Preparation and Evaluation of Aminoglycoside-Based Nanogels and Microgels for Gene Delivery and DNA binding

by

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ABSTRACT

Many therapeutics administered for some of the most devastating illnesses can be toxic and result in unwanted side effects. Recent developments have been made in an alternative treatment method, called gene therapy. Gene therapy has potential to rectify the genetic defects that cause a broad range of diseases. Many diseases, such as cancer, cystic fibrosis, and acquired immunodeficiency (AIDS) already have gene therapy protocols that are currently in clinical trials. Finding a non-toxic and efficient gene transfer method has been a challenge. Viral vectors are effective at transgene delivery however potential for insertion mutagenesis and activation of immune responses raises concern. For this reason, non-viral vectors have been investigated as a safer alternative to viral-mediated gene delivery. Non-viral vectors are also easy to prepare and scalable, but are limited by low transgene delivery efficacies and high cytotoxicity at effective therapeutic dosages. Thus, there is a need for a non-toxic non-viral vector with high transgene efficacies. In addition to the hurdles in finding a material for gene delivery, large-scale production of pharmaceutical grade DNA for gene therapy is needed. Current methods can be labor intensive, time consuming, and use toxic chemicals. For this reason, an efficient and safe method to collect DNA is needed. One material that is currently being explored is the hydrogel. Hydrogels are a useful subclass of biomaterials, with a wide variety of applications. This class of biomaterials can carry up to a thousand times their weight in water, and are biocompatible. At smaller dimensions, referred to as micro- and nanogels, they are very useful for many biomedical applications because of their size and ability to swell.

Based on a previously synthesized hydrogel, and due to the advantages of smaller dimension in biomedical applications, we have synthesized aminoglycoside antibiotic based nanogels and microgels. Microgels and nanogels were synthesized following a ring opening polymerization of epoxide-containing crosslinkers and polyamine-containing monomers. The nanogels were screened for their cytocompatibilities and transfection efficacies, and were compared to polyethylenimine (PEI), a current standard for polymer-mediated transgene delivery. Nanogels demonstrated minimal to no toxicity to the cell line used in the study even at high concentrations. Due to the emerging need for large-scale production of DNA, microgels were evaluated for their binding capacity to plasmid DNA. Future work with the aminoglycoside antibiotic-based nanogels and microgels developed in this study will involve optimization of nanogels and microgels to facilitate in better transgene delivery and plasmid DNA binding, respectively.

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Chapter 1

INTRODUCTION

1.1 Introduction to Hydrogels.

Hydrogels are an attractive subclass of biomaterials with a wide variety of applications. They have been used in soft contact lenses, as bioadhesives for wound treatment, as coatings on catheters, pills, capsules, and even inside the capillary wall in capillary electrophoresis. In sheet form, hydrogels have been used as a reservoir in transdermal drug delivery patches and 2D electrophoresis gels [1]. Hydrogels are three-dimensional hydrophilic polymer networks that have the ability to swell while maintaining an intact structure. These hydrophilic polymer networks can absorb up to thousands of times their dry weight in water [2]. Their high water content makes them biocompatible.

Hydrogels can be fabricated over a wide range of sizes, and at smaller dimensions exhibit desirable qualities. Hydrogels in the micron range are referred to as microgels, and in the nanoscale are nanogels. These smaller dimension hydrogels have been studied extensively in the biomedical field, because of their tunable chemical and physical structure, good mechanical properties, high water content, and biocompatibility [3]. Microgels and nanogels are generally made from with natural or synthetic polymers. Common natural polymers used are chitosan and alginate, and synthetic are polyethylenimine, poly(vinyl) alcohol, poly(vinyl) pyrrolidone, poly(ethylene)glycol, poly-N-isoprpylacrylimide [4]. Furthermore, the reduced size of nanogels make them

highly attractive for tissue regeneration and for drug delivery because they can reach specific sites in the body not easily accessible by microgels and macroscopic hydrogels.

1.2 Nanogels as Drug / Gene Delivery Vehicles

1.2.1 Considerations in Designing Nanocarriers. The main goal in drug delivery is to unload an effective dose of therapeutic agent at the targeted site, while minimizing the adverse side effects. Therefore, as a nanocarrier design engineer it is important to understand the design features that are beneficial for effective drug delivery. Some of the characteristics of an ideal nanocarrier are that they have (1) longevity in the blood, (2) co-loading capabilities, (3) stimuli-sensitivity, (4) sight-specific delivery, as shown in figure 1.

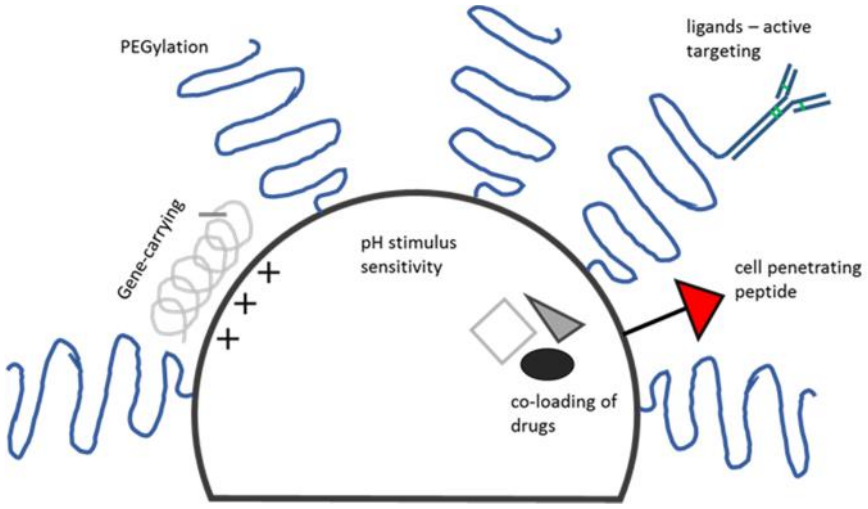

Figure 1. Schematic representation of ideal nanocarrier for gene / drug delivery. Based on [7].

One of important property in a good delivery vehicle is the ability to circulate in the blood for a long time. Nanocarriers face many hurdles as they travel to their destination, such as opsonization and clearance by the reticuloendothelial system (RES).

Opsonization is the immune response that results from the adsorption of serum proteins known as opsonins [5]. Surface properties can be optimized to avoid opsonization. It has been demonstrated that vehicles that are uncharged and have hydrogen bond donor groups can reduce protein adsorption [6]. Another surface modification that increases longevity in blood is the use of poly(ethylene)glycol (PEG) or other hydrophilic polymers (polyvinyl pyrrolidone, polyacryl amide, polyhydrazones, polymorpholines) [7]. Modification of nanocarriers with these polymers prevents interaction with solutes and reduction of uptake by the RES. The additional benefit of nanocarriers with long-circulation is that they can participate in a phenomenon called the enhanced permeability and retention effect. By this phenomena, particles can slowly accumulate in solid tumors due to the defective vasculature, resulting in passive targeting.

Another design aspect to consider is the ability to load multiple therapeutic agents. For instance, in comparison to a single drug, combination chemotherapy regimens in cancer treatment have shown improved anticancer activity compared to a single drug [8]. Combination therapies improve drug solubility and bioavailability and can potentially prevent multidrug resistance [9]. For these reasons nanocarriers with high loading capacity and with multifunctional groups are advantageous.

Stimuli-sensitivity in a nanocarriers can further improve efficacy of delivery. One of the most extensively studied stimuli-sensitive nanocarriers are acid-sensitive carriers, due to the characteristic excess acidity of tumor regions. In comparison to normal tissues, tumors typically exhibit a weak acidity with pH of 6.8. Upon cellular uptake, drug-loaded nanocarriers will be subjected to an intracellular pH of 5.9–6.2 in early endosomes, and pH of 5.0–5.5 in late endosomes and lysosomes [10]. Thus, nanocarriers responsive to

these pH alterations may provide an alternative type of targeting strategy to further improve therapeutic efficacy by cancer site-specific delivery and controlled release.

The ability to tailor a nanocarrier to reach its intended site is advantageous because it can reduce the accumulation and toxicity in healthy tissues. Many therapeutic agents delivered systemically pose risk of harsh side effects. One example is the anticancer drug doxorubicin. This drug is very effective at causing cell death in cancer cells, however causes significant risk for congestive heart failure [11]. To engineer specificity to delivery vehicles, vehicles can be coated or conjugated with ligands for a particular cell surface receptor. Cancer cells commonly over express cell surface receptors for nutrients and growth factors, such as epidermal growth factor, transferrin, and folic acid. All have been used for active targeting of delivery vehicles [12]. With the combination of an optimally engineered nanocarrier, a suitable therapeutic, and an appropriate disease application, the benefit of a nanocarrier over the equivalent non-targeted system can be substantial.

1.2.2 Nanogel Swelling Characteristics. One of the unique properties of nanogels is its capability to swell or collapse in response to environmental stimuli. The high swelling capacity allows nanogels to load more cargo than normally observed for other nanosize pharmaceutical carriers such as polymeric micelles, liposomes, and polymeric nanoparticles. This is because swollen nanogels are mainly comprised of water and this space provides more room for incorporation of therapeutic molecules [13]. Along with desirable high loading capacity, swelling and collapse of nanogels also optimize these carriers for effective drug/gene release, based on its response to environmental stimuli. Understanding the swelling characteristics makes these carriers

tunable for desired stimuli response. In summary, the two key advantages of swelling properties in nanogels is their high loading capacity and tunability for responsive drug/gene release.

It is widely accepted that swelling capacity in nanogels is a balance between a change in osmotic pressure and the polymer elasticity. The characteristics in nanogel formulation that govern swelling include cross-linking density, chemical structure and charge density. Nanogel engineers can tune these properties to swell in presence of stimuli such as pH, temperature, ions, glucose, etc., and to swell to a desired capacity. Lower crosslinking density can increase swelling size and lead to homogenous swelling. In fact, in one study, it was found that high cross-linking reduced swelling size significantly and lead to inhomogeneous deformation of nanogel [14].

Presence of chemical moieties such as amino groups, carboxylic groups, some synthetic polymers can give nanogels the stimuli-responsive swelling characteristics. In pH-responsive nanogels functional groups that are weak acids (carboxylic groups) and weak bases (amino groups) can ionize in pH values above its pKa, or below its pKb respectively. Ionization of the functional groups causes the electrostatic repulsion, thereby increasing the size of the nanogel. As stated in the previous section, pH-responsive swelling is advantageous because many defective tissues have lower pH values than normal tissue. Also, beneficial is along the GI tract, pH has drastic change between the stomach (pH=2) and the intestine (pH= 5-8) [15].

Chemical structure of the nanogel can also influence temperature response. These types of nanogels are generally made of synthetic polymers such as poly-N-isopropylacrylamide [16], poly(ethylene oxide-b-propylene oxide-b-ethylene oxide) and

poly(ethylene oxide)/(d,l-lactic acid-co-glycolic acid) [17]. Molecular weights of these synthetic polymers can dictate the transition temperature at which the nanogel exhibits swelling or collapse. One example of this phenomenon is PEG, which a very popular stabilizing agent. PEG at 2000 Da, which is considered lower molecular weight PEG moiety, has a transition temperature around 170 – 180°C [18], whereas infinite molecular weight PEG exhibits transition temperature around 100°C [19]. Swelling occurs in PEG because when temperature rises, hydrogen bonds between the PEG units in the nanogel and water molecules break.

1.2.3 Microparticles for DNA purification. With recent developments in gene therapy, there is an increasing need for large-scale production of pharmaceutical-grade plasmid DNA. The problem with traditional DNA purification methods, such as saccharose and cesium chloride–diethidium bromide density gradient ultracentrifugation, is that they are difficult to scale up, are time-consuming, and often use chemical reagents that can be harmful for the operator. For this reason, adsorption chromatography is advantageous because it uses chemicals that are safe and it is scalable. There have been several chromatographic methods such as size exclusion, hydrophobic interaction, reversible phase, and anion exchange based particulate supports that have been reported for purification of DNA [20]. Although, limited research has been done to determine the efficacy of microgel particles for DNA purification purposes, the cationic property of many hydrogel particles may make it a good candidate for these applications.

Chapter 2

SYNTHESIS AND EVALUATION OF AMINOGLYCOSIDE-BASED NANOGEL FOR GENE DELIVERY

2.1 Introduction

As stated in the previous chapter, nanogels are a useful class of drug delivery vehicles, primarily because of their size and swelling capabilities. If tuned properly, nanogels can be made to be “intelligent” devices for drug delivery. Intelligent nanocarriers are devices that respond to environmental stimuli, delivering therapeutics to their intended sites. Recently, a new class of aminoglycoside antibiotic-based polymers were synthesized and demonstrated for their transgene efficacy. Cell viability studies revealed that these polymers were less toxic than 25 kDa pEI, and lead polymers demonstrated high luciferase expression in transfections studies [21]. Among the antibiotics analyzed in the study was amikacin-hydrate, as shown in Fig. 2

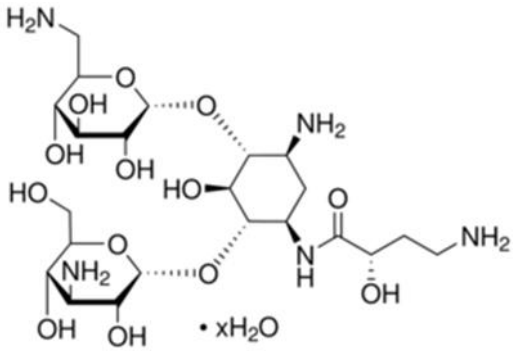

Figure 2. Structure of amikacin-hydrate

Amikacin is an aminoglycoside used to treat various bacterial infections. Amikacin works by binding to the bacterial 30S ribosomal subunit, causing misreading of mRNA, thus leaving the effected bacterium unable to synthesize proteins which are vital

for its own growth [22]. In the molecular structure of amikacin, there are four primary amines. Potta et al. [21] synthesized an amikacin-based hydrogel, among others hydrogels, using ring-opening polymerization between amines of amikacin and epoxides of poly (ethylene glycol) diglycidyl ether (PEGDE).

Using the chemistry from Potta et al [21], we have developed aminoglycoside-based nanogels. We hypothesize that amines present on the chemical structure of the nanogels will give the nanogel cationic properties and expect the nanogels to have the ability to bind DNA. In this work, we have synthesized three amikacin-based nanogels with varying cross-linking densities, and evaluated their size, zeta, and cytocompatibility. Of these three nanogels, we have evaluated one nanogel, referred to herein as AM1, for its transfection efficacy as a starting point to optimize these nanogels for effective gene delivery. The cell line used in this experiment is PC3 (prostate cancer cells). Without optimization, these nanogels have low transfection efficacy, however due to their minimal to no cytotoxicity even at high treatments demonstrates that they have potential to be effective nanocarriers for gene and drug delivery purposes. The following preparation of nanogels is determined as the current best known method for synthesis of amikacin-based nanogels, and serves as a starting point for its optimization.

2.2 Materials and Methods

2.2.1 Materials. Mineral oil (light), Amikacin hydrate, Acetone, Hexane, poly(ethyleneglycol) diglycidyl ether (PEGDE), were procured from Sigma Aldrich (St. Louis, MO) and used as received without any modification. Evonik ABIL®EM90 was given as a gift from Dr. Youngbull.

2.2.2 Synthesis of Cationic Nanogels. Nanogels were synthesized via inverse miniemulsion polymerization [23]. Organic phase (2.2 ml of 10% abil em 90 in mineral oil) was mixed with aqueous phase (25 mg Amikacin hydrate, 250 µl nanopure water, and various volumes of PEGDE, see Table 1*)* using a magnetic stir bar at 850 rpm, 5 min. Emulsion was ultrasonicated three times at 40% amplitude for 30 cycles 1s on/off, over ice. Emulsion was spun at 170 rpm, for 10 min in an oil bath maintained at 70^{o}C.

Table 1:

Amine/Epoxide Molar ratio and volume of PEGDE added to make AM1, AM2, AM3

Sample	**Molar ratio of amine/epoxide**	**Volume of PEGDE added (µl)**
AM1	1:1.5	28.125
AM2	1:2	37.5
AM3	1:3	56.25

Following polymerization, nanogels were pelleted down by centrifugation (700 g, 2 min) and the supernatant was discarded. The nanogel pellet was washed twice: once in acetone, and once in a 1:1 (v/v) acetone/hexane mixture before being dispersed in 11 ml of nanopure water.

2.2.3 Amine concentration. Reactive primary amines on nanogels were determined by the ninhydrin assay. Preparation of sample was done by adding 100 µl of ninhydrin reagent to 200 µl of 1.6 mg/ml AM2 nanogel suspension (dialyzed and undialyzed). The resulting sample was exposed to boiling water for 10 minutes and then allowed to cool to room temperature. Samples were stabilized with 500 µl ethanol before

performing absorption measurements at 570 nm using a plate reader (Bio-Tek Synergy 2). Amine content was calculated using a glycine standard curve.

2.2.4 Zeta Potential and Particle Size Determination of Nanogels and Nanoplexes. The surface charge of the nanogels and nanoplexes was determined using the Zetasizer Nano ZS (Malvern Instruments Ltd., UK) at a temperature of 25 °C. The nanoplexes were prepared in nanopure water with increasing pGL 4-5 plasmid DNA/polymer (w/w) ratios from 1:1.26 to 1:628. The particle size of the nanoplexes was evaluated by dynamic light scattering measurement. Here, complexes with varying ratios were measured using the Zetasizer Nano ZS (Malvern Instruments Ltd., UK) at 25 °C.

2.2.5 Cells and cell culturing. PC-3 cells (prostate cancer cell line) were obtained from the American Type Culture Collection (Manassas, VA). Cells were cultured in serum containing media (RPMI 1640, 10% FBS, 1% penicillin, 1% streptomycin) and were grown in an incubator under humidified air containing 5% CO_2 at 37°C.

2.2.6 In vitro transfection activity. PC3 cells were seeded in 24 well plate at cell density of 50,000 cells per well with RPMI medium containing 10% FBS and incubated at 37 °C for 24 hours. Nanoplexes were prepared at ratios 1:1, 1:2, 1:5, 1:10, and 1:25 with pE-GFP plasmid DNA and equilibrated for 30 min. The cells were then treated with prepared nanoplexes in serum-free RPMI media. At 6 hours, the medium was replaced and cells were further incubated with the samples for 48 hours in RPMI containing FBS. Cells were compared using emissions from fluorescence microscopy (AxioObserver D1, Carl Zeiss MicroImaging Inc., Germany).

2.3 Results and Discussion

2.3.1 Nanogel properties. Three formulations of nanogels (AM1, AM2, and AM3) were synthesized and studied for size, zeta, and amine content. These nanogel formulations differ only by crosslinker. Table 2 shows the size, zeta potential, and amine content of undialyzed nanogels. All studies in this document were done with undialyzed nanogels unless otherwise stated. Size of AM1, AM2, and AM3 all were in the acceptable range for nanocarriers. Nanogel carriers in the size range of 10–100 nm will have improved blood circulation time and are permeable tissues such as tumors [24]. AM1 is significantly larger than AM2 and AM3, which maybe an indication of the large swelling capabilities of AM1. As reported earlier, decreased cross-linking density, as observed with AM1 leads to larger swelling. Zeta potential was unaffected by cross-linking density.

Table 2:

Properties of Nanogels

	Zeta Potential (mV) ±sd	**Average diameter (nm) ±sd**	**Amine Content (mM)**
AM1	52.16 ± 6.60	60.36 ±50.64	--
AM2	54.38 ± 4.90	43.98 ±17.34	83.12
AM3	56.4 ± 4.92	42.61 ±21.87	--

AM2 was dialyzed and analyzed for size, zeta and amine content. All characteristics were reduced to half, and standard deviations were decreased significantly. The average size was 25.44 ± 5.53 nm, 27.37 ± 2.21 mV, and amine content was 45.07

mM. From these values, it is understood that dialysis is an important step to remove any unwanted impurities and to have a controlled and consistent study.

2.3.2 Cytocompatibility of Undialyzed Nanogels. Cytocompatibility of three nanogel formulations was determined in PC3 cell lines using MTT assay. It was found that all three nanogels were cytocompatible with PC3 cells for all masses studied. In comparison to the dead control, the average cell viabilities for AM1, AM2, AM3 are 98.6 $\pm$5.3%, 102 $\pm$ 8.8%, and 99.4 $\pm$ 8.7%, respectively. Figure 3 shows the cytocompatibility study of the three nanogels in PC3 cell line.

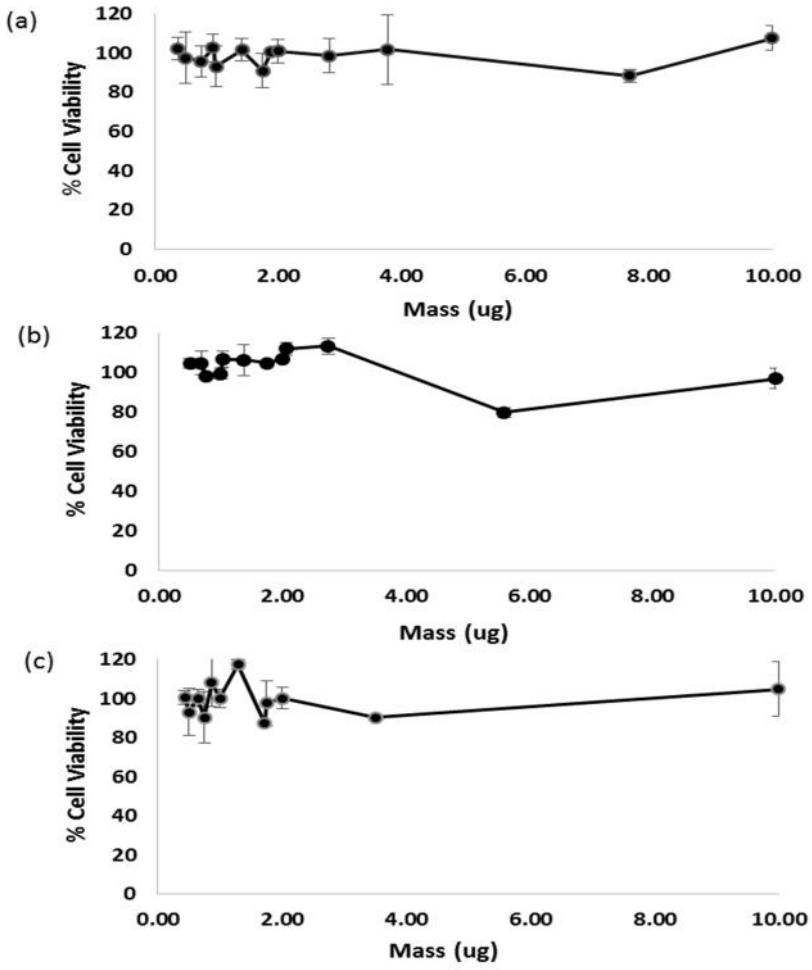

Figure 3. Cytocompatibility studies for (a) AM1, (b) AM2, and (c) AM3 in PC3 cell line

2.3.3 Nanoplex Properties and Transfection Efficacy. Stability of a nanoplex is determined by its surface charge which is indicated by the zeta potential value. The zeta value was recorded at 25 °C in nanopure water and is shown in Table 3. Based on these readings, we can assume nanoplexes formed because only one peak was observed. Generally, when the complexes do not form, multiple peaks would be observed, each indicating the zeta potential of the separate entities. Table 3 also shows the surface charge and size of the nanoplexes made of varying AM1 and pDNA masses.

Table 3:

Zeta potential (mV), hydrodynamic diameter (nm) and polydispersity index of AM1 and DNA complexes at different weight ratios.

Samples (polymer:DNA weight ratios)	**Zeta Potential (mV) ±sd**	**Average diameter (nm) ±sd**
AM1:DNA 1.3 : 1	-23.30 ±6.23	89.69 ± 39.77
AM1:DNA 3.1 : 1	-6.96 ±5.01	94.39 ± 18.30
AM1:DNA 6.3 : 1	+ 13.60 ±4.60	--
AM1:DNA 15.7 : 1	+ 31.50 ±4.21	91.70 ± 49.59
AM1:DNA 31.4 : 1	+ 27.17 ±2.40	38.48 ± 2.89
AM1:DNA 62.9 : 1	+ 32.60 ±2.33	--
AM1:DNA 125.7 : 1	+ 36.05 ±2.00	--
AM1:DNA 314.3 : 1	+ 42.23 ±2.09	41.56 ± 5.53
AM1:DNA 628.6 : 1	+ 46.00 ±2.50	52.86 ± 4.82

From ratios between 1.3 and 3.1 the nanoplexes are negatively charged, whereas for ratios of 6.29 and above, the surface charge were found to increase from +13.6 mV to

+46.0 mV as in Table 3. As the polymer concentration increased, the zeta potential value also increased. Nanoplexes acquired the positive surface charge from the secondary ammonium bases present in AM1.

Intact compaction and condensation of pDNA within the polymer was asserted by the measurement of the particle size by dynamic light scattering performed at 25 °C. The mean hydrodynamic diameters of the complexes of different weight ratios are given in Table 3. Size was seen in the range from 38.48 to 94.39 nm. At lower charge and lower DNA/polymer ratios (1:1.3 to 1:6.3), the size is almost two times larger than DNA/polymer ratios (1:31.4 to 1:628.6), which exhibit more positive surface charge. As explained in the previous chapter, swelling of ionizable nanogels are dictated by electrostatic repulsion. Our results, go against this assertion, in that at a more neutral state, swelling is occurring.

Fig. 4 shows the transfection efficiency of the AM1 when coupled to DNA in ratios of 1:10 and 1:25. Nanoplex sample of 1:25 is representative of 1:1 and 1:2.

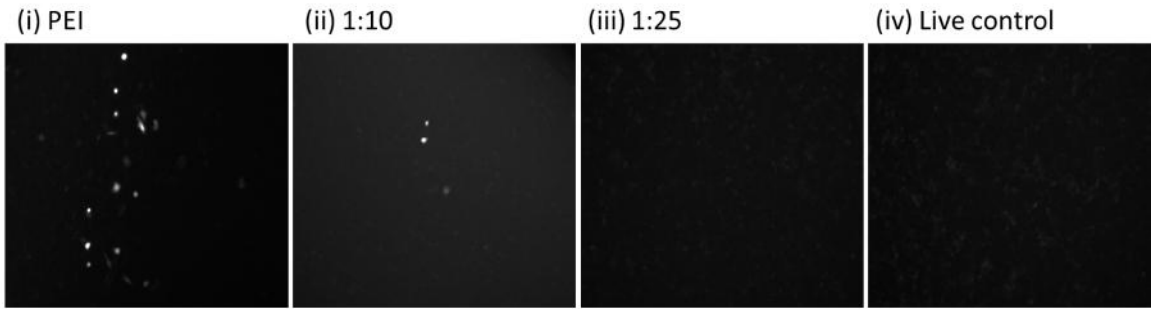

Figure 4. Fluorescence microscopy images of PC3 cells treated with (i) PEI, (ii) 1:10 nanoplex, (iii) 1:25 nanoplex, and live control showing transfection efficacies. Successful transfer is indicated by white spots.

When compared to the control reagent PEI, AM1 showed minimal to no levels of transfection in PC3 cells. Table 3 shows that at DNA to polymer weight ratios of 1:10,

nanoplexes are cationic, which is good for uptake by the negatively charged cell surface. However Table 3 shows the zeta potential of nanoplexes in water, and transfection study was done in serum-free media, so results are not a conclusive explanation for the limited uptake seen in 1:10 samples. As observed in the previous section, dialysis may improve transfection because this would eliminate complexation of DNA with free amines that may be present in the sample. Additionally, conjugation of the nanogels with a cell penetrating ligand may also increase uptake.

Conclusion. Thus far, we have synthesized three cationic aminoglycoside antibiotic-based nanogels with varying crosslinking densities. Due to their positive surface charge, these nanogels are able to complex with plasmid DNA to form nanoplexes. In addition to our characterization of the nanogels, we have found that dialysis can improve purification of nanogels. Purified nanogels may have improved properties, and load more DNA because complexation with competing free amines would be avoided. Although these nanogels have not demonstrated effective transgene delivery, if optimized, can serve as a potential nanocarrier because of their low toxicity profile.

Chapter 3

PREPARATION AND DNA ADSORPTION ISOTHERM OF AMINOGLYCOSIDE-BASED MICROGELS

3.1 Introduction

With recent developments in gene therapy, there is an increasing need for large-scale production of pharmaceutical-grade plasmid DNA. Many diseases, such as cancer, cystic fibrosis, and acquired immunodeficiency (AIDS) have gene therapy protocols that are currently in clinical trials.

For this reason, we have developed a novel amino-glycoside based microgel, which may have the ability to bind high quantities of DNA in adsorption column chromatography. These microgels use the same chemistry used as in described in the previous chapter and in Potta et al [21]. It is hypothesized that due to the presence of primary amines on amikacin, microgels will be cationic and can be neutralized with anionic pDNA. In this chapter we describe our methodology to synthesize aminoglycoside antibiotic-based microgels, determine the hydrodynamic diameter and report preliminary results on its pDNA binding ability.

3.2 Materials and Methods

3.2.1 Materials. Mineral oil (light), Amikacin hydrate, Acetone, poly(ethyleneglycol) diglycidyl ether (PEGDE), sorbitan monooleate (span 80) were procured from Sigma Aldrich (St. Louis, MO) and used as received without any modification.

3.2.2 Synthesis of Amino-glycoside based Microgel Particles. Microgels were prepared by suspension polymerization. Aqueous and oil phase were prepared separately. Aqueous phase was comprised of a homogenous solution of 50 mg of amikacin hydrate, 75 µl of PEGDE and 500 µl of nanopure water. Oil was 80 ml of 1 wt% span 80 in mineral oil. Aqueous solution was dispensed dropwise at a rate of 10 µl/min and maintained at 70°C, with constant stirring. Polymerization reaction was carried out for 15 minutes after the last drop. Following polymerization, microgels were pelleted down by centrifugation (5430 g, 10 min) and the supernatant was discarded. The microgel pellet was washed twice in roughly 0.5% tween 20 in nanopure water and followed by nanopure water until solution appeared clear.

3.2.3 DNA Batch Isotherm. pGL4.5 plasmid DNA was transformed into E coli and expanded, extracted, and purified using a Qiagen Maxi Prep kit according to manufactures protocols. A solution of 200 µl of pDNA in EB buffer at varying concentrations (75.75, 227.5, 455, and 910 ng/µl) was combined with ~2mg of dehydrated microgels in 2 ml eppendorf tubes. The mixture was rotated for at 25°C for 24 hour to allow equilibrium to be reached. Equilibrium plasmid concentration was then measured by reading the 260 nm absorbance. The amount of adsorbed plasmid, q_e, was calculated by mass balance of supernatant content, before and after equilibrium (C_e), and experimental data was plotted. Maximum binding capacity (q_{max}) was obtained from the best fit of the experimental data to a Langmuir isotherm curve from experiments done in duplicate.

$$q_e = \frac{q_{max} \cdot K \cdot C_e}{1 + K \cdot C_e}$$

3.3 Results and Discussion

3.3.1 Microgel synthesis. Microgel particles were suspended in nanopure water and allowed to swell for at least 15 minutes before analysis. In swollen state, nanogels appear to be spherical in shape, as shown in Figure 5. Hydrodynamic diameter was to be 12.6 $\pm$ 8.18 µm, measured by confocal microscopy.

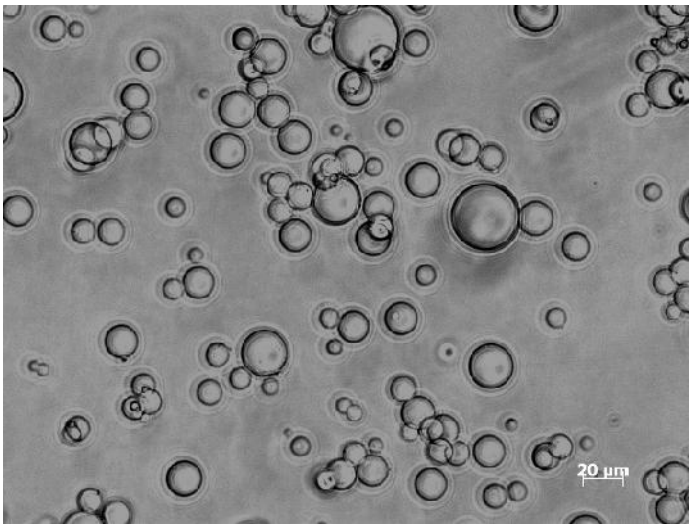

Figure 5. Image of microgels in nanopure water. Microgels were prepared in mineral oil using sedimentation polymerization method.

3.3.2 Plasmid Adsorption. Batch adsorption data was measured for the plasmid adsorbing to novel microgel beads. Microgel beads used in experiment were from 3 batches of beads per experiment synthesized by methods mentioned above. The results plotted in Fig. 6 show that the solid-phase plasmid concentration against liquid phase plasmid concentration. All experiments were carried out in EB buffer. The data were fit to the Langmuir isotherm, yielding an estimated capacity q_{max} of 35 mg/g.

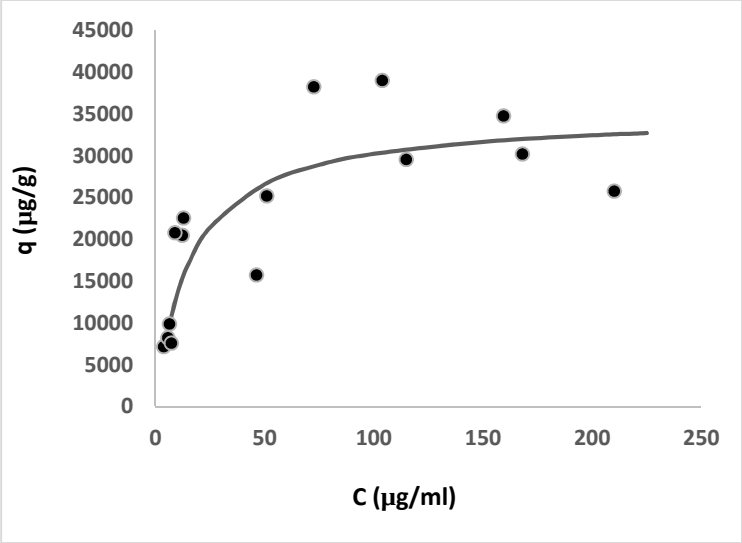

Figure 6. Batch Adsorption isotherms for adsorption of plasmid DNA to novel microgel beads. Experimental data (points) and fitted Langmuir isotherm (line) are for plasmid binding with 12 µm microgel beads equilibrated for 24h in EB buffer.

3.4 Conclusions. Successful synthesis of aminoglycoside antibiotic-based microgels was achieved. Microgels made were roughly 12 microns in diameter when suspended in nanopure water. From preliminary batch adsorption isotherm data, microgels demonstrates ability to bind to DNA. However many improvements need to be made to increase loading capacity, in order for this material to be effective for DNA purification.

FUTURE WORK

4.1 Chapter 2 Future Work

From size, zeta potential, and amine content results of AM2, we have determined that AM1 and AM3 need to be purified via dialysis using a 1000 Da MWCO membrane against water. This will ensure that all contaminants and excess amines, which are soluble in water, are removed from the nanogel solution. The excess amines may present a problem in accurate size determination of the nanogel samples. They also can bind to the DNA occupying binding sites where the nanogel usually binds. With all of the excess monomer removed from the polymer solution, it is speculated that only the polymer will bind to the DNA. This could lead to more efficient condensation of the DNA and allow higher transfection efficacy due to smaller particle sizes. The size, zeta potential, amine content, and transfection efficacy would have to be re-evaluated with dialyzed nanogels. Although purifying the nanogels further will only improve cytocompatibility, it would be beneficial to test the cytocompatibility of dialyzed nanogels at the higher masses to give higher confidence to the MTT results.

Size and zeta of polyplexes should be repeated in serum-free and serum-containing media. Understanding the effect of serum free and serum containing media can explain whether charge of the polyplex is effecting the uptake of polyplex. If charge is not the culprit, another possibility could be that the nanoplexes are too stable and the vector is unable to release the pDNA for expression by the cell. In the case that the charge is not found to be the culprit, altering the formulation to make them degradable may alleviate this issue. As the nanogel degrades, it is expected to be released from the carrier, freeing it for expression by the cell. Degradable component must be

cytocompatible in order to be beneficial. Loading capacity of these vectors needs to be determined using the gel retardation assay. Polyplexes in ratios between 1:1 to 1:50 would need to be prepared and loaded onto agarose gel. When voltage is applied, the nanoplex that does not move is considered to be the fully loaded nanoplex. One commonly reported item that poses a threat to the toxicity of nanogels is the presence of surfactant. We currently use 10% abil em 90, which is relatively a large amount. It would be a good idea to determine whether there is still a presence of the surfactant.

4.2 Chapter 3 Future Work

It would be useful to develop size controlled microparticles of various sizes, and analyze binding effects based on size. Elution is a pretty important step, which often uses harsh and toxic chemicals. And, this problem can be exacerbated in large scale production. Elution of DNA binding beads need to be studied. To determine crosslinking degree of the microgels, amine content should be determined. This information would also be valuable if any conjugation for optimization were to be done.

REFERENCES

1. Hoffman, A., Hydrogels for biomedical applications. *Advanced Drug Delivery Reviews*, 64 (Supplement), 18-23.
2. Yoshimura, T., Matsuo, K., Fujioka, R. (2006). Novel biodegradable superabsorbent hydrogels derived from cotton cellulose and succinic anhydride: Synthesis and characterization. *Journal of Applied Polymer Science*, 99(6), 3251-6.
3. Oh, J., Drumright, R., J. Siegwart, D., Matyjaszewski, K. (2008). The development of microgels/nanogels for drug delivery applications. Progress in Polymer Science, (33)4, 448-477
4. Hamidi, M., Azadi, A., Rafiei, P., Hydrogel nanoparticles in drug delivery. Advanced Drug Delivery Reviews, 60(15), 1638-1649
5. Vinogradov, S., Batrakova, E., Kabanov, A. (2004). Nanogels for Oligonucleotide Delivery to the Brain Bioconjugate. *Chemistry* 15(1), 50-60.
6. Yang, S. Liu, Y., Wang, Y., Cao, A. Biosafety and Bioapplication of Nanomaterials by Designing Protein–Nanoparticle Interactions. *Small*, 9(9-10), 1613-6829.
7. Mussi, S., Torchilin,V. (2013)Recent trends in the use of lipidic nanoparticles as pharmaceutical carriers for cancer therapy and diagnostics. *J. Mater. Chem*, 1(39), 5201-5209.
8. Tang, B. Cheng, G., Gu, J., Xu, C. (2008) Development of solid self-emulsifying drug delivery systems: preparation techniques and dosage forms, *Drug Discovery Today*, 13(13–14), 606-612.
9. Cao, N., Cheng, D., Zou, S., Ai, H., Gao, J., Shuai, X. (2011) The synergistic effect of hierarchical assemblies of siRNA and chemotherapeutic drugs co-delivered into hepatic cancer cells. *Biomaterials*, 32 (8), 2222-2232.
10. Torchilin, V. (2009) Multifunctional and stimuli-sensitive pharmaceutical nanocarriers, *European Journal of Pharmaceutics and Biopharmaceutics*, 71(3),431-444.
11. Zhan, F., Chen, W., Wang,Z., Lu,W., Cheng,R., Deng,C., Meng,F., Liu,H., and Zhong,Z. (2011) Acid-Activatable Prodrug Nanogels for Efficient Intracellular Doxorubicin Release. *Biomacromolecules*, 12(10), 3612-3620
12. Brannon-Peppas,L., Blanchette, J. (2012) Nanoparticle and targeted systems for cancer therapy, *Advanced Drug Delivery Reviews*, 64(Supplement) 206-212.

13. Kabanov, A., Vinogradov, S. (2009), Nanogels as Pharmaceutical Carriers: Finite Networks of Infinite Capabilities. *Angew. Chem. Int. Ed*., 48, 5418–5429.

14. Tamura, G., Shinohara, Y., Tamura, A., Sanada, Y., Oishi, M., Akiba, I., Nagasaki, Y., Sakurai, K., Amemiya, Y. (2011). Dependence of the swelling behavior of a pH-responsive PEG-modified nanogel on the cross-link density *Polymer Journal* 44**,** 240–244

15. Chang, C. Wang, Z. Quan, C. Cheng, H. Cheng, S. Zhang, X. Zhuo, R. (2007). Fabrication of a novel pH-sensitive glutaraldehyde cross-linked pectin nanogel for drug delivery. *Journal of Biomaterials Science*, 18 (12), 1591-1599

16. Kanazawa,H. ,Yamamoto, K. Matsushima, M. Takai, N. Kikuchi, A. Sakurai, Y. Okano, T. (2006). Temperature-Responsive Chromatography Using Poly(N-isopropylacrylamide)-Modified Silica. *Analytical Chemistry,* 68 (1), 100-105.

17. Daniel-da-Silva, A. Ferreira, L. Gil, A. Trindade, T. (2011) Synthesis and swelling behavior of temperature responsive κ-carrageenan nanogels. Journal of Colloid and Interface Science (355)2, 512-517.

18. Yamaoka, T., Tabata, Y. and Ikada, Y. (1994), Distribution and tissue uptake of poly(ethylene glycol) with different molecular weights after intravenous administration to mice. *J. Pharm. Sci.*, 83, 601–606.

19. Nagahama, K., Hashizume, M., Yamamoto, H., Ouchi, T., Ohya, Y., (2009). Hydrophobically Modified Biodegradable Poly(ethylene glycol) Copolymers that Form Temperature-Responsive Nanogels. *Langmuir*, 25 (17), 9734-9740

20. Perçin, I., Sağlar, E., Yavuz, H., Aksöz, E., Denizli, A., (2011). Poly(hydroxyethyl methacrylate) based affinity cryogel for plasmid DNA purification, *International Journal of Biological Macromolecules*, 48(4), 577-582

21. Potta, T., Zhen, Z., Grandhi, T. Christensen, M., Ramos, J., . Breneman, C., Rege, K. (2014). Discovery of antibiotics-derived polymers for gene delivery using combinatorial synthesis and cheminformatics modeling, *Biomaterials*, 35(6),1977-1988

22. Kondo, J., François, B., Russell, R. J., Murray, J. B., & Westhof, E. (2006). Crystal structure of the bacterial ribosomal decoding site complexed with amikacin containing the γ-amino-α-hydroxybutyryl (haba) group. *Biochimie*, 88(8), 1027-1031.

23. Raemdonck, K., Naeye, B., Buyens, K., Vandenbroucke, R. E., Høgset, A., Demeester, J. and De Smedt, S. C. (2009), Biodegradable Dextran Nanogels for

RNA Interference: Focusing on Endosomal Escape and Intracellular siRNA Delivery. *Adv. Funct. Mater*, 19, 1406–1415.

24. Ganta, S., Devalapally, H., Shahiwala, A., Amiji, M. (2008). A review of stimuli-responsive nanocarriers for drug and gene delivery. *Journal of Controlled Release*, 126(3) 187-204