both β–1,6 and β–1,3-glucan are encapsulated at greater than 10ug/ 5mg of particles with encapsulation efficiencies of 1.2% and 3.7% respectively. B–1,3-glucan was encapsulated more efficiently; this is likely because of it's more favorable solubility in water. Figure 7.3 shows the results for kinetics of release over several days.

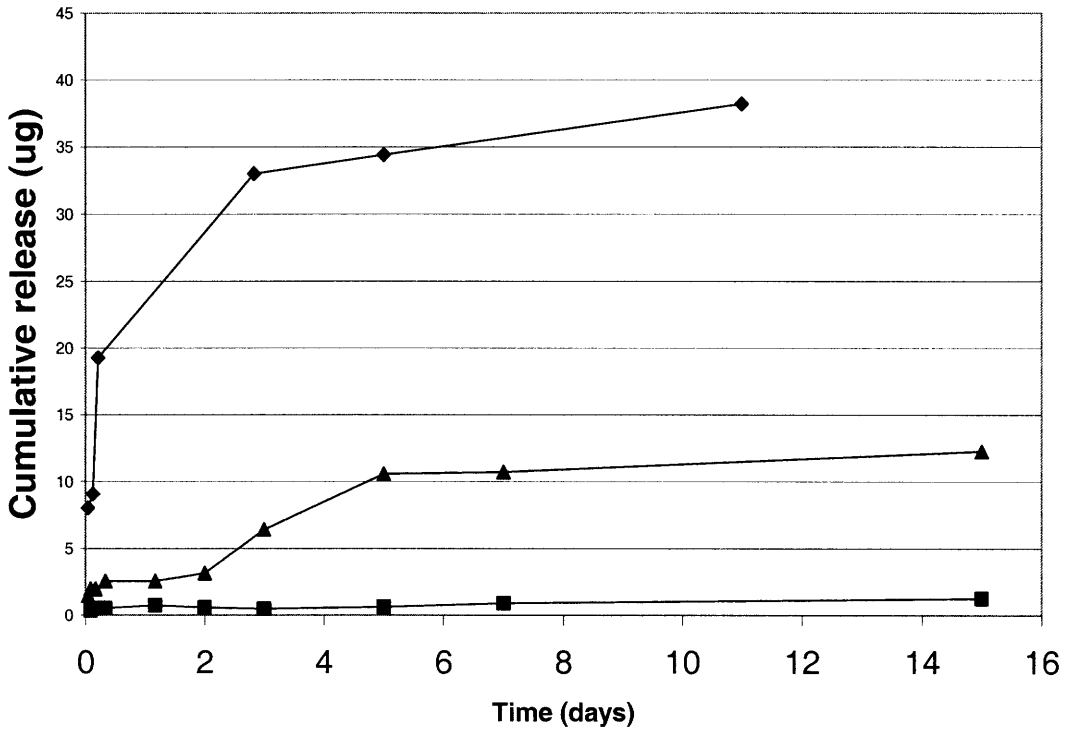

Figure 7.3 Release kinetics of encapsulated glucan

Particles containing β-1,6 glucan (triangles), β-1,3 glucan (diamonds) and blank (square) particles release their payload with the above kinetics

Both β−1,3-glucan and β−1,6-glucan are released in an initial burst release which lasts for a few hours. This burst is followed by a phase of slower more steady release over the next several days. As the particles were degrading over the time periods shown in Figure 7.3.

7.3.3 Glucan surface display

Since surface display of β−1,6-glucan is required for compliment mediated activation [11] the surface of the particles were tested for the presence of the glucan. As show in the encapsulation kinetics a burst release occurred within the first few days of incubation in aqueous media at 37^0C. To show that a significant amount of β−1,6-glucan remained on the surface of particle after the completion of this burst release phase, particles were incubated for 3 days and then stained with anti-β−1,6-glucan antibody and read with FACS analysis, results of FACS analysis are shown in Figure 7.4.

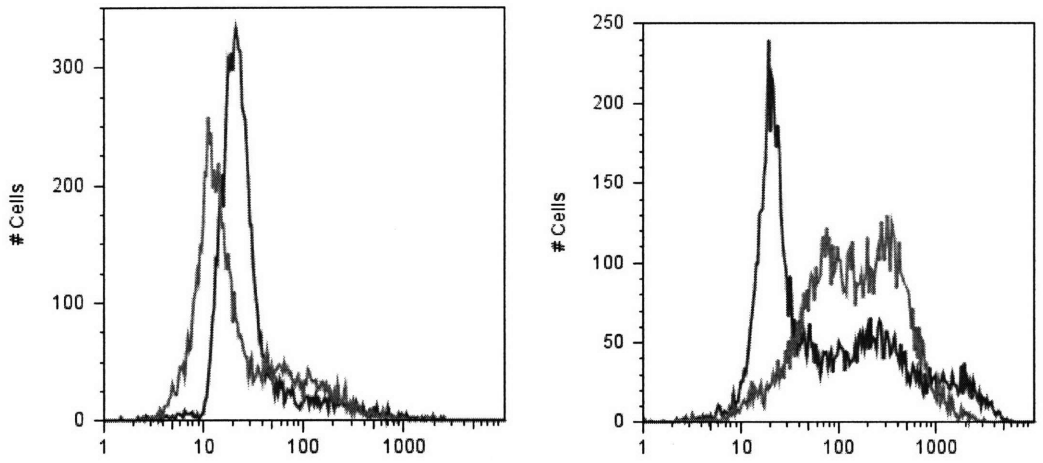

Figure 7.4 Microparticles displaying b-ı,6-glucan induce expression of Reactive Oxidative Species (ROS) in Human Neutrophils in vitro

Human Neutrophils were treated with particles (a.) and particles incubated in phosphate buffered saline for three days (b.). Particles containing B 1,6 glucan (green) induce a significant increase in ROS expression only after being incubated in PBS for three days.

As you can see 43.3% of the particles were stained positive for the presence of β−1,6-glucan on their surface after three days of incubation verses 1.5% for the control. This result suggests that the particles will also be capable of binding complement C3 and therefore activating neutrophils to increase expression of reactive oxygen species.

Activation of Human Neutrohpils

To test the ability of the particles to induce ROS in primary neutrophil they were first opsionized and then incubated with human blood. To understand the dynamics of burst release phase the particles were tested at time zero or after incubation in 37^0C buffer for three days. Results are shown in Figure 7.5.

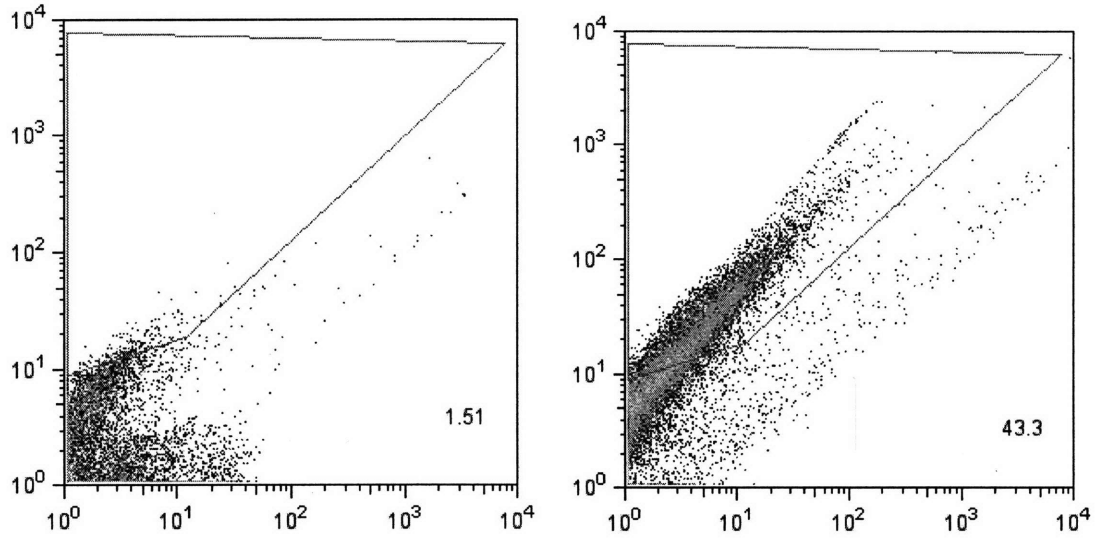

Figure 7.5 b-1,6-glucan is displayed on the surface of micro-particles after three days

Particles were incubated for three days in PBS at 37^0C then analyzed for surface expression of β–1,6-glucan with β–1,6-glucan specific antibody analyzed by FACS analysis.

It was shown that β–1,3-glucan weakly activates human neutraphil both at time zero and after three days of incubation. Microparticles encapsulating β–1,6-glucan however activate ROS in nutriphils after 3 days of biodegradation as induced by incublation in aqueous buffer. We believe that a short period of incubation actually increases the expression of β–1,6-glucan on the surface of the particles since the initial surface erosion of the surface surfactant layer and polymer allows for the exposure of the glucan molecules. Figure 7.6 shows surface morphology of particles after 3 days of incubation. Other factors which may contribute to the additional activation are size differences due to aggregation, differences in surface properties such as charge and surface geometry (data not shown).

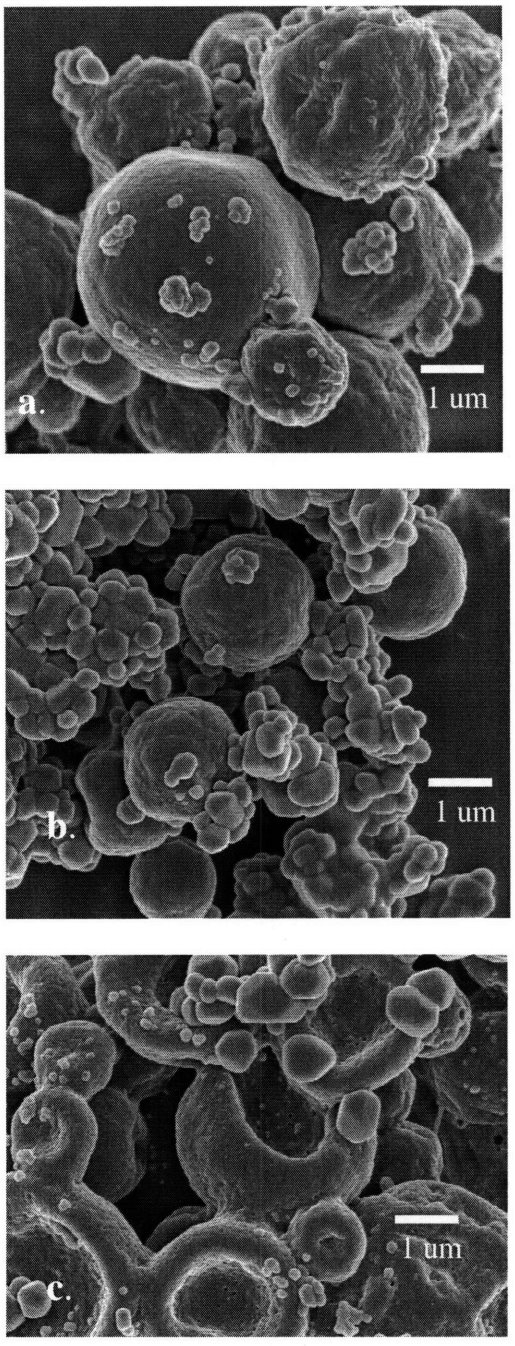

Figure 7.6 Degradation of microparticles after 3 days

SEM micrographs of microparticles incubated with mixing at 37^0 C for the indicated times. Crystals of salt from residual buffer can be seen as samples were dehydrated prior to analysis. (a) blank or (b) encapsulating β–1,6-glucan (c) encapsulating β–1,3-glucan

7.3.4 Treatment of candida albicans in vivo

To examine the utility of this approach in activating the immune system against a microbial infection we used an animal model of candida albicans blood stream infection. Mice were treated with a lethal dose of candida infection and also treated at the same time with microparticles encapsulating either β–1,6-glucan or β–1,3-glucan. Mice were then monitored for survival. Data is show in Figure 7.7. In both the non-treated control and the sample treated with particles encapsulating β–1,3-glucan all mice eventually died. However, in the case of mice dosed with β–1,6-glucan containing particles 3/5 mice survived the experiment.

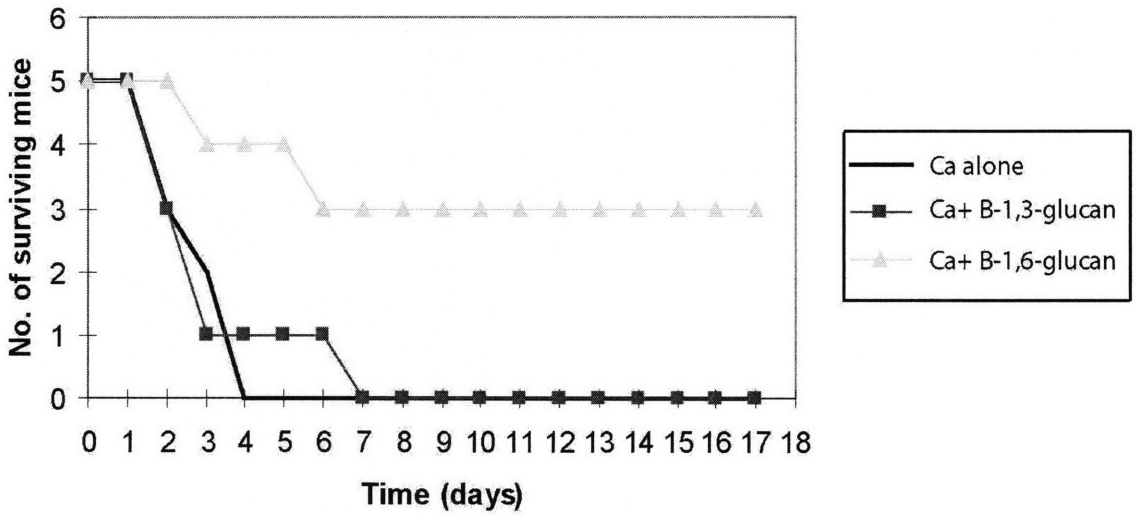

Figure 7.7 Microparticles displaying b-1,6 glucan treat Candida albicans in mice

At time zero mice were dosed with a lethal dose of candida albicans ($1X10^6$ units) and with $1X10^5$ microparticles containing either β–1,3-glucan, or β–1,6-glucan and examined daily for survival.

7.4 Discussion

The successful encapsulation and surface disply of β−1,6-glucan has been demonstrated and response to in vivo Candida infection has been illustrated. We believe this approach may potentially be valuable as a method of treating patients with neutrapenia as a result of many common immune disorders.

β−1,6-glucan was also encapsulated in particles with diameters less than 500 nanometers. Theses nanoparticles were not effective inducing ROS experession in vitro (data not shown); also soluble β−1,6-glucan did not induce activation of ROS (data not shown). Therefore, we believe that the microparticle approach demonstrates the best proof of principle. There are however some limitations to using intravenous delivery of microparticles [17, 18] related to complications with blood clotting and regulatory limitations of microparticle infusions. This is a consideration with intravenous delivery of microparticles; however, particles in the size range demonstrated here <5um have been used in other approaches such as contrast agents [18].

As shown by Rubin-Bejerano et al. the mechanism of neutriphil activation by β−1,6-glucan is complement C3 dependent and not shared by β−1,3-glucan [11]. We therefore used encapsulated β−1,3-glucan as a negative control for the in vivo application in Candida infection to demonstrate that this effect was specific to the C3 binding mechanism. Chitin microparticles were shown to activate macrophage to increase ROS production[19]. However this mechanism was specific to Chitin and did not occur with chitosan or latex beads.

This simple proof of concept validates the use of β−1,6-glucan as a method of modifying immune response against Candida during infection. We believe that this approach may

also be relevant for the prevention of infection in immune compromised patients or those with acute injury at high risk of infection such as burns, surgical wounds or battle-field wounds. Additional formulations may also be developed for local delivery to wounds or ulcers which may become infected.

Current treatments of Candida infections include iv amphotericin B, iv or oral fluconazole [20]. However these approaches have become less and less effective recently as more and more patients with compromised immune systems from HIV, chemo therapy induced neutropenia has increased. In each of these immune compromised situations the normal balance between host and pathogen is disrupted and shifted into the favor of the pathogen. Every current anti-microbial therapy focuses on treating the microbes directly [6] by either disrupting membrane dynamics[21], or enzymatic function[22]. Unfortunately these approaches to killing fungi can lead to selection of drug resistant strains of fungi [23]

We suggest a new approach to activating the host to more effectively battle the pathogen infiltration. This approach should not lead to increased resistance against treatment, and will treat the actual problem (neutropeania) instead of the symptom (fungal infection).

7.5 Conclusions

We demonstrated effective encapsulation of b-glucan in biodegradable PLGA microparticles, which effective surface display of the glucan after days in aqueous media. This particles were shown to induce ROS expression when incubated with human neutriphils. Mice treated with a lethal dose of Candida albicans were able to recover from the infection when treated with particles displaying β−1,6-glucan, but not β−1,3-glucan. Overall we believe this proof of concept study may lead to additional exciting approaches to anti-microbial treatment and prevention.

7.6 References

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