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The In Vitro Characterization of Chitosan-PLGA Nanoparticle/ Plasmid DNA Complexes

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The *In Vitro* Characterization of Chitosan-PLGA Nanoparticle/Plasmid DNA Complexes

by

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Table of Contents

List of Tables

List of Figures

List of Abbreviations

PLGA	Poly(D,L-lactide-co-glycolide)
PEI	Polyethylenimine
AFM	Atomic Force Microscopy
SPM	Scanning Probe Microscopy
DLS	Dynamic Light Scattering
pDNA	Plasmid DNA
PBS	Phosphate Buffered Saline
OG	OliGreen™
RFU	Relative Fluorescence Units
ACN	Acetonitrile
CHO	Chinese Hamster Ovary

Abstract

Poly(D,L-lactide-co-glycolide)-chitosan nanoparticles have become a popular choice for the delivery of nucleic acids to cells for various genetic manipulation techniques. These particles are biocompatible with tuneable size and surface charge, possessing an overall positive surface charge that promotes complex formation with negatively charged nucleic acids. Thorough studies have been performed on the morphology and surface properties of these particles in recent years; however, most of this work neglects the study of these properties once nanoparticle/nucleic acid complexes have been formed. This study begins to examine some of the overall properties of the PLGA-chitosan nanoparticle/plasmid DNA complex after formation. Specifically, work has been done to determine the ideal ratio of plasmid DNA:nanoparticles for drug delivery purposes, and begins examining methods for elucidating the location of the pDNA within the complex.

Using the fluorescent intercalating dye OliGreen® to label free plasmid DNA, the ability of PLGA-chitosan nanoparticles to form complexes for drug delivery purposes can be determined. With this analysis method, the ideal plasmid:nanoparticle ratio for complete uptake of all plasmid was determined to be 1:100. In conjunction with these results, future testing is necessary to determine both the location of plasmid DNA within the complex and the transfection efficiency of the system as a whole. This knowledge provides a further understanding of complex composition, which will aid future investigations into the functionality of the system *in vitro.*

Chapter 1: Background and Introduction

Drug Delivery for Applications in Gene Therapy

Gene therapy is the use of nucleic acids as drugs for the correction of deficient genes. Treatments include the replacement of a dysfunctional gene with a working copy, correction of a faulty gene, or knockdown of an unwanted gene [4]. Although gene therapy is a popular thrust in the research community, there is still much work to be done before it becomes common in a clinical setting. Currently, much of the research focus is on the treatment of genetic disorders (i.e. diabetes [5, 6], genetic blindness [7]) and various forms of cancer [8-10], and is taking place in animal models.

Much of the progress being made in the development of these treatments is still being done in laboratories around the world. The treatment of children with SCID-X1 is one of a relatively small quantity of treatments that proceeded to the clinical trial stage. Unfortunately, this study was a major setback in genetic engineering, ending tragically in 2002 when two of the patients had immune reactions to the treatment and developed leukemia [4, 11, 12]. Such instances have slowed the movement of gene therapy treatments from the laboratory to the clinic. As of September 2008 there were a total of 1,472 approved clinical trials in progress around the world, the majority of them being in either phase I or phase I/II (see Figure 1) [1].

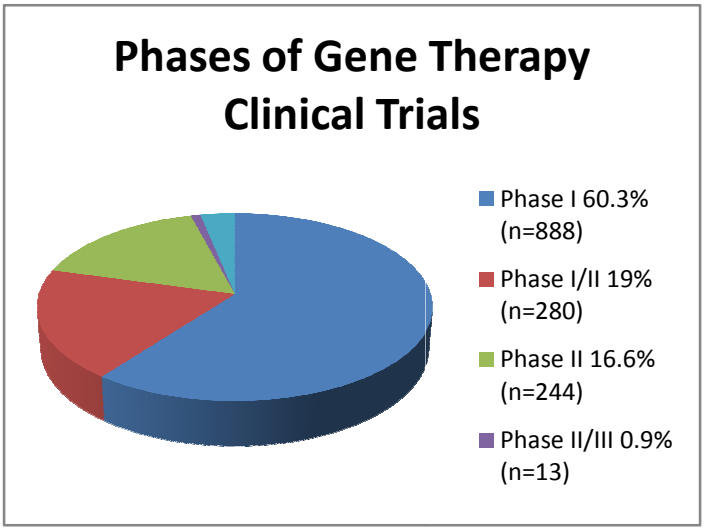


Figure 1.1: *Approved gene therapy clinical trials around the world and their respective phases [1].*

Despite the ongoing research in gene therapy, the number of clinical trials remains minimal because many of the pitfalls associated with treatments have not been thoroughly worked out. The genetics behind the treatment must be thoroughly understood. In addition, once an effective drug has been synthesized, it must be delivered to the correct location within the body efficiently and without causing any immune response [4].

There are two delivery systems that can be employed in gene therapy treatments. The first involves physical injection of the genetic material to a specific site within the body. For treatments targeting skin and muscle tissues this is an effective method; however, injections to more interior tissues and organs would be overly invasive [13]. In these cases, a delivery vector (either viral or nonviral) must be used to transport the drug to a specified location. In viral vectors, the genetic material of the virus is removed and replaced with the nucleic acid drug to be delivered to the patient [13-15]. This method of delivery is highly efficient; however, the risk of immune response is great and in severe cases can lead to patient death.

Nonviral vectors consist of liposomes, lipid reagents (Lipofectamine™, DOTAP, Effectene™ FuGene®), and polymeric nanoparticles. Because of their biocompatibility and ability to reach more interior target tissues, these delivery systems have the potential to circumvent the issues surrounding both direct injection and viral vectors [13-17]. Polymeric nanoparticles have an advantage over both liposomes and lipid reagents because of their ease of manipulation, control over DNA release profiles, and biological stability *in vivo* [18, 19]. In addition, the properties of the particles can be easily modified (i.e. by addition of functional polymer groups) to increase uptake or improve release of genetic material [16, 17, 20].

Nanoparticle/Plasmid DNA Complex Formation

In order to deliver the nucleic acid drug to its target location within the body, it must be loaded onto a delivery vector. This process involves the formation of a complex between the nucleic acid and nanoparticles that is based upon differences in overall charge between the two species [15]. The negative charge of nucleic acids necessitates a polymeric nanoparticle with an overall positive charge, and the materials chosen for nanoparticle synthesis must meet these requirements.

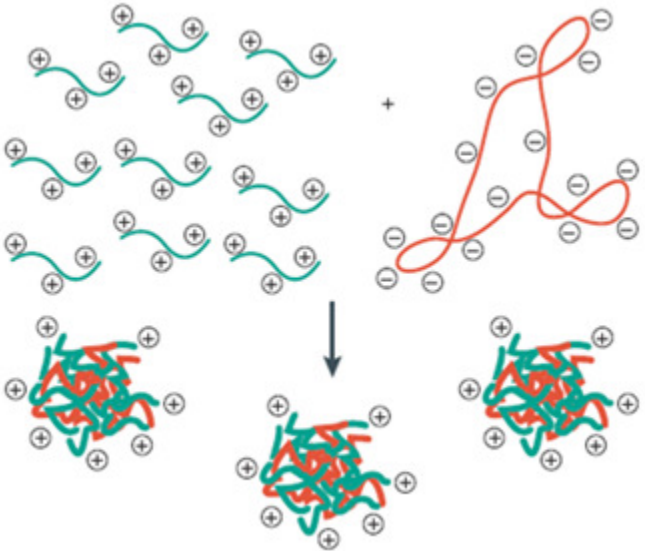


Figure 1.2: *Spontaneous complex formation between a positively charged polymeric nanostructures and negatively charged nucleic acids. An excess of the nanostructure yields an overall positively charged complex [Pack 2005].*

Once placed in solution together, the two species spontaneously form complexes because of these charge differences, a phenomenon that is demonstrated in Figure 2. Poly(D,L-lactide-co-glycolide) (PLGA), whose structure is shown in Figure 1.3A, is a popular polymer for nanoparticles in drug delivery applications because of its biocompatibility and biodegradability

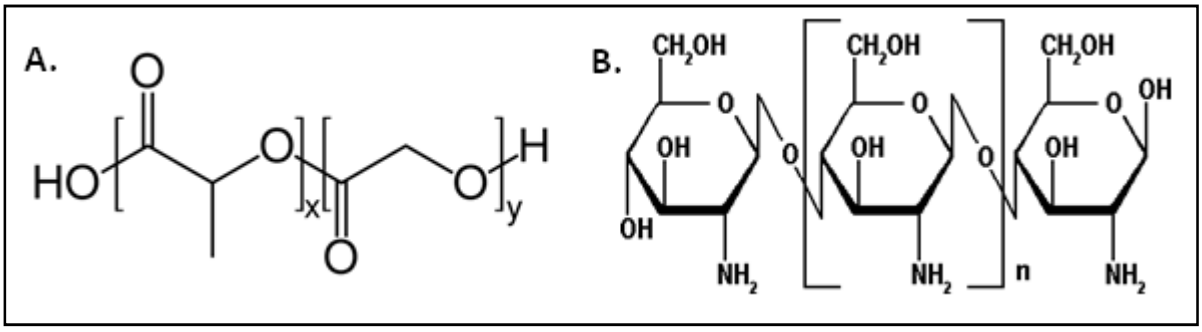


Figure 1.3: *Chemical structure of* ***A.*** *PLGA. 'X' represents the lactic acid units, 'Y' the glycolic acid units, and* ***B.*** *chitosan*

in vivo [21]; however, the most stable method for PLGA nanoparticle synthesis produces a negatively charged particle [22, 23]. Methods for complex formation between negatively

charged particles and nucleic acids exist, but involve the use of organic solvents which could cause damage to the nucleic acid [24, 25]. The addition of a cationic substance to the surface of the nanoparticles after synthesis can circumvent this problem by producing an overall positively charged particle. Commonly used materials include polyethylenimine (PEI), polylysine, gelatin, and chitosan [26, 27]. Chitosan is a favorable material because of its biocompatibility, biodegradability and ability to increase cell permeability [26]. The structure of chitosan is shown in Figure 3B. The adsorption of this material onto the surface of PLGA nanoparticles yields a positively charged particle that can easily complex with nucleic acids for drug delivery [28]. In addition, when compared with uncoated particles the addition of chitosan has been shown to increase the uptake of the nanoparticles into cells *in vitro* [29].

In addition to stability and biocompatibility, PLGA-chitosan nanoparticles can be tailored to fit the specific needs of the target delivery site/system. The efficiency with which the complexes can be transfected into target cells is dependent on the size and charge of the particles, and varies with differing cell types. Modifications to the process parameters during nanoparticle synthesis can produce predictable changes in particle size and overall surface charge, allowing for the production of nanoparticles possessing the desired characteristics [8, 26, 30].

Analysis of PLGA-chitosan/Nucleic Acid Complexes

It is important to assess the characteristics of nanoparticles after synthesis to ensure that they possess the desired properties for complex formation and delivery. In addition, an understanding of the characteristics of the complex is crucial to understanding the behavior of the system during both *in vitro* and *in vivo* delivery [26]. The ability of the complex to protect nucleic acids from enzyme degradation, loading efficiency of nucleic acids into the complex, and

overall size and zeta potential are important properties that can be characterized. Zeta potential is the best means for estimating the overall surface charge of a particle or complex. In a charged particle, a layer of charged ions forms around the particle (see Figure 4). Further away from the particle, ions are distributed evenly throughout the medium. The difference in charge between the surrounding medium and the charged layer around the particle is described as the zeta potential [31].

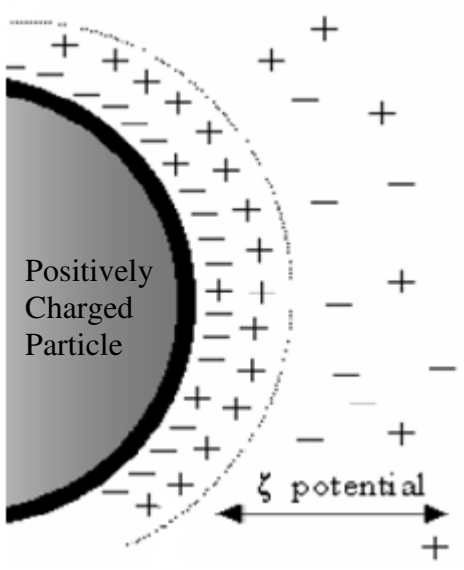


Figure 1.4: *Zeta potential for a nanoparticle is measured by detecting the difference between the ions surrounding the particle and the surrounding medium[2].*

Surprisingly, much of the characterization that has been done to date has been on the nanoparticles themselves; it is only recently that analysis of the complexes as a whole has become more common [8, 26, 30]. There are several assays that can be used to elucidate the properties of the complex including various forms of microscopy (i.e. SPM and AFM), gel electrophoresis, dynamic light scattering (DLS), and fluorimetry.

Microscopy

Until recently, microscopy techniques were used mainly to determine average particle size and surface morphology after synthesis. Although important, these values are more significant for the entire system, which must be able to effectively pass through cell membranes for treatment purposes. More thorough studies have begun to emerge which employ these same microscopy techniques for the analysis of nanoparticle/nucleic acid complexes. In addition to revealing the overall size and uniformity of the complex [32], imaging can provide clues regarding the adsorption of nucleic acid to the nanoparticle surface. Using atomic force

microscopy (AFM), Kumar, Bakowski and Lehr became the first group to obtain a high resolution AFM image of PLGA-chitosan nanoparticles complexed with DNA. The image clearly reveals the interaction of the negatively charged DNA with the positively charged surface of the nanoparticles and definitively confirms the formation of the complex [33]. Similar techniques have become more common for other nanoparticle delivery systems such as PLA and gold [34, 35].

Scanning probe microscopy (SPM) has also been employed to study the size and morphology of particle/nucleic acid complexes. Nafee et al. has produced nanoparticles of varying properties by modifying the process parameters during synthesis. Using SPM, along with other analysis techniques, they were able to study the effects of nanoparticle characteristics on the ability of the particles to form complexes with nucleic acid. The images allowed them to visualize changes to morphology and size with these modifications [26].

Gel Electrophoresis

Gel electrophoresis is an invaluable technique in particle/nucleic acid complex analysis because of the wide variety of complex properties that can be determined from it. Most importantly, an electrophoresis gel can be used to determine whether a specific polymeric nanoparticle is capable of complex formation with the desired nucleic acid. Within a particle/nucleic acid complex, only the remaining free nucleic acid will migrate through the gel and produce a visible band. The technique is applied to a series of particle:nucleic acid ratios to determine the quantity of nanoparticles that is most appropriate for the complete uptake of the desired quantity of nucleic acid, and is performed frequently in particle and complex analysis [17, 22, 33, 34, 36]. Calculations made with results obtained from this analysis are often

performed to determine the loading efficiency of nanoparticles as a delivery vector using the following equation:

$$Loading\ Efficiency = \frac{weight\ of\ nucleic\ acid\ in\ complex}{weight\ of\ nucleic\ acid\ in\ formulation\ system} \times 100\%$$ [37]

Free nucleic acid within the body will be quickly degraded by enzymes, rendering them useless as a gene therapy drug. In addition to serving as a delivery vector, the nanoparticles must be able to protect the nucleic acids from enzyme degradation once they have been introduced into an *in vivo* system. The ability of polymeric nanoparticle systems to protect pDNA from enzyme degradation has been widely explored [17, 34, 37-39], and PLGA-chitosan nanoparticles are among those delivery systems that have been found capable of nucleic acid protection. Gel electrophoresis is the most common method for assessing nuclease degradation within complexes. DNAse I is introduced to the samples to destroy any exposed or uncomplexed nucleic acid. The remaining nucleic acid can be dissociated from the complex and run on an electrophoresis gel. Results of this assay will indicate whether the nucleic acid was destroyed by the enzyme activity.

Dynamic Light Scattering

Dynamic Light Scattering (DLS) is a common method for determination of particle (or complex) size and zeta potential, and nearly all studies that characterize nanoparticle/nucleic acid complexes apply this technique [26, 34, 37-39]. A thorough knowledge of these characteristics is essential to evaluating the effectiveness of a specific nanoparticle as a delivery system. In DLS analysis, a laser is shone into a sample containing the nanoparticles or particle/nucleic acid complexes. The scattering of that light after it hits the complexes varies with time as those complexes move through the solution. In addition, the scattering from one particle interferes

with the scattering from surrounding particles. These measurements can be auto-correlated to produce a size distribution of the complexes found within the sample solution [40]. A schematic of this process is depicted in Figure 5 below.

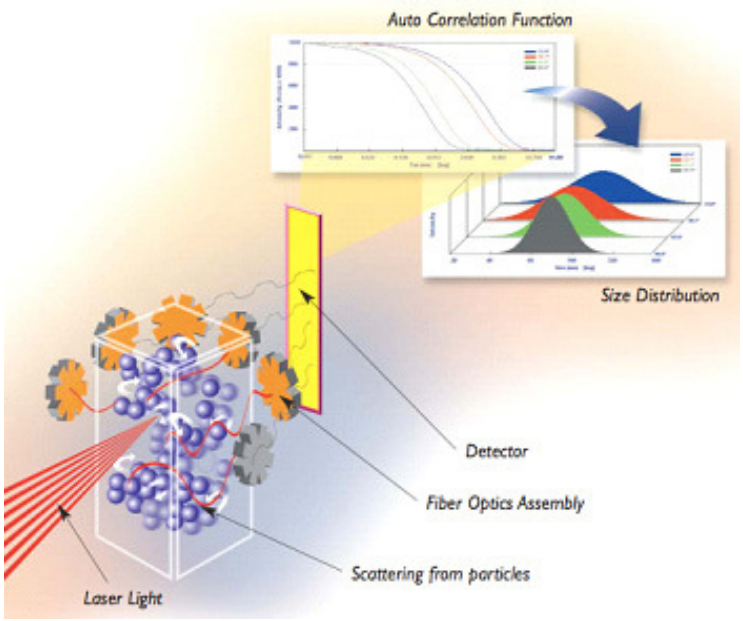


Figure 1.5: *Dynamic light scattering--incident laser light passing through a sample creates interference that can be correlated to particle size [41].*

In addition, DLS is the most common method for measuring the zeta potential of the complexes. An electric field is applied across the complexes in addition to the incident laser beam, and changes in light intensity based on movement through the field can be correlated to the zeta potential of the sample [40].

Fluorimetry

Fluorescence intensity is an important measurement for nucleic acid quantification. The ability to correlate nucleic acid presence with fluorescence intensity is a useful property when analyzing particle/nucleic acid complexes. Intercalating fluorescent dyes (i.e. OliGreen, SYBR Green) emit a signal when they have intercalated within the strands of a nucleic acid, and can be

used to provide a measurable intensity in fluorescence-based assays. Any nucleic acid that has not become part of the complex will take up the dye and emit a strong fluorescent signal. As a result, the most common application for fluorimetry is the quantification of unbound nucleic acid within a complex sample [8, 38]. Fluorimetry can be used for many of the same applications as gel electrophoresis, as both of them work by characterizing the nucleic acid in a given sample. Similar to electrophoresis, this assay can determine the optimal particle:nucleic acid ratio for the uptake of all of the desired quantity of nucleic acid [42]. The ability of the complexes to protect their nucleic acids from nuclease degradation can also be determined with this assay, by first separating formed complexes from free nucleic acid and then breaking up the complexes to release the nucleic acid within them [39]. Several methods for complex dissociation exist and vary depending on the polymeric nanoparticle being used [17, 34, 42]

***In Vitro* Transfection of Nanoparticle/Nucleic Acid Complexes**

A thorough analysis of the complexes *in vitro* will provide a platform from which the delivery system can be optimized for cellular transfections. *In vivo* analysis of these delivery systems is not feasible unless *in vitro* transfection studies are effective. In addition, no clinical trial would be approved without successful *in vitro* cellular studies and *in vivo* animal analyses. There are a relatively few number of studies that have been done regarding the cellular uptake of PLGA-chitosan/nucleic acid systems into cells, and their successes have been minimal. Nafee and Taetz et al. have published two studies on the characterization, optimization, and transfection of these complexes into A549 human lung carcinoma cells. Their results demonstrate that delivery of the nucleic acid 2'-*O*-methyl RNA is improved with PLGA-chitosan nanoparticles as a delivery vehicle; however, the uptake of the complexes into the cells is not significantly high [8, 26]. A study performed by Guan et al. demonstrated similar results in the delivery of plasmid

DNA (pDNA) into 293FT cells. Uptake of the complexes into the cells was again minimal, and insignificant when compared with the commercial transfection agent Lipofectamine™ [22]

Development of Particles for Specific Delivery Purposes

Current literature focused on the feasibility of the chitosan-PLGA nanoparticles for nucleic delivery provides useful information on preparation methods, the final characteristics of synthesized nanoparticles, and their behavior *in vivo* (i.e. cytotoxicity and transfection efficiency) [34, 42, 43]. The ability of nanoparticles to limit nuclease degradation of encapsulated pDNA has also been well documented [17, 34, 37-39]. The previously mentioned analysis techniques are used frequently for the analysis of synthesized nanoparticles, but there is a gap in information regarding particle characteristics and their effect on DNA binding. More recent studies have begun to address this deficiency [8, 26, 30]. Studies have demonstrated that the modification of particle characteristics such as particle size and overall surface charge directly affected nucleic acid binding efficiency. Variations in chitosan type and content, PLGA type, and particle preparation methods (i.e. centrifugation, freeze-drying) have been used to change these nanoparticle characteristics and have been shown to directly affect the binding efficiencies of the particles to antisense oligonucleotides [8, 26] and small interfering RNA [30], for example. With this knowledge, it is becoming easier to develop a complete delivery system that can be tailored to a particular application. Applying the previously discussed techniques toward the analysis of the entire complex provides a more thorough understanding of the system and allows for the most effective particle/nucleic acid complex to be formed.

Research Significance and Goals

Any potential delivery system must be fully characterized and its properties understood to optimize it for delivery to *in vitro* and *in vivo* systems. The goal of this project is to characterize

the pDNA/chitosan-PLGA complexes using fluorescence tagging of the pDNA to determine the optimal ratio of particle:pDNA for complete uptake of the pDNA sample into the complex. The intercalating dye OliGreen was used to tag the free pDNA within the samples. Optimization of this assay involved experimentation to determine the appropriate pDNA concentration and OliGreen volume to obtain ideal fluorescence signals. In addition, the assay has been used in conjunction with nuclease degradation and complex dissociation to elucidate the loading efficiency and location of the pDNA within the complex. Further research is necessary to draw definitive conclusions regarding the degradation and dissociation experiments, and to develop a dissociation method that is compatible with the OliGreen fluorescence assay. With this understanding of the *in vitro* properties of the complexes, their uptake and transfection efficiency into cells in culture can be studied. Future work on this project aims to continue with characterization of the complexes and to determine their efficiency as a nucleic acid delivery system.

Chapter 2: Fluorescence-based Characterization of Plasmid DNA Uptake by PLGA-chitosan Nanoparticles

Introduction

Polymeric nanoparticles are important drug delivery vectors because of their biocompatibility and ease of manipulation [18, 19]. In particular, PLGA-chitosan nanoparticles are becoming a popular choice because of their tuneable size and surface charge, biocompatibility, and stability *in vivo* [21, 29]. In the development of any delivery system, it is important to thoroughly analyze the properties of the vectors and complexes. It is especially important to fully characterize the interaction between the nanoparticles and nucleic acid so that delivery can be optimized.

Characterization of the nanoparticles after synthesis is a common process; however, analysis of the complexes after formation is only recently becoming popular [8, 26, 30]. With any synthetic delivery vector, it is necessary to prove the ability of the particles to complex with nucleic acids. In addition, complex size and morphology, zeta potential, and nucleic acid protection are several useful characteristics that should be determined. The ideal nanoparticle:nucleic acid ratios for a specific delivery system must be determined before any *in vitro* transfection studies can be performed. These analyses can be accomplished using a variety of techniques including several types of microscopy, dynamic light scattering, gel electrophoresis and fluorimetry.

The fluorescence-based assay is an effective qualitative analysis of nucleic acid uptake that uses a DNA intercalating dye to determine the relative amounts of free plasmid within a given sample [8, 38]. In conjunction with gel electrophoresis, fluorimetry measurements are an effective means for determining the ability of the PLGA-chitosan nanoparticles to form

complexes while elucidating the optimal mass of nanoparticles required to complex with a given mass of DNA.

Materials and Methods

Materials

PLGA-chitosan nanoparticles were synthesized in Dr. Cristina Sabliov's lab, and plasmid DNA was obtained from Dr. Todd Monroe's lab. Lipofectamine™ 2000 Transfection Reagent and Quant-iT™ OliGreen® ssDNA Reagent were purchased from Invitrogen Corporation (Grand Island, New York). Phosphate Buffered Saline (PBS) and OptiMEM were purchased from Hyclone Laboratories, Inc. (Logan, UT). Fluorescence measurements were made using the Perkin Elmer LS 55 Luminescence Spectrometer (Waltham, MA). Gel electrophoresis was performed using ReadyAgarose™ Mini Gels (1% Agarose, 8-well) purchased from BioRad Laboratories (Hercules, CA).

Optimization of Plasmid Concentration for Fluorescence Measurements

Plasmid DNA was obtained at an initial concentration of 1ug/ul. Serial dilutions were made using water to final concentrations of 0.1ug/ul, 10ng/ul, 1ng/ul and 0.1ng/ul. 4ul of pDNA and 25ul of OliGreen® (OG) at 1X concentration were added to a 1.5ml Plastibrand® PMMA cuvette (Sigma Aldrich, St. Louis, MO) and incubated in the dark for 5 minutes. After incubation, the volume of the cuvette was brought up to 500ul using OptiMEM. Fluorescence intensity was measured in relative fluorescence units (RFU) using FL WinLab (excitation 490nm, emission, 520nm). Appropriate pDNA concentration was determined based on obtained intensities. The assay was repeated with both increased and decreased volumes of OG to determine optimal OG volume.

Lipofectamine/pDNA Complex Formation and Determination of pDNA Uptake

Complexation of Lipofectamine and pDNA was done in OptiMEM. pDNA at a concentration of 1ng/ul was combined with lipofectamine, also at 1ng/ul. The two were combined in varying pDNA:Lipofectamine ratios (1:0.1, 1:1, 1:10, 1:100). The solution was vortexed thoroughly and allowed to incubate at room temperature for 30 minutes before any analysis was performed. Fluorescence intensity of each complex was measured in RFU as previously mentioned. Free pDNA and OptiMEM were analyzed as positive and negative controls, respectively.

Media Selection

The high autofluorescence of OptiMEM interferes significantly with the fluorescence of the OG once it has intercalated with the pDNA. In an effort to eliminate this background noise, the autofluorescence of PBS was tested as an alternative. Sucrose was added to the PBS (5% w/v) so that the media would be compatible with live cells in future testing. The autofluorescence of several solutions of PBS and OptiMEM was measured to determine the contribution of each to the background intensity. 100% OptiMEM and 100% PBS were measured as controls. In addition, solutions containing 25% OptiMEM/75% PBS, 10% OptiMEM/90% PBS, and 5% OptiMEM/95% PBS were analyzed. Cuvettes were filled with 500ul of test solution and 25ul of OG, incubated for 5 minutes in a dark space, and measured as previously described.

Nanoparticle Preparation and Complex Formation

PLGA-chitosan nanoparticles were obtained from Dr. Cristina Sabliov. A stock solution was prepared by dissolving 1mg of dried particles in 1ml of water in a 1.5ml centrifuge tube. The tube was vortexed for 15 minutes at 1000rpm. After initially dissolving particles in water

further dilutions were made using PBS/sucrose solution. Formation of complexes was accomplished by combining pDNA and nanoparticles in PBS/sucrose in several nanoparticle:pDNA ratios (1:1, 10:1, 20:1, 50:1, 100:1). The samples were thoroughly vortexed and allowed to complex for one hour at room temperature before analysis.

Determination of Unbound pDNA

The samples were analyzed for unbound pDNA using the fluorescence-based assay previously described. In summary, each complex was incubated with 25ul of OG for 5 minutes. PBS was added and analysis was completed. Each complex was analyzed five times and the resultant intensities averaged. Free pDNA and PBS/sucrose were also analyzed as positive and negative controls, respectively. In addition, both the free pDNA and complexes were analyzed on the pre-cast gels. The samples were run in TAE buffer at 60V for 60 minutes. The gel was then stained with SYBR Gold for imaging.

Results

Fluorescence-based Assay Optimization Using Lipofectamine™

In order to optimize the OG assay for analysis of complexes, the sensitivity of the assay to changes in pDNA concentration first had to be determined. Serial dilutions of pDNA, initially at 1ug/ul were made and their fluorescence intensity determined. Each sample contained equal volumes of pDNA at the various concentrations. The results from the assay are shown in Figure 2.1. Based on the fluorescence readings obtained, the optimal concentration of pDNA was chosen to be 1ng/ul. Higher concentrations produced intensities that exceed (or come close to) the maximum signal that can be read by the fluorimeter. Readings of 999.999 were obtained frequently using higher concentrations, indicating that the fluorescence of the OG is too high.

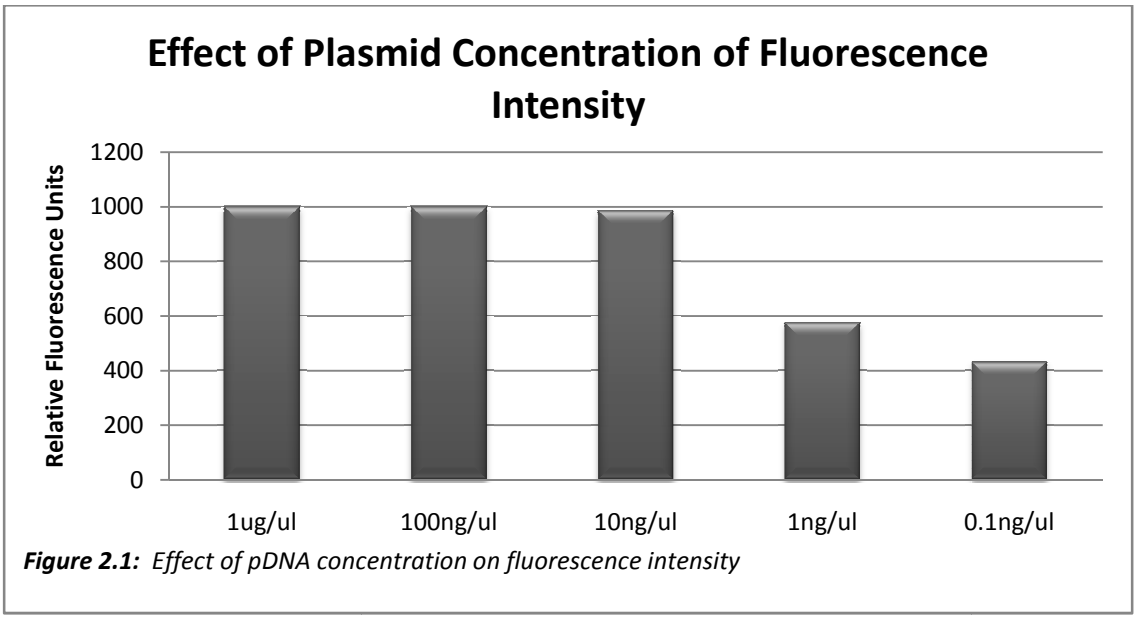


Figure 2.1: *Effect of pDNA concentration on fluorescence intensity*

Further tests were performed at the optimal pDNA concentration to confirm that 25ul was a sufficient OG volume to bind to all of the available free plasmid. Figure 2.2 shows the relative fluorescence intensities of all OG volumes tested. Volumes below 25ul began to show a decrease in average fluorescence while volumes greater than 25ul do not have any effect on the intensity of the signal.

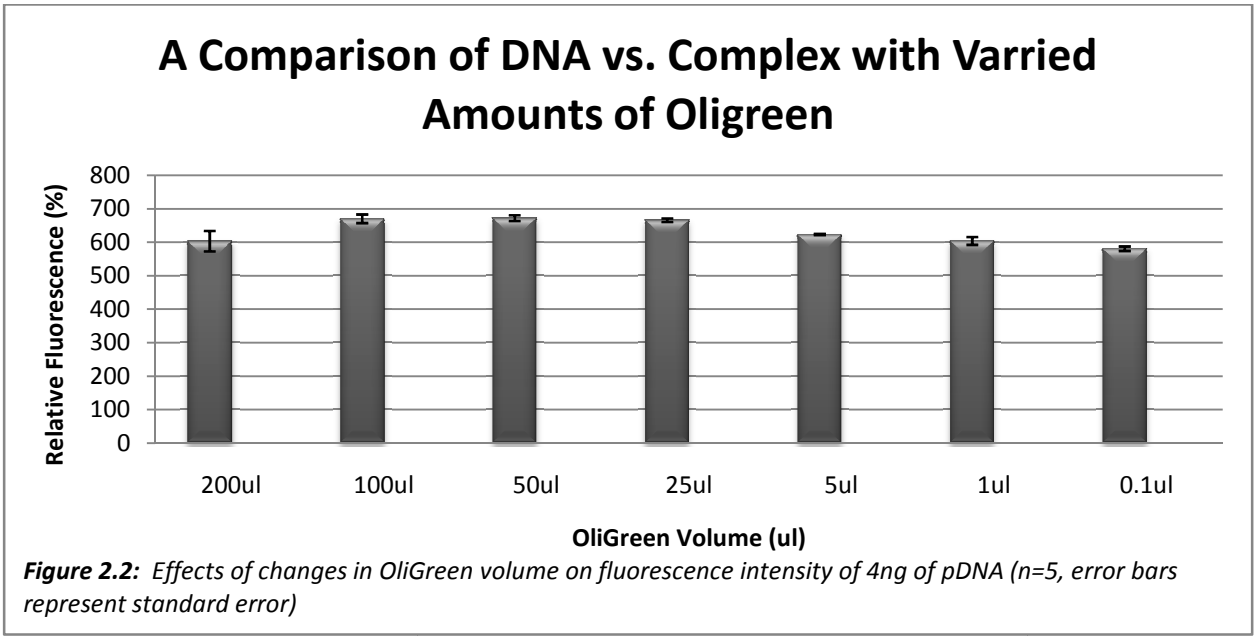


Figure 2.2: *Effects of changes in OliGreen volume on fluorescence intensity of 4ng of pDNA (n=5, error bars represent standard error)*

Running negative controls using OptiMEM yields a high fluorescence indicating the tendency of the media to autofluoresce. As Figure 2.3 shows, the signal decreases significantly with an increasing percentage of PBS, and is near zero for PBS alone. As a result, all subsequent tests were performed in PBS.

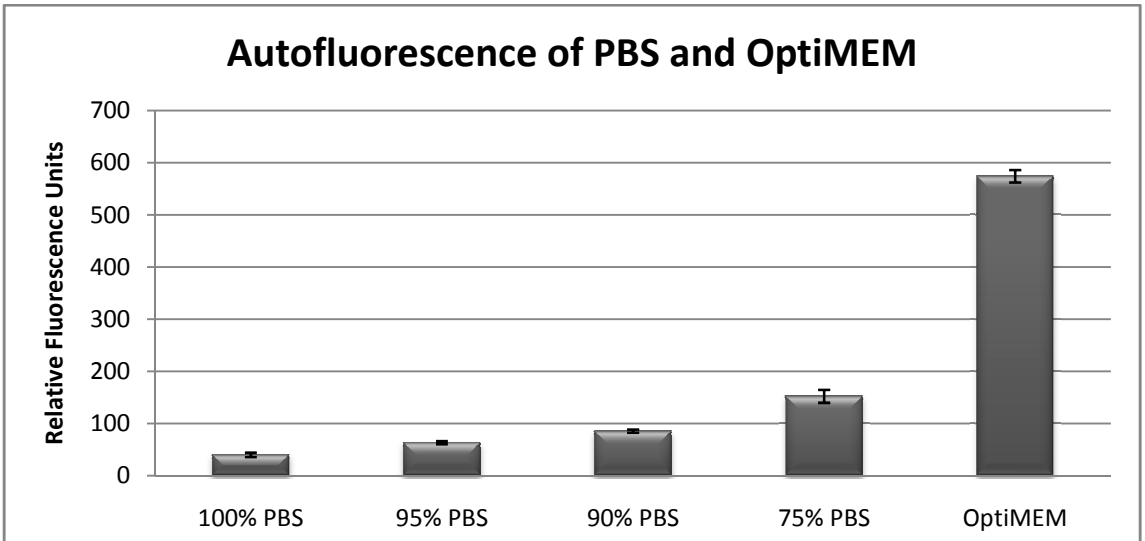


Figure 2.3: *Relative intensity of PBS with increasing percentage of OptiMEM (n=5, error bars represent standard error.)*

Optimization of the pDNA concentration and OG volume allowed the assay to be used for pDNA characterization in complex formation. Once complexed with a common transfection agent, Lipofectamine, the assay could be used to analyze the free DNA at several Lipofectamine:pDNA ratios. Figure 2.4 depicts the results of these analyses. Increasing amounts of Lipofectamine directly correspond to decreasing signal intensity. Once the ratio of Lipofectamine:pDNA increases to 100:1, there is no detectable fluorescence when compared to the negative control. At smaller ratios (0.1:1, 1:1) the fluorescence is comparable to pDNA.

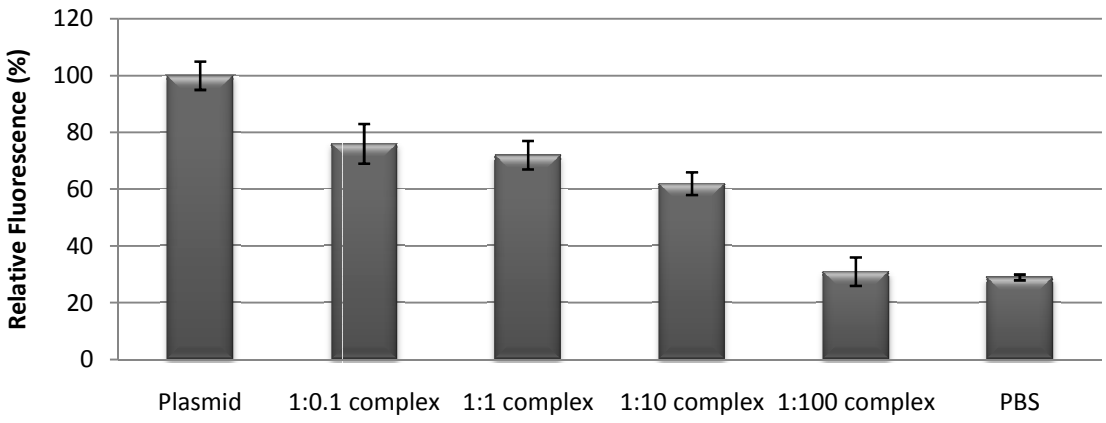


Figure 2.4: *Effect of the increase of pDNA:Lipofectamine ratios on relative fluorescence compared with a pDNA positive control (n=5, error bars represent standard error).*

PLGA-chitosan Nanoparticle/pDNA Complex Analysis

The trend seen in Figure 2.4 is also present when Lipofectamine is replaced with the PLGA-chitosan nanoparticles (see Figure 2.5). The fluorescence of the free pDNA is comparable to that of the complexes at a 1:1 nanoparticle:pDNA ratio. As the mass of the nanoparticle increases, the relative fluorescence decreases. Once the nanoparticle:plasmid ratio reaches 100:1, the signal is comparable to that of the negative control.

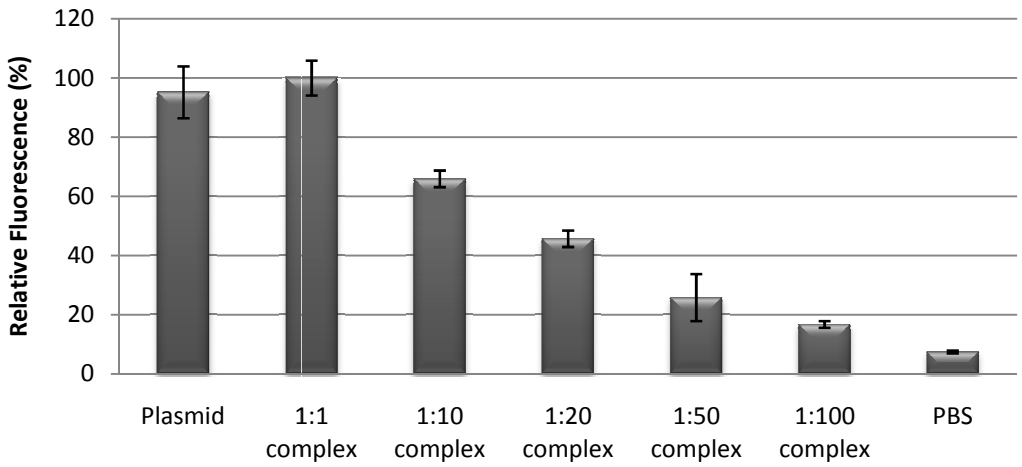


Figure 2.5: *Effect on the increase of pDNA:nanoparticle ratios on relative fluorescence compared with a pDNA positive control (n=5, error bars represent standard error.)*

Gel electrophoresis was used to determine the electrophoretic mobility of the complexes compared with free pDNA. The image of the gel and contents of each lane can be seen in Figure 2.6. The pDNA banding pattern is seen in lanes 2, 3, and 4 indicating that free DNA has migrated through the gel. In subsequent lanes, the absence of banding indicates that no free pDNA was available to move through the gel.

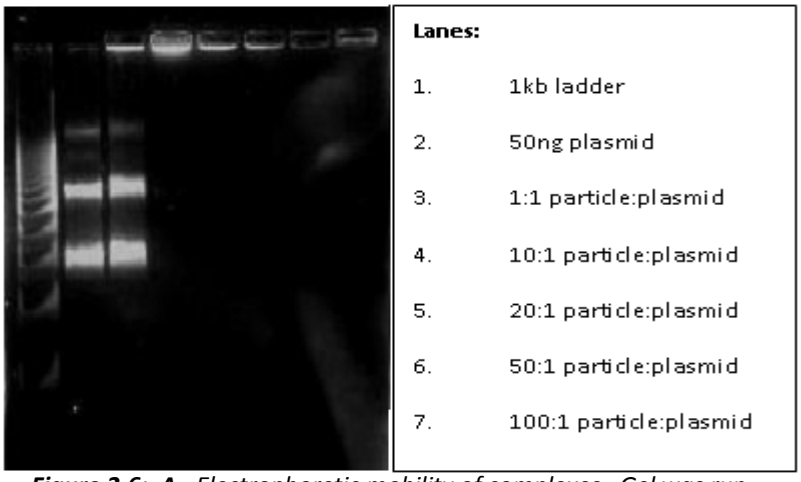


Figure 2.6: ***A.*** *Electrophoretic mobility of complexes. Gel was run at 60V for 60 minutes and imaged with a 3.5 second exposure time.* ***B.*** *Contents of each lane*

Discussion

In order to use this fluorescence-based assay for analysis of nanoparticle:pDNA complexes, optimization is essential. Although tedious, the determination of appropriate pDNA mass and concentration, OG volume, and media are imperative to the success of measurements. Based on the results from the free pDNA experiments, 1ng/ul was determined to be the ideal concentration for characterization. At this concentration, only 4ng of pDNA will be needed for each measurement, making it convenient to perform several repetitions with minimal sample size. In addition, experiments to minimize the volume of OG required have been helpful in optimizing the assay.

The high autofluorescence of OptiMEM limits its usefulness in this assay. The average fluorescence signal of 575 RFU is significantly greater than that of PBS. This background noise interferes with the increase in signal experienced after the OG has intercalated into the pDNA. Without a clear signal change due to intercalation, it would be difficult to detect free pDNA within a sample containing nanoparticle/pDNA complexes. The autofluorescence of PBS is significantly less than that of the OptiMEM. Its average intensity is 35 RFU, making it an ideal media in which to take fluorescence measurements. With virtually no background signal, it will be easier to detect changes in the fluorescence of the OG due to pDNA intercalation.

These optimization steps allowed for the creation of a baseline from which further analysis could be performed. The signal emitted by 4ng of free pDNA can be used as a control to analyze other samples that contain equivalent amounts of plasmid. Lipofectamine is a known transfection agent that forms a complex with nucleic acids. As more lipofectamine is added to a sample, more of the pDNA can be taken up into the complex. This reduces the mass of free pDNA within the solution. Less of the OG is able to intercalate into the plasmid, effectively

reducing the fluorescence emitted. At lower lipofectamine:pDNA ratios, the fluorescence is comparable to that of the free plasmid; however, at 100:1, the intensity is nearly equivalent to that of the negative control, indicating that all of the plasmid has complexed with the lipofectamine. These results indicate that this assay can be used to determine the complexing efficiency of a given vector by effectively measuring the free pDNA remaining in the sample.

Optimization and proof of concept experimentation prepared this assay for use in the analysis of our PLGA-chitosan nanoparticles. A similar trend to that of lipofectamine/pDNA complexes was observed, indicating that the particles are effective in binding with the pDNA. The results show low fluorescence once the particle:plasmid ratio reaches 50:1, indicating very little free pDNA remaining in the sample. Comparing the intensity of the 100:1 complexes to that of the negative control suggests that all of the pDNA has been complexed.

These results can be further confirmed by the gel electrophoresis mobility studies. The nanoparticle/pDNA complexes are too large to travel through the gel and remain in the wells. Any free pDNA within these samples will migrate through the gel. Consequently, this technique can be used in conjunction with the fluorescence-based assay to confirm particle/pDNA complex formation. The results indicate that complex formation begins to occur at particle:plasmid ratios of 10:1. The differences between the gel electrophoresis and fluorescence studies are most likely a difference in sensitivity between the two assays, but both confirm that complexation is able to occur with our PLGA-chitosan nanoparticles.

Chapter 3: Determination of pDNA Location Within PLGA-chitosan Nanoparticle/pDNA Complexes

Introduction

Recent research on PLGA-chitosan nanoparticle synthesis and characterization for drug delivery purposes has begun to focus more thoroughly on complex characterization and the effects of particle properties on DNA binding [8, 26, 30]. Despite these steps forward in the synthesis and characterization of a successful drug delivery system, the location of DNA within the particle-DNA complexes remains unknown. Surface images have been captured by scanning probe microscopy only in the role of verifying morphology of the occurring particle-DNA complexes [26]. By releasing and quantifying the pDNA from the complex, it is possible to determine whether the majority of the DNA has been taken up within the complex, or is simply associated on the outside. It is the intent of this study to optimize a fluorescence-based assay that can be used to provide insight into the location of the pDNA within the complexes.

Materials and Methods

Materials

All fluorescence readings were taken using the FL WinLab computer program on the Perkin Elmer LS 55 Luminescence Spectrometer (Waltham, Massachusetts). Gel electrophoresis was performed on ReadyAgarose™ Mini Gels (1% Agarose, 8-well) purchased from BioRad Laboratories (Hercules, CA). Quartz cuvettes for ACN fluorescence assay were obtained from Starna Cells (Atascadero, CA). All other materials were available in Dr. Monroe's lab.

Complex Dissociation

Nanoparticle/pDNA complexes (100:1) were exposed to various treatments for dissociation. Free pDNA and PBS were also treated as positive and negative controls, respectively.

Acetonitrile: Because ACN interferes with the PMMA cuvettes, a quartz cuvette was used for these experiments. Each sample was treated with 200ul of ACN, vortexed thoroughly, and allowed to incubate at room temperature for 30 minutes. *Sodium Chloride and Triton X-100*: These treatments were performed 5 times for both the complexes and positive and negative controls. The total solution of each sample was added to a 15ml centrifuge tube. 1ml of treatment solution was added to the samples in 200ul aliquots with thorough vortexing between each addition. NaCl was added in concentrations of 1, 3, or 5M and TritonX-100 was added as 10% and 25% solutions (v/v) in H_2O. The samples were allowed to incubate at room temperature for 30 minutes before analysis, and were vortexed again immediately prior to analysis.

Hydrochloric Acid: HCl was added dropwise to the samples until pH strips indicated a drop in pH to 5.0. The samples were vortexed and again allowed to incubate at room temperature for 30 minutes. *NaCl/TritonX-100:* 4M or 2M NaCl was combined with 25% TritonX-100 (v/v in water). The solution was vortexed thoroughly and allowed to incubate at room temperature until all of the bubbles had risen to the top. The solution was then added to samples in 200ul aliquots as described above. In addition, this solution was added as a treatment after the pH was reduced using HCl. All of the treatments applied to the complexes are summarized in Figure 3.1 below.

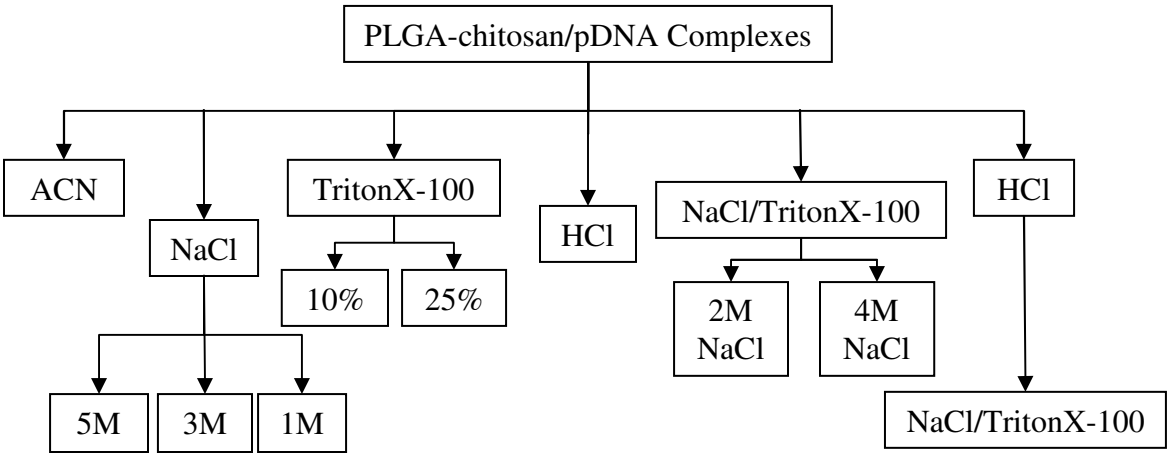


Figure 3.1: *Solutions added to PLGA-chitosan/pDNA complexes for dissociation experiments*

Complex Analysis Using Fluorescence and Gel Electrophoresis

Analysis of treated complexes was performed using the fluorescence-based OG assay. In summary, each sample was incubated in PMMA cuvettes with 25ul of OG and incubated for 5 minutes in the dark. After incubation, the sample volume was raised to 500ul using PBS/sucrose solution and analyzed for fluorescence at excitation and emission wavelengths of 490 and 520nm respectively.

Additional analysis on complexes treated with ACN and NaCl was performed with gel electrophoresis. Each lane contained 50ng of pDNA and was treated with 5ul of ACN or 5M NaCl. The gel was run in 1X TAE buffer at 60V for 60 minutes.

Results

Acetonitrile and NaCl: Because the ACN interfered with the PMMA cuvettes, all analyses involving this chemical were performed in a quartz cuvette. As a result, only one repetition of each experiment was performed. The relative intensity of each ACN treated sample can be seen in Figure 3.2. Fluorescence readings for each treated sample were comparable to that of the PBS negative control, which contained neither ACN nor pDNA. Appreciable signal

was emitted by the pDNA positive control which contained no ACN. Similar effects were observed when samples were treated with NaCl (data not shown). The pDNA positive control continued to emit a significant signal, but no detectable signal was measured in any cuvette containing NaCl.

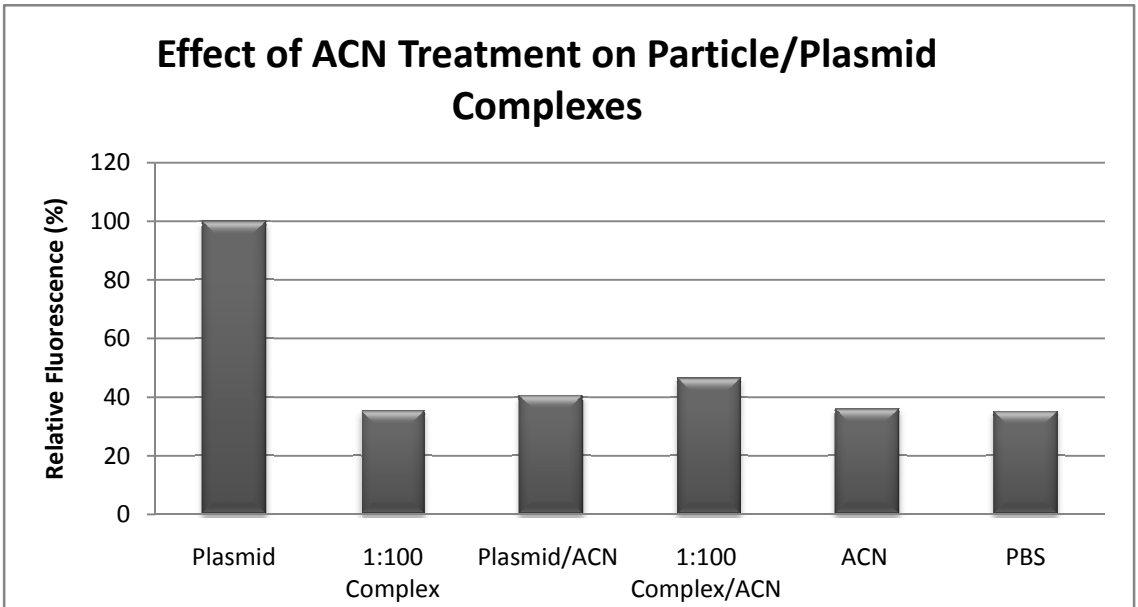


Figure 3.2: *Relative fluorescence of ACN treated pDNA and particle/plasmid complexes compared with untreated pDNA (n=1).*

Triton X-100: The addition of Triton to the samples had varying effects that were inconsistent and unpredictable. Upon addition to complexes and free pDNA, the 25% Triton solution caused a massive increase in fluorescence intensity that resulted in an intensity that was above the range of the spectrometer. The addition of 10% Triton solution produced values that were vastly inconsistent and ranged in intensity from 59-999 RFU.

HCl: After confirmation of pH using test strips, the complexes were analyzed to determine whether any dissociation had occurred. Figure 3.3 depicts the relative fluorescence of the complexes in reference to the free pDNA sample. The addition of HCl had no effect on the

fluorescence of pDNA. The fluorescence intensity of the complexes after treatment is comparable to the fluorescence of the negative control containing no pDNA.

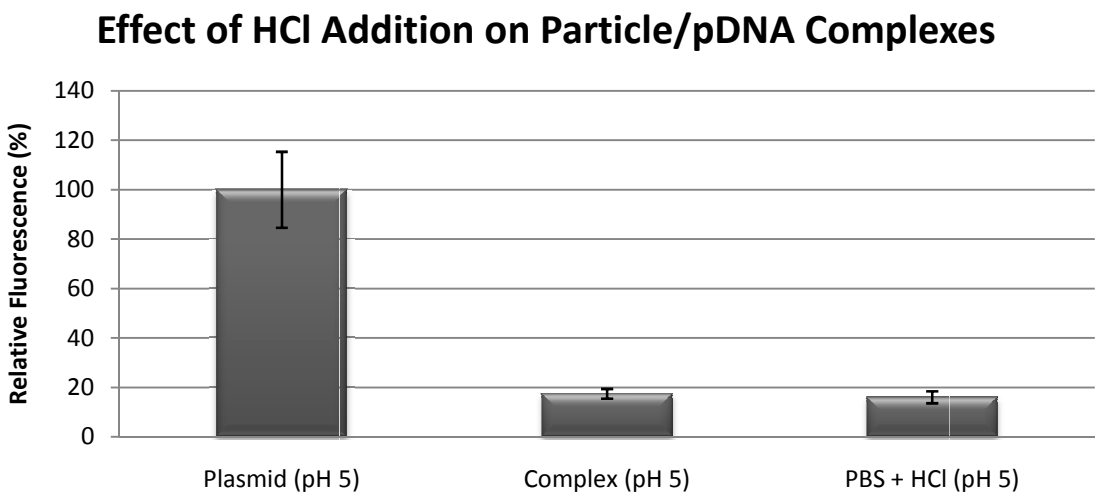


Figure 3.3: *Relative fluorescence of HCl treated PLGA-chitosan/pDNA complexes compared with pDNA positive control (n=5, error bars represent standard error).*

NaCl/Triton X-100: The combination of NaCl and Triton X-100 had similar effects on the fluorescence as the NaCl alone. The high intensity of the NaCl/Triton negative control solution indicates a high autofluorescence. In addition, inconsistent results were again obtained for the samples containing particle/pDNA complexes, indicated by the high standard error. These results can be observed in Figure 3.4.

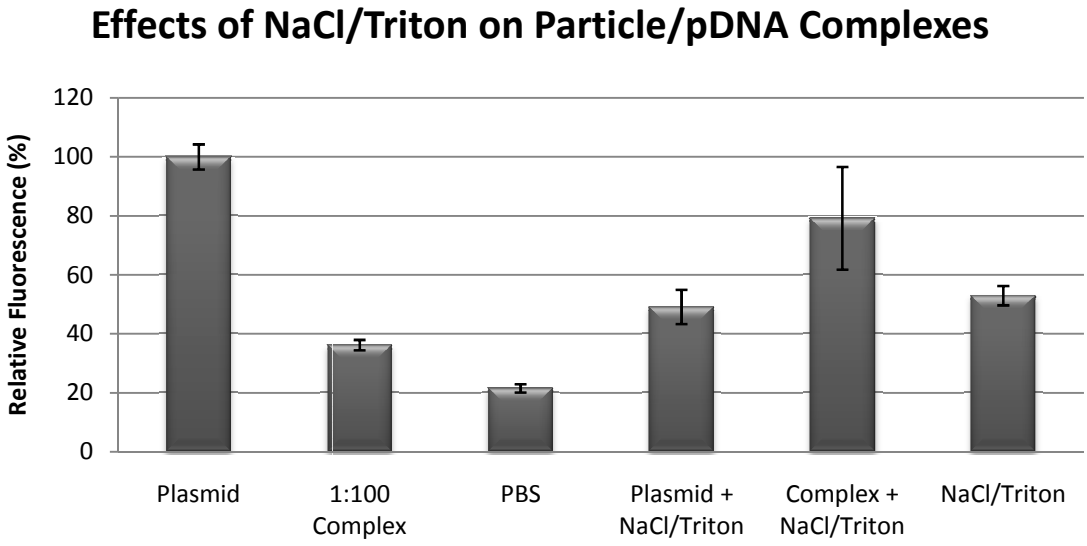
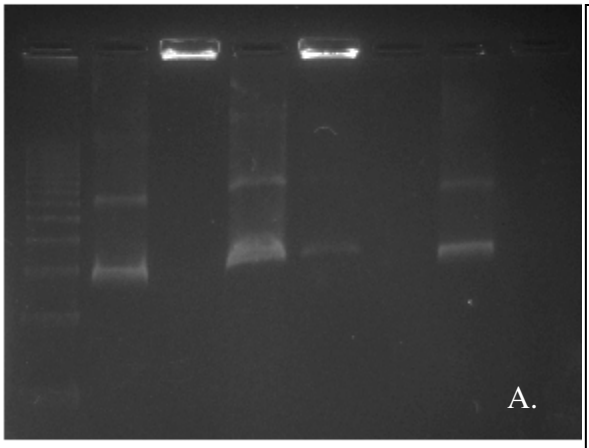


Figure 3.4: *Relative fluorescence of samples treated with 4M NaCl/25% Triton X-100 solution. The high autofluorescence of NaCl/Triton compared with PBS should be noted (n=5, error bars represent standard error.*

The addition of HCl prior to treatment with NaCl/Triton lowered the pH to 5.0 but had no effect on the intensities observed in Figure 3.4 above.

Gel electrophoresis used to detect the electrophoretic mobility of complexes treated with ACN and 5M NaCl were performed. Lane assignments and imaging can be seen in Figure 3.5. Plasmid samples treated with NaCl and ACN have banding patterns identical to those of the pDNA with no treatment. A light band can also be seen in lane 5 (complex + NaCl) indicating that some free plasmid was available to travel through the gel.



B.

Lanes:	
1.	1kb ladder
2.	50ng pure plasmid
3.	100:1 particle:plasmid
4.	50ng plasmid + 5M NaCl
5.	100:1 particle:plasmid + 5M NaCl
6.	
7.	50ng plasmid + ACN
8.	100:1 particle:plasmid + ACN

Figure 3.5:* A.** *Electrophoretic mobility of treated pDNA and complexes. Gel was run at 60V for 60 minutes and imaged with a 3.2 second exposure time.* ***B. *Contents of each lane*

Discussion

The PLGA-chitosan nanoparticles and pDNA associate with one another based on differences in surface charge. Within this complex, pDNA can associate either on the inside or outside. In order to determine the location of the pDNA, it is possible to break apart the complexes and quantify the DNA that is released. Prior to dissociation, any plasmid that is located on the outside of the complex can be removed by nuclease degradation. Theoretically, any remaining plasmid that is released during dissociation can then be quantified using fluorescence spectroscopy. The complexes were treated with several solutions in an attempt to dissociate them and free any pDNA.

A comparison of plasmid treated with ACN and NaCl with untreated plasmid DNA indicates that these solutions are incompatible with the OG assay. These samples contained pDNA in equal quantities to that of the positive control, but emitted signals comparable to the

negative control which contained no nucleic acid. As a result, it is impossible to conclude whether the ACN and NaCl treatments are capable of dissociating the PLGA-chitosan/pDNA complexes using this assay. Similarly, the Triton X-100 interfered with the intensity of the pDNA signal. Instead of eliminating the fluorescence, the Triton seemed to enhance it, producing inconsistent measurements that were higher than that of the pDNA positive control. Investigation into these changes in OG fluorescence found that multiple 'contaminants' have been proven to directly interfere with OG signal intensities [3]. A list of these compounds and their effects are found in Table 1.

Table 3.1: *Effects of several compounds that commonly contaminate nucleic acid preparations on the signal intensity of the OliGreen ssDNA quantitation assay [3]*

Compound	Concentration	% Signal Change*
Salts		
Ammonium Acetate	50mM	13% decrease
Sodium Acetate	30mM	3% decrease
Sodium Chloride	100mM	25% decrease
Zinc Chloride	1mM	43% decrease
Magnesium Chloride	5mM	34% decrease
Urea	2M	47% decrease
Organic Solvents		
Phenol	0.2%	19% decrease
Ethanol	10%	19% increase
Chloroform	2%	2% increase
Detergents		
Sodium dodecyl sulfate (SDS)	0.01%	73% increase

Triton X-100	0.1%	11% increase
Proteins		
Bovine Serum Albumin (BSA)	2%	20% increase
IgG	0.1%	37% increase
Other Compounds		
Poly(ethylene glycol)	1%	29% increase
Agarose	0.1%	8% increase
ATP	0.1%	30% increase

** The compounds were incubated at the indicated concentrations with OliGreen reagent in the presence of 660 ng/mL of a 24-mer M13 sequencing primer. All samples were assayed in a final volume of 200 µL in 96-well microplates. Samples were excited at 485 nm, and the fluorescence intensity was measured at 520 nm.*

A combination of NaCl and the detergent Triton X-100 was chosen in order to overcome the effects that these solutions have alone on the OG signal. According to Table 1 NaCl will lead to a 25% decrease in OG fluorescence at 100mM, while a 0.1% Triton solution will yield an 11% increase. It was hoped that a combination of the two would balance out the signal and produce a useable fluorescence measurement. After experimentation, the solution was found to have a high autofluorescence. In addition, the fluorescence intensities were inconsistent between repetitions and no difference was detected between the free pDNA and complexes.

HCl was added to the samples in an attempt to dissociate the complexes by introducing more ions in the solution. The results indicate that the drop in pH had no effect on the fluorescence of the OG; however there was no change in the intensity of the complexes when compared with that of the negative control, indicating that decreasing the pH to 5 was not sufficient to dissociate the complexes. Further decreases in pH were not investigated in order to protect the plasmid from strongly acidic conditions. A combination of HCl addition and

treatment with a 4M NaCl/25% Triton X-100 solution was also added to the complexes. The results of this experiment were similar to those obtained from the NaCl/Triton treatments alone, and continue to indicate that these solutions are interfering too strongly with the OG fluorescence.

Because many of the treatments for complex dissociation were incompatible with the OG assay, samples treated with ACN and 5M NaCl were also run on an electrophoresis gel. Bands were visible on all lanes containing free pDNA indicating that the addition of ACN and NaCl did not interfere with the results. A faint band appeared in Lane 5, indicating that some pDNA may have been released from the complex with the 5M NaCl treatment; however, it appears that the complexes were not sufficiently dissociated. The complexes treated with ACN exhibited no electrophoretic mobility. These results, in conjunction with the fluorescence studies suggest that neither ACN nor NaCl is capable of dissociating the PLGA-chitosan/pDNA complexes.

Chapter 4: Conclusions and Future Directions

Conclusions

The characterization of PLGA-chitosan nanoparticles and nanoparticle/nucleic acid complexes is essential to ascertaining their effectiveness as a drug delivery system. In addition to the physiochemical characteristics of the nanoparticles and complexes, the binding interactions between the particles and pDNA must be determined. Using a DNA intercalating fluorescent dye, the unbound pDNA present after complex formation can be quantified. The intensity of the fluorescent signal emitted by OG after intercalation into free pDNA can be used to determine the optimal PLGA-chitosan:pDNA ratio for the delivery system. Gel electrophoresis is a second technique that can be applied to characterize binding efficiencies of the nanoparticles and has been used to confirm the effectiveness of the fluorescence-based assay. Optimization of the OG assay was performed, and the assay was found to be sensitive enough to accurately characterize nanoparticle/pDNA complexes using only 4ng of pDNA. As a result, this system is capable of a more sensitive characterization using smaller quantities of sample than gel electrophoresis.

Using the fluorescence-based OG assay, the optimal nanoparticle:pDNA ratio was determined to be 100:1. Further analysis can be performed on these complexes to determine the location of the pDNA in relation to the nanoparticles. Using the OG assay, this must be accomplished first by dissociating the complexes to release the nucleic acid within them. Attempts to dissociate the particles include the addition of ACN, NaCl, HCl and Triton X-100. These treatments proved to be incompatible with the assay by interfering with signal emission of OG. Further analysis using gel electrophoresis confirmed that neither ACN nor NaCl were sufficient for complex dissociation. Experiments have been conducted to determine the effects

of several compounds on the OG assay [3]. These results have found that NaCl leads to a significant decrease in the fluorescence of OG, which was confirmed by this work. Additionally, Triton X-100, has been proven to lead to a significant increase in OG signal. In addition, attempts were made to combine the two solutions to cancel the effects on OG fluorescence. The OG assay has been optimized for analysis of pDNA uptake in PLGA-chitosan nanoparticles. Further work will be required to find a treatment capable of dissociating the complexes, especially one that is compatible with the OG assay.

Future Directions

The work that has been accomplished in this study has lead to the optimization of a useful fluorescence-based assay that can be applied to the characterization of PLGA-chitosan nanoparticle/pDNA complexes. The determination of pDNA binding efficiency and optimal nanoparticle:nucleic acid ratio are the important first steps in complex characterization. In addition, this work seeks to elucidate the location of the pDNA within those complexes. One approach to accomplishing this is to compare the pDNA on the outside to the pDNA on the inside by dissociating the complex.

The work presented in Chapter 3 describes previous attempts to dissociate the PLGA-chitosan nanoparticle/pDNA complexes. Future work in this regard will continue to attempt to release the pDNA. In studies using chitosan nanoparticles, complex dissociation has been accomplished using enzyme degradation [39]. The particles were loaded with the drug chloriquine, and a combination of chitosanase and lysozyme was used to digest the particles, releasing the drug. The same enzymes have been used to release DNA from PEGylated chitosan-PLA nanoparticles [34]. In both cases, after particle digestion the released compounds can be further analyzed. The digestion of the chitosan from the PLGA-chitosan nanoparticles

after they have complexed with pDNA is a promising treatment for dissociating the complexes. In addition, it may be possible to first separate the chitosan and pDNA from the PLGA using a liquid-liquid extraction. Once the PLGA has been removed, the chitosan can be digested using chitosanase/lysozme. The freed pDNA can then be quantified using the fluorescence-based assay and gel electrophoresis. It would be necessary to determine whether the OG is compatible with the chitosanase treatment before the results of the fluorescence intensity can be used. It may also be possible to dissociate the complexes by enzyme degradation of the chitosan without removal of the PLGA [8].

Should the OG assay be incompatible with the addition of chitosanase, other intercalating dyes will be investigated (i.e. SYBR Green). Once particle dissociation is accomplished, studies will be performed to quantify the amount of pDNA that has been taken into the complexes. The pDNA located on the outside of the complex can be thoroughly digested using DNAse I. Following nuclease digestion, the complexes can be dissociated, releasing the remaining pDNA that was previously encapsulated by the PLGA-chitosan nanoparticles. It is also possible to create a standard curve in order to correlate the fluorescence intensity with mass of pDNA present in each sample. This will be done so that the amounts of freed pDNA can be quantified. At this point, the mass of pDNA that associates on the outside of the complex versus the mass that associates on the inside can be determined. With this knowledge it will be possible to determine the location of the majority of the pDNA within the complexes.

Chapter 2 describes a fluorescence-based assay for the characterization of the pDNA binding efficiency of PLGA-chitosan nanoparticles. Using this assay, the ideal particle:pDNA ratio for the uptake of all desired pDNA was determined to be 100:1. With this knowledge, the system can be introduced into cells for *in vitro* transfection studies. These studies are important

when characterizing the effectiveness of a particular drug delivery system. After complex formation, the uptake of the complexes and their transfection efficiency can be determined in Chinese Hamster Ovary (CHO) cells. Successful transfection of the PLGA-chitosan nanoparticle/pDNA complexes into these CHO cells is an indication of the effectiveness of PLGA-chitosan nanoparticles as drug delivery vectors.

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