“SMART” POLYELECTROLYTE NANOMEDICINE FOR CYSTIC FIBROSIS PNEUMONIA

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ABSTRACT OF THE THESIS

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By JESSICA MCDONALD

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Bacterial biofilms build up in the sputum of cystic fibrosis patients, causing chronic respiratory infections and eventual lung failure. This mucus layer contains several bacterial species including gram-negative *Pseudomonas aeruginosa* and gram-positive *Staphylococcus aureus*, which are hard to eliminate, as multi-drug resistant strains, such as *MRSA*, develop. Three significant antimicrobial drugs known to fight cystic fibrosis lung infections include polymyxin B (PB), tobramycin (TB), and cathelicidin LL-37. Encapsulating such antimicrobials in self-assembling, amphiphilic polyelectrolyte surfactants (PSs) is hypothesized to enhance mucus penetration and biofilm eradication.

Numerous PS-PB, PS-TB and PS-LL37 formulations were prepared, varying the poly(alkylacrylic acid) backbones, the graft percentage of the Jeffamine® pendant chains, and the drug to polymer charge ratio. These “GRAPLON” nanomedicines were then characterized based upon their hydrodynamic diameter, zeta potential and release kinetics. Optimal formulations were aerosolized via a 3-jet Collision nebulizer, and aerosol droplet size distributions were determined for proper pulmonary delivery. Finally, the antimicrobial and anti-biofilm activities of particular formulations against clinical isolates from cystic fibrosis patient sputum samples were examined, and their minimum
inhibitory concentrations (MICs) and minimum biofilm eradication concentrations (MBECs) were determined.

The “GRAPLON” nanomedicine, PMAA-g-10%J:TB, exhibited the most potential as an effective antimicrobial agent to eradicate cystic fibrosis lung infections. Complexes with PMAA-g-1%J and PPAA-g-1%J appeared to aggregate, as exhibited by their large hydrodynamic diameters. This was due to the nature of the PSs having less Jeffamine® graft, or fewer ethylene oxide groups, which typically resist aggregation. Nanoparticle formulations encapsulating LL-37 produced large and polydispersed solutions. The high molecular weight and amphiprotic nature of LL-37 may have caused weaker binding to the PSs, leading to mutual self-association, rather than complexation. LL-37 controlled release studies further exemplified the challenges with this peptide, as the concentrations of its membrane dialysis assay samples were below the limit of HPLC detection.

Small and stable complexes, less than 200 nm in hydrodynamic diameter and greater than |5| mV in zeta potential, were observed with the formulations PMAA-g-10%J:PB, PMAA-g-5%J:PB, PMAA-g-10%J:TB and PMAA-g-5%J:TB, with PSs dialyzed in 1X PBS. PMAA-g-10%J:TB exhibited a controlled release over the first 6 hours, demonstrating the release kinetics of the Higuchi model. PMAA-g-10%J:PB aerosolized easily, without mechanical damage, and its aerosol size distribution exhibited droplet sizes appropriate for pulmonary delivery, or under 5 µm in diameter.

Antimicrobial and anti-biofilm activity studies utilized nanoparticles conjugated with PMAA-g-10%J due to its high density of Jeffamine®, which is a copolymer of ethylene oxide and propylene oxide, and is hypothesized to confer particle stabilization
and mucus penetration. As expected, the MICs of drugs were retained following PS encapsulation into nanoparticles. PMAA-g-10%J:TB consistently exhibited greater antimicrobial activity against the clinical isolate and commercial *P. aeruginosa* strains, as compared to PMAA-g-10%J:PB; therefore, the MBECs of tobramycin were investigated. As hypothesized, the MBECs of clinical isolate samples treated with PMAA-g-10%J:TB were less than or equal to the MBECs of analogous samples treated with free tobramycin. The greater anti-biofilm activity of PS-TB was due to the PS’s high detergency and membrane permeability, which enhanced mucus penetration. Therefore, due to its consistently small and stable nanoparticle formations, its Higuchi-like release kinetics, and its consistently high antimicrobial and anti-biofilm activities, PMAA-g-10%J:TB was concluded as the most optimal “GRAPLON” nanomedicine for cystic fibrosis treatment.
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TABLE OF CONTENTS

ABSTRACT OF THE THESIS.................................................................ii

ACKNOWLEDGEMENT.................................................................v

List of Tables..................................................................................ix

List of Figures..................................................................................xi

CHAPTER 1: INTRODUCTION.........................................................1

1.1 Target Issue: Bacterial Biofilms in The Sputum of Cystic Fibrosis Patients…1

1.2 Previous Therapeutic Approaches.............................................2

1.2.1 CFTR Modulators.................................................................2

1.2.2 Antimicrobials for Treating Lung Infections............................4

1.3 Proposed Nanoparticle Delivery System ....................................6

1.3.1 “GRAPLON” Nanomedicine Development............................6

1.3.2 Proof of Concept.................................................................8

1.3.3 Cystic Fibrosis “GRAPLON” Nanomedicines.........................10

1.4 Impact.....................................................................................12

CHAPTER 2: MATERIALS & METHODS........................................14

2.1 Formulation and Characterization of Aqueous Nanocomplex Solutions.....14

2.1.1 Preparation of Drug-Encapsulating Graft Copolymer

Nanocomplexes (“GRAPLON” Nanomedicines)...............................14

2.1.2 Characterization of Nanoparticle Hydrodynamic Diameter and

Zeta Potential............................................................................19

2.2 Controlled Release of Antimicrobial Drugs..................................20

2.2.1 Membrane Dialysis Assays..................................................20
2.2.2 HPLC Analysis ................................................................. 22
2.3 Formulation and Characterization of Aerosol Droplets .............. 24
  2.3.1 Formation of Aerosols via Collision Nebulizer .................. 24
  2.3.2 Aerosol Droplet Size Distribution .................................. 25
2.4 Antimicrobial Activity of “GRAPLON” Nanomedicines .............. 26
  2.4.1 Production of Tryptic Soy Agar Plates ............................. 26
  2.4.2 Streaking Tryptic Soy Agar Plates .................................. 27
  2.4.3 Bacteria Inoculation ..................................................... 28
  2.4.4 Serial Broth Dilution Method for Minimum Inhibitory
        Concentrations ........................................................... 28
2.5 Anti-Biofilm Activity of “GRAPLON” Nanomedicines .............. 42
  2.5.1 Bacteria Inoculation ..................................................... 42
  2.5.2 Seeding the 96-Well Plate at a Density of 1x10^6 cells/well ....... 43
  2.5.3 Washing the 96-Well Plate and Adding the Serially Diluted
        Antimicrobial Drug ....................................................... 45
  2.5.4 Applying alamarBlue Reagent and Reading the 96-Well Plate ... 48
CHAPTER 3: RESULTS & DISCUSSION .............................................. 49
  3.1 Average Hydrodynamic Diameter and Zeta Potential of Nanoparticles ...... 49
  3.2 Controlled Antimicrobial Drug Release ................................ 59
    3.2.1 Controlled LL-37 Release ........................................... 59
    3.2.2 Controlled Tobramycin Release ................................... 62
  3.3 Aerosol Droplet Size Distribution ..................................... 66
  3.4 Minimum Inhibitory Concentrations ................................... 67
3.5 Minimum Biofilm Eradication Concentrations ........................................73

CHAPTER 4: CONCLUSIONS & FUTURE WORK ........................................80

APPENDIX .................................................................................................85

REFERENCES .............................................................................................95
List of Tables

Table 1. Parameters for PS and antimicrobial drug calculations…………………………17

Table 2. Parameters for each controlled release study……………………………………21

Table 3. Useful and discarded TSA plates, streaked with 20 clinical isolates from cystic fibrosis patient sputum samples collected at Rutgers Robert Wood Johnson Hospital….29

Table 4. Antimicrobial drug concentrations due to serial dilution for all trials………35

Table 5. Inoculated bacteria volume calculations for a density of 1x10^6 cells/well……37

Table 6. Bacteria solution volume data, based upon OD values of each clinical isolate sample, to seed 96-well plates at a density of 1x10^6 cells/well………………………….44

Table 7. Nanoparticle formation data table. *Indicates use for controlled release study, **indicates use for antimicrobial study, ***indicates use for aerosolization, and ****indicates use for anti-biofilm study. Polydispersity values closer to 1 signify less accurate sizing and quality factor values less than 1 indicate low quality data…………50

Table 8. (A) LL-37 standard solution peak areas, displaying inaccurate detection of concentrations below 15.6 µg/mL. (B) LL-37 controlled release study 1 sample peak areas, used to calculate membrane dialysis assay sample concentrations via the standard curve in Figure 6. All membrane dialysis assay samples were below the limit of detection achieved with this setup………………………………………………………………….60

Table 9. LL-37 controlled release study 2 sample peak areas, used to calculate membrane dialysis assay sample concentrations via the standard curve in Figure 6. Most membrane dialysis assay samples were below the limit of detection achieved with this setup, and their values were attributed to noise, as seen by the similar peak areas of the blanks…..61

Table 10. (A) Tobramycin (TB) standard solution peak areas, used to create the standard curve in Figure 7. (B) Tobramycin (TB) controlled release study sample peak areas, used to calculate membrane dialysis assay concentrations via the standard curve in Figure 7. Correction calculations for the 3 mL dialysate dilutions were performed to obtain actual concentration values for PMAA-g-10%J:TB release……………………………………64

Table 11. Minimum inhibitory concentrations for various bacteria samples and antimicrobial drug formulations………………………………………………………..68

Table 12. Summary of minimum inhibitory concentration results…………………………73
Table 13. Visual results of the remaining biofilms after 24 hour incubation with the serially diluted drug, washing the plate with 1X PBS and 30 minute incubation with alamarBlue. Similar biofilm conservation was observed for tobramycin and PMAA-g-10%J:TB

Table 14. Results summary of minimum biofilm eradication concentrations (MBECs) for various bacteria samples using tobramycin and PMAA-g-10%J:TB
List of Figures

**Figure 1.** Carbodiimide coupling reaction, grafting pendent polyetheramine chains (with molecular weights of $10^3$ Da to $10^4$ Da) to poly(alkylacrylic acid) backbones (with molecular weights of $10^3$ Da to $10^6$ Da). PAAA backbones were produced via free radical polymerization methods, and nanoparticle size and binding were dependent on pendent chain and PAAA backbone molecular weight. A novel graft copolymer, or polyelectrolyte surfactant, was produced from this reaction.

**Figure 2.** Clear PS solution (left) and slightly turbid “GRAPLON” solution (right).

**Figure 3.** Antimicrobial drug serial dilution 96-well plate set-up for (a) 2 samples in triplicate or 3 samples in duplicate (b) 4 conditions in duplicate.

**Figure 4.** Bacteria 96-well plate set-up for (a) 2 samples in triplicate (b) 3 samples in duplicate (c) 1 sample per plate (d) 2 samples per plate. Different colors specify different bacteria samples.

**Figure 5.** Bacteria 96-well plate set-up for (a) 2 samples in triplicate (b) 4 samples in duplicate. Different colors specify different bacteria samples.

**Figure 6.** LL-37 standard curve, used to obtain unknown LL-37 concentration values from membrane dialysis assay sample peak areas via the equation $\frac{\text{Peak area} - \text{y-intercept}}{\text{slope}}$, where the y-intercept was -76478 and the slope was 8582.

**Figure 7.** Tobramycin standard curve, used to obtain unknown tobramycin concentration values (x) from membrane dialysis assay sample peak areas (y) via the equation $y = -8.87x^2 + 52395x + (2x10^7)$.

**Figure 8.** Tobramycin release from PMAA-g-10%J:TB, described by the Higuchi model, in which the amount released was proportional to $t^{1/2}$.

**Figure 9.** PMAA-g-10%J:PB aerosolization log-normal size distribution, displaying a mean size of about 100 nm. Electrical mobility diameters ($10^1$ – $10^3$ nm) were computed via SMPS and aerodynamical diameters ($10^3$ – $10^4$ nm) were computed via APS. DDS(1), in black, was aerosolized using a desiccation chamber, and DDS(2), in red, was aerosolized using a nebulizer only.

**Figure 10.** Bar graphs of relative fluorescence units vs. tobramycin concentration, displaying the quantitative proliferation of bacteria for various clinical isolate samples and tobramycin formulations. Minimum biofilm eradication concentrations were exhibited at concentration values less than 5% of the maximum fluorescence.
Figure A1. Hydrodynamic diameter distribution plots and their corresponding correlograms

Figure A2. Zeta potential distribution plots
CHAPTER 1: INTRODUCTION

1.1 Target Issue: Bacterial Biofilms in The Sputum of Cystic Fibrosis Patients

Cystic fibrosis is a genetic disease that causes a buildup of mucus, chronic respiratory infections, and eventual lung failure. The overproduction of mucoid alginate results in the growth of multi-drug resistant bacterial biofilms, or microcolonies, within the sputum of cystic fibrosis patients. As biofilms develop, airways become severely obstructed and inflamed. This accumulation and lack of clearance of mucus leads to a constant cycle of deterioration and repair of the pulmonary epithelium, eventually causing subepithelial fibrosis and weakened respiratory function.¹

Cystic fibrosis results from a defect in the cystic fibrosis transmembrane conductance regulator (CFTR), which is expressed in a number of tissues. Defective CFTR proteins cause a decrease in chloride secretion, thus altering ionic transport. In turn, water absorption increases and mucus composition changes. As such, CFTR defects cause a variety of health problems in addition to pulmonary difficulties, including malfunction of the GI tract, pancreas and liver. Impairment of such organs may lead to many health issues including but not limited to vitamin malabsorption, poor growth, autodigestion, gallstones and hepatobiliary disease.² More than 70,000 people suffer from cystic fibrosis, and most patients do not live past the age of 44. 90% of cystic fibrosis patients die from respiratory failure; therefore, when considering treatments, the lung is the principal target.¹

There are about 2,000 different CFTR mutations, which cause this life-threatening disease; therefore, cystic fibrosis is divided into six different classes of mutations.
including those that interfere with protein synthesis (class I), those that affect protein maturation (class II), those that alter channel regulation (class III), those that affect chloride conductance (class IV), those that reduce CFTR function at the apical membrane (class V), and those that decrease the stability of CFTR at the plasma membrane (class VI). Since so many different mutations cause cystic fibrosis, there isn’t a cure or therapy for all; instead, this disease is currently managed with symptomatic treatments, all whose end goal is to improve the clearance of mucus, decrease inflammation and fight infection.3

1.2 Previous Therapeutic Approaches

1.2.1 CFTR Modulators

Many therapeutic approaches for cystic fibrosis have been explored, but are incomplete due to the heterogeneity and complexity of this disease. Mutation-specific therapies have been investigated, but they are mostly inconclusive due to the variability of cystic fibrosis mutations, delivery challenges, toxicity and off-target effects. Targeting and manipulating other channels, such as the epithelial sodium channel (ENaC) and the calcium-dependent chloride channel (TMEM16A), may counteract the defective CFTR by improving the balance of airway surface liquid (ASL), which, in turn, may increase mucociliary clearance and the quality of life for patients; however, a proof of concept for this theory has yet to be established.4

Correctors, such as lumacaftor and tezacaftor, move malfunctioning CFTR to the cell surface, and potentiators, such as ivacaftor, strengthen its activity by improving channel function. Ivacaftor improves lung function for class III and IV patients by about
10%, reducing frequency of pulmonary infections by about 55% and sweat chloride concentration by about 60 mmol/L, ultimately improving quality of life.\textsuperscript{3,4} Still, this drug does not treat four other classes of cystic fibrosis patients. Combining lumacaftor and ivacaftor reduces the frequency of pulmonary infections, which ultimately improves lung function; however, this therapy produces many unwanted side effects, including chest tightness and shortness of breath. Combining tezacaftor and ivacaftor improves lung function with fewer side effects; nonetheless, this therapy is only applicable to specific mutations.\textsuperscript{4} There have been other therapeutic attempts including fully functional CFTR gene therapy and various pharmacotherapies, which activate CFTR function in a mutation-specific manner; however, clinical trials for gene therapy failed, and the pharmacotherapies are limited by the knowledge of CFTR and how much it varies from patient to patient.\textsuperscript{3}

miRNA-binding block oligonucleotides (MBBOs) inhibit the binding of certain microRNAs, improving CFTR channel activity. However, the delivery of oligonucleotides is limited due to mucus obstruction, and miRNA regulate the expression of many genes; therefore, inhibiting them may lead to off-target side effects.\textsuperscript{5} GET peptides, or non-viral gene vectors, coupled with DNA to form nanoparticles show significant potential, as they penetrate barriers, target specific cells and undergo endosomal degradation very effectively, especially when PEGylated. However, the low gene transfection efficacy of non-viral vectors requires that further research be done in this area.\textsuperscript{1} DNA viruses, such as adenovirus and adeno-associated virus (AAV), have higher transfection efficacy and move through mucus easily. Nonetheless, there are
disadvantages to this gene delivery method, as the viruses have low transduction efficiencies, immune responses and basolateral pulmonary receptors.²

1.2.2 Antimicrobials for Treating Lung Infections

Cystic fibrosis treatments include single or combination therapies of various mucolytics, anti-inflammatories, bronchodilators and antibiotics. Chest physiotherapies, hypertonic saline and dornase alfa break up mucus secretions in the lung, and prednisone, ibuprofen and N-acetyl cysteine treat inflammation.²

Many antibiotics are used to prevent, eradicate and control infection. Flucloxacillin and ciprofloxacin are two antibiotics used for prophylactic treatment of cystic fibrosis infections. Flucloxacillin treats the most commonly found gram-positive bacteria, *Staphylococcus aureus*, and ciprofloxacin treats the most commonly found gram-negative bacteria, *Pseudomonas aeruginosa*. Inhaled, oral and intravenous forms of antibiotics are used to eradicate and control infection, though oral and intravenous forms lead to increased side effects. Tobramycin is an aminoglycoside antibiotic, which eradicates *P. aeruginosa*. It is delivered via a dry powder inhaler or nebulizer, and is often paired with dornase alfa, an inhaled mucolytic drug that reduces mucus viscosity by destroying its DNA. Oral azithromycin and inhaled aztreonam are two antibiotics, which also treat *P. aeruginosa* infections, often in conjunction with tobramycin.⁶ Oral and intravenous vancomycin treat *S. aureus* infection and inflammation; however, *P. aeruginosa* often protects *S. aureus* biofilms from this antibiotic. Such methicillin-resistant *S. aureus (MRSA)* strains are common in cystic fibrosis infections, thus compromising the effectiveness of the antibiotics used to treat its symptoms.⁷

Colistimethate sodium, including cationic antimicrobial peptides (CAPs) polymyxin B
and E (colistin) effectively destroys *P. aeruginosa*, but is prone to nephrotoxicity and systemic biodegradation in vivo. Therefore, nanocarriers are necessary for safe and proper drug delivery of such antimicrobial peptides.\(^8\)

Nanostructured lipid carriers (NLCs) deliver drugs and nucleic acids. They have high drug stability and advantageous pharmacokinetics, as they transport across barriers and deliver to target organs very well. Even more, they mitigate drug resistance and allow for lower drug doses. PEGylation improves pharmacokinetic properties by enhancing solubility, protecting from degradation, increasing circulation times and allowing for controlled release. Therefore, PEGylation of NLCs increases mucus penetration and lung delivery. Meanwhile, liposomes are nanocarriers of hydrophilic, lipophilic and amphiphilic drugs. They, too, increase the pharmacokinetics and in vivo efficiency of drugs, as they transport across barriers well, provide selective delivery to target organs and decrease drug toxicity. Examples of drugs encapsulated by liposomes include alpha-tocopherol for the treatment of lung edema and prostaglandin E2 (PGE2) for the treatment of idiopathic pulmonary fibrosis. PGE2 has proven to decrease the amount of hydroxyproline, a cystic fibrosis indicator, which, in turn, reduces lung damage.\(^2\)

Finally, nanoencapsulated colistin interacts with the outer membrane of *P. aeruginosa*, ultimately decreasing its stability and increasing its permeability and leakage. Still, it is presumed that diffusion to the lung will be limited in vivo by the current formulation’s larger particle size.\(^8\) Additionally, colistin may be toxic to the kidneys if administered systemically and a method of inhaling the drug has yet to be approved in America.\(^9\) Therefore, it is necessary to develop a lung-specific drug delivery
system, which penetrates the thick and dehydrated mucus of cystic fibrosis patients and eradicates drug resistant bacterial biofilms, without producing advert side effects.

1.3 Proposed Nanoparticle Delivery System

Though previous therapeutic approaches are promising, there are many limitations to these treatments due to the heterogeneity and complexity of CFTR mutations, as well as selective lung delivery challenges. Antibiotics are currently used to treat infection and its symptoms; however, it is common for MRSA strains to develop, rendering these antimicrobials ineffective. Therefore, a novel medicine that may penetrate and destroy the biofilms of *P. aeruginosa*, *S. aureus* and their multi-drug resistant strains in the sputum of cystic fibrosis patients, without toxicity, is desired.

1.3.1 “GRAPLON” Nanomedicine Development

Antimicrobial peptides have proven to treat infection well by killing bacterial cells via membrane disruption; however, their use has been limited, due to their tendency to degrade and inactivate in biological fluids, such as blood plasma. Self-assembling graft polymer-lipid oligonucleotide nanocomplexes (“GRAPLON” nanomedicines) were developed to encapsulate and deliver CAPs, such as linear decapeptide KSL-W and cyclic lipopeptide polymyxin B (PB), without hydrolytic degradation. The pH-sensitive polyelectrolyte surfactants, or graft copolymers, were produced by grafting pendent chains (amine-terminated Jeffamine® polyetheramine or amine terminated poly(ethylene oxide)) to poly(alkylacrylic acid) backbones (poly(propylacrylic acid) (PPAA) or
poly(methacrylic acid) (PMAA)), which stimulate membrane penetration and endosomal escape, via carbodiimide coupling at a molar ratio of 1:100, as seen in Figure 1.\textsuperscript{10}

\[\text{Poly(alkylacrylic acid)} + \text{Polyethyamine} \rightarrow \text{Graft copolymer}\]

where: R=H, -CH\text{3} (methyl) or -CH\text{2}-CH\text{2}-CH\text{3} (propyl); (EO) x=31, (PO) y=10; n=300; and j=3-30

\textbf{Figure 1.} Carbodiimide coupling reaction, grafting pendent polyethyamine chains (with molecular weights of $10^3$ Da to $10^4$ Da) to poly(alkylacrylic acid) backbones (with molecular weights of $10^5$ Da to $10^6$ Da). PAAA backbones were produced via free radical polymerization methods, and nanoparticle size and binding were dependent on pendent chain and PAAA backbone molecular weight. A novel graft copolymer, or polyelectrolyte surfactant, was produced from this reaction.\textsuperscript{10}

These patented graft copolymers were formulated to be surface-active and electrostatically self-assembling via the use of Hildebrand cohesive energy (solubility) parameters and the hydrophilic:lipophilic balance (HLB). The equation, \(\log(\text{CAC}) = a + b \times \text{(HLB)}\), relates the HLB to the critical aggregation concentration (CAC), which was obtained through surface tension studies using the DuNooy tensiometer.\textsuperscript{11} There is a thermodynamic relationship between the HLB and the detergent activity of the polyelectrolyte surfactant against mucus.\textsuperscript{12} This correlation was used to enhance the antimicrobial activity of the nanocomplexes against bacterial biofilms. The HLB of the polyelectrolyte surfactant is a function of graft density and pendent chain amphiphilicity.\textsuperscript{13} Amphiphilic pendent chains have both hydrophobic and hydrophilic properties. The extent of this was governed by the alkyl group of the hydrophobic poly(alkylacrylic acid) backbones (methyl for PMAA or propyl for PPAA), the ratio of
hydrophilic ethylene oxide (EO) to hydrophobic propylene oxide (PO) in the pendent chains, and the graft density of the pendent chains.\textsuperscript{13, 14} The pendent chain Jeffamine® M-2070 polyetheramine was utilized, which is primarily poly(ethylene glycol) (PEG) based. PEG both stabilizes the copolymer blend and increases the circulation time of the drug delivery system in vivo. Jeffamine® M-2070 (J) has an EO/PO mole ratio of 31/10; hence, it is more hydrophilic and, therefore, avoids interaction with serum proteins, which typically promote aggregation and opsonization.\textsuperscript{10}

1.3.2 Proof of Concept

The polyelectrolyte surfactants (PSs), indicated by the labels PPAA-g-j\%J and PMAA-g-j\%J, where j = 1, 5 or 10, self-associated into drug-containing nanoparticles via hydrogen bonding, and exhibited controlled drug release via reversible electrostatic binding forces.\textsuperscript{10, 15} The self-assembly and stability of the PS-CAP nanoparticles were confirmed by measuring particle size and zeta potential via laser light scattering methods of the Malvern ZetaSizer Nano instrument. Nanoparticles with sizes less than 200 nm and zeta potentials greater than $|5|$ mV were considered stable. Nanoparticle size depended on PS graft density and PS-CAP charge ratio. Various Jeffamine® densities and charge ratios were examined in previous studies by increasing the amount of KSL-W from 30 to 80 µg/mL. Particle sizes decreased and became more mono-dispersed as more KSL-W was added to achieve a charge ratio of 0.5. Meanwhile, PMAA-g-10\%J:PB at a charge ratio of 0.5 exhibited an average particle size of 142 nm and an average zeta potential of -18 mV. Small, nano-sized particles are integral for the proper delivery of the encapsulated drug.\textsuperscript{16} Polymyxin B lifetimes were extended more than three-fold, as degradation of polymyxin B was hindered when encapsulated in PS. This was useful in
lowering the previously high, toxic doses of biodegradable polymyxin B. Preliminary studies showed that ungrafted PMAA:PB created the largest particles, PMAA-g-5%:PB created smaller particles and PMAA-g-10%:PB created the smallest particles.¹⁰

The controlled release of CAPs from PS-CAP nanoparticles was examined via membrane dialysis assays and HPLC analysis. Degradation rates, release profiles and binding efficiencies were measured via HPLC, which recorded KSL-W concentration for each condition and formulation. PS graft density is a factor determining both nanoparticle size and controlled release of the encapsulated antimicrobial drug. As graft density increased, net anionic charge decreased and the electrostatically bound CAPs were released faster. In previous studies, free KSL-W exhibited a half–life of about 4 hours and 100% release. PPAA-g-10%:KSL-W exhibited approximately 80% release and PPAA-g-1%:KSL-W exhibited about 30% release. Nanocomplexes with a charge ratio of 0.5 displayed half-lives of about 24 hours and 100% binding efficiency. Meanwhile, PMAA-g-10%:PB at a charge ratio of 0.5 exhibited polymyxin B binding levels from 70-90%. Ungrafted PMAA:PB exhibited the lowest polymyxin B release, PMAA-g-5%:PB exhibited higher polymyxin B release and PMAA-g-10%:PB exhibited the highest polymyxin B release.¹⁰

Preliminary studies using the DuNouy tensiometer and cone-and-plate viscometry displayed that PMAA-g-10%:PB nanoparticles reduced the shear-dependent viscosities of two mucus-imitating hydrogels, non-ionic hydroxyethylcellulose (HEC) and anionic carboxymethylcellulose (CMC). At a low shear rate of 5/s, CMC shear viscosity was 100 Pa-s when treated with polymyxin B and 35 Pa-s when treated with PMAA-g-10%:PB nanoparticles.¹⁶
The lowest concentration of antimicrobial peptide that prevents bacterial growth is known as the minimum inhibitory concentration, or MIC. Lower MIC values indicate greater drug effectiveness. MICs are useful in determining dosages of antimicrobial agents, as biofilms are broken down by dosages 10-1,000 times greater than the MICs. The higher the drug dosage, the greater the chance of adverse side effects in vivo. Therefore, it is important to obtain low MICs for lower antibiotic dosages and, thus, lower possibilities of unwanted side effects.\(^{17,18}\) It was hypothesized that the antimicrobial activities of CAPs would be preserved when complexed with PSs. Therefore, CAPs and PS-CAPs were expected to exhibit the same MIC values, as was seen in previous PMAA-g-j%J:PB studies. Additionally, preliminary studies measured MICs of the KSL-W:PMAAg1 nanocomplexes against \textit{S. aureus} via the CLSI broth microdilution method. The results of this experiment concluded that the nanocomplexes retained some or all (25% - 100%) of the KSL-W peptide’s antimicrobial activity. Less activity was seen in the more protected nanocomplexes, exemplifying the tradeoff between these two properties. Anti-biofilm activities of PS-CAP nanoparticles were expected to increase, as compared to free CAPs, since the PS increases biofilm penetration and decreases drug degradation. Therefore, MBECs of PS-CAP nanoparticles were expected to be lower than MBECs of the free peptide, as was seen in PMAA-g-j%J:PB studies.\(^{10}\)

\subsection{Cystic Fibrosis “GRAPLON” Nanomedicines}

Amphiphilic, anionic PSs, which encapsulate antimicrobials and self-assemble via “smart” surface-active chemistry into novel nanocomplexes, are hypothesized to distribute across previously limited physiological barriers and kill gram-positive \textit{S.}
*aureus*, gram-negative *P. aeruginosa*, and their multi-drug resistant organism (MDRO) strains. The anionic PS component of the nanoparticle is postulated to improve mucus penetration via an increase in membrane permeability and detergency, and the cationic drug cargoes are expected to exhibit proper controlled release after distributing through the small pores of the lungs. Such drug cargoes include polymyxin B, human cathelicidin LL-37 and tobramycin. Polymyxin B and LL-37 are two promising CAPs hypothesized to eradicate cystic fibrosis infections, and tobramycin is a cationic aminoglycoside widely used in treatment methods today. Polymyxin B (PB) and tobramycin (TB) are effective against gram-negative *P. aeruginosa*, while LL-37 has shown recent advances in research, eradicating gram-positive *S. aureus* and *MRSA*.6, 8, 19

The novel PS-CAP and PS-TB “GRAPLON” nanomedicines are to be aerosolized at the proper droplet size, via a clinical nebulizer, and sprayed into the sputum of cystic fibrosis patients, exhibiting enhanced mucus penetration and destruction of biofilms. This local pulmonary delivery of the nanoparticles directly into the lungs prevents nephrotoxicity due to systemic exposure, as well as increases the drug concentration at the site of infection, thus lowering dosage and increasing efficacy. The “GRAPLON” nanomedicines are hypothesized to exhibit higher activity against *MRSA*, as well as lower dosages and frequencies of administration, lowering patient pain and anxiety.

The main aims for this study include synthesizing PS-PB, PS-LL37 and PS-TB nanocomplexes and determining their particle size, zeta potential and release kinetics. Optimal solutions are to be aerosolized via a 3-jet Collision nebulizer to determine the aerosol droplet size distributions of the nanoparticles, and confirm their ability to passively target the lungs of cystic fibrosis patients.20, 21 Lastly, antimicrobial and anti-
biofilm activities of the “GRAPLON” nanomedicines are to be measured against clinical isolates from cystic fibrosis patient sputum samples collected at the Rutgers Robert Wood Johnson Hospital, to determine effective drug load concentrations and confirm the validity of this treatment.

1.4 Impact

Cystic fibrosis lung infections, characterized by thick mucus build-up, are caused by irregular chloride ion and water exchange in the epithelial cell linings, as a result of defective CFTR proteins. The pathogenic species in such lung infections may include *Pseudomonas aeruginosa* (80% prevalence, 17.9% of which are multi-drug resistant), *Staphylococcus aureus* (70.7% prevalence, 25.9% of which are methicillin-resistant (MRSA)), *Stenotrophomonas maltophilia* (12.9% prevalence), *Mycobacterial species* (12.7% prevalence) and *Burkholderia cepacia* (2.5% prevalence). Infections often involve multiple species of bacteria, making treatment difficult. Current treatments, including antibiotics, mucolytics, CFTR modulators, ibuprofen and pancreatic enzyme replacement therapy, have only extended the median life expectancy to 44 years.

*P. aeruginosa* is currently treated with nebulized tobramycin (Novartis’ TOBI®), inhaled dry powder tobramycin (TOBI Podhaler®), oral azithromycin, inhaled aztreonam, or inhaled colistimethate sodium (Colobreathe® and Colomycin®). Colistimethate sodium (CMS) is a cationic antimicrobial peptide that is approved by the European Medicines Agency, as its inhalation does not lead to substantial systemic exposure and, therefore, nephrotoxicity. Still, it is not approved by the U.S. FDA, as it is highly susceptible to systemic biodegradation, which is problematic in regards to dosage.
CMS includes the peptides polymyxin B and polymyxin E (colistin), which kill gram-negative bacteria, such as *P. aeruginosa* and *S. maltophilia*, but not gram-positive *S. aureus*. Therefore, polymyxin B encapsulated in polyelectrolyte surfactant is a promising formulation, which may destroy *P. aeruginosa*, without systemic nephrotoxicity or biodegradation.

Though there are several treatments to control cystic fibrosis lung infections, a cure does not exist to completely eradicate them, as the bacterial biofilms are multi-drug resistant (MDR) due to fluctuations in metabolic gene expression and limited diffusion through extracellular polymeric substances (EPS). The build-up of sticky, thick mucus in cystic fibrosis lung infections causes inflammation, tissue damage and hindered antibiotic delivery; therefore, mucolytics are important components in increasing the effectiveness of antibiotics, as they thin the mucus and facilitate its clearance. More effective mucolytics, which act directly on biofilms, are in high demand, as patients are unresponsive to current medicines, such as dornase alfa (Pulmozyme®). There are several promising cystic fibrosis medicines currently being tested in clinical trials. AlgiPharma’s OligoG®, an oligosaccharide, and NovaBiotic’s Lynovex®, a cysteamine, are proposed to exhibit combined mucolytic and antibiotic activities. Inhalable, liposomal nanoparticle formulations of commercial antimicrobial agents, such as amikacin (Insmed’s Arikayce®), a fosfomycin/tobramycin mixture (CURx’s FTI®) and vancomycin (Savara’s AeroVanc®), are expected to enhance delivery through mucus and exhibit controlled antibiotic release into biofilms. Still, liposomes form long-chain phospholipid bilayers, which are not very detergent and, thus, fail to break up mucus well. In contrast, biosurfactants, including glycolipids, short-chain phospholipids
and lipopeptides (polymer B and E), retain high detergency against cystic fibrosis mucus and effectively dissolve *P. aeruginosa* biofilms.\textsuperscript{40, 41} Therefore, PS-CAP “GRAPLON” nanomedicines show potential in today’s pharmaceutical market. Due to the established efficacy of tobramycin in modern medicine, PS-TB is a promising formulation as well.

**CHAPTER 2: MATERIALS & METHODS**

**2.1 Formulation and Characterization of Aqueous Nanocomplex Solutions**

2.1.1 *Preparation of Drug-Encapsulating Graft Copolymer Nanocomplexes*\textsuperscript{ (“GRAPLON” Nanomedicines)}

First, a 20 mL screw cap scintillation vial was placed on an analytical electronic balance (A&D Weighing) and the tare function was selected so that the reading was 0.000 grams. The desired amount of polyelectrolyte surfactant (PS), synthesized via carbodiimide coupling, was weighed out into the vial. PSs were stored in a -20°C refrigerator/freezer (Fisher Scientific). 5 mL of 0.10 NaOH, prepared by mixing 100 µL of 10 M NaOH stock (Sigma Aldrich) with 10 mL of HPLC-grade water (Fisher Chemical), was added into the vial, along with a small, Teflon-coated magnetic stir bar. The vial was sealed with a screw cap, covered in aluminum foil to protect its contents from ambient light and placed on a magnetic stirrer (Thermolyne Cimarec 2), set at a speed of 200 rpm, for 16-18 hours.

After this 16-18 hour dissolution process, the solution was removed from the vial, using a 20G1½, 0.9mm x 25mm syringe (Becton Dickinson), and injected into two separate 10,000 Da MWCO, 3 mL Slide-a-Lyzer Dialysis Cassettes (Thermo Scientific,
Waltham, MA) in 2.5 mL aliquots. The dialysis cassettes were soaked in deionized water (diH₂O) for 10 minutes before this injection. Each dialysis cassette was then placed in separate 500 mL beakers (Kimble Kimax), each filled with 200 mL of either HPLC-grade water or 1X phosphate buffer saline (PBS). 1X PBS was prepared by mixing 100 mL of OmniPur 10X PBS Liquid Concentrate (MilliporeSigma) in 900 mL of diH₂O. The beakers were sealed with parafilm (Pechiney Plastic Packaging, Chicago, IL) and covered with aluminum foil to protect the solutions from ambient light. The 200 mL of HPLC-grade water or 1X PBS was changed at 1, 3, 6, 24 and 30-hour time points, and the beakers were resealed and recovered each time.

After three days, the solutions within the dialysis cassettes were removed using a syringe and released into a screw cap vial. 2 mL of this solution were added to 2 mL of HPLC-grade water in another screw cap vial. This vial was sonicated in a 4°C ice-water bath (Fisher Scientific) for 20 minutes. Meanwhile, another screw cap vial was placed on an electronic balance, which was then set to tare so that the reading was 0.000 grams. The desired amount of antimicrobial drug was weighed out into the vial and, subsequently, 1 mL of HPLC-grade water was added to form an aqueous solution. Tobramycin sulfate salt (Sigma Aldrich) and polymyxin B sulfate salt (Sigma Aldrich) were stored in the 4°C Isotemp Plus Refrigerator (Fisher Scientific). LL-37 (Biomatik) was stored in the -20°C refrigerator/freezer. The cap was screwed onto the vial and the solid drug particles were fully dissolved in the solution using a Vortex Genie 2 (Fisher Scientific). This vial was then covered with aluminum foil to keep its contents out of ambient light, and an ice-water bath was prepared in a beaker on top of a magnetic stirrer. After 20 minutes had passed, the PS solution was removed from the sonicator and 2 mL of it was transferred
into another screw cap vial containing a small, Teflon-coated magnetic stir bar. This vial was placed in the ice-water bath beaker and the magnetic stirrer was set to a speed of 8000 rpm. The aluminum foil covering the drug solution vial was removed and 800 µL of the solution was drawn up using a 100-1000 µL pipette (Eppendorf). The 800 µL drug solution was added to the spinning PS solution, at a rate of 1 drop every 5 seconds, forming a final “GRAPLON” solution of about 2.85 mL in volume, which then mixed on the magnetic stirrer for another 10 minutes. Larger solution volumes were required for aerosolization use, in which case 4 mL of the PS solution was added to 4 mL of HPLC-grade water, and after 20 minutes of sonication, four times the amount of desired antimicrobial drug was added to 4 mL of HPLC-grade water. 3.2 mL of drug solution was then added, drop-wise, to the entire 8 mL of PS solution, totaling to a final bulk “GRAPLON” volume of about 11.25 mL.

After 10 minutes of mixing, the magnetic stir bar was removed from the “GRAPLON” solution, which was then placed in the 4°C ice-water bath to sonicate for another 20 minutes, along with the leftover PS solution. After 20 minutes, both solutions were removed from the sonicator, covered with aluminum foil to protect from ambient light, and warmed up to room temperature for 1 hour. As seen in Figure 2, the PS solution was clear, whereas the “GRAPLON” solution was slightly turbid. After 1 hour, 100 µL of each solution were added to two separate collection tubes (Ambion), each containing 900 µL of HPLC-grade water. These two solutions were injected into two separate zeta cells (Malvern Zetasizer DTS1070 cell) via 1 mL syringes (Henke Sass Wolf), and their hydrodynamic diameters and zeta potentials were measured in triplicate at a refractive
index of 1.5, absorption of 0.01, equilibration time of 120 seconds and temperature of 37.5°C using the Malvern Zetasizer Nano ZS90 (Malvern Instruments, UK).

Figure 2. Clear PS solution (left) and slightly turbid “GRAPLON” solution (right).

The desired amounts of PS and antimicrobial drug were determined by several parameters including the molecular weights and number of negative charges per mole of the parent polymer (PMAA or PPAA) and graft copolymer (1%, 5% or 10% density), the molecular weight of the graft (Jeffamine® M-2070), the molecular weight and number of positive charges per mole of the antimicrobial drug of choice (polymyxin B, LL-37 or tobramycin), and the desired drug to polymer charge ratio. Table 1 provides the values of such parameters, which were used to calculate how much of one component was needed to achieve a desired charge ratio.

Table 1. Parameters for PS and antimicrobial drug calculations.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parent polymer MW (PPAA)</td>
<td>71000 g/mol</td>
</tr>
<tr>
<td>MW of parent monomer (PPAA)</td>
<td>114 g/mol</td>
</tr>
<tr>
<td>Parent polymer MW (PMAA)</td>
<td>77000 g/mol</td>
</tr>
<tr>
<td>MW of parent monomer (PMAA)</td>
<td>86.1 g/mol</td>
</tr>
<tr>
<td>Graft copolymer MW (1%)</td>
<td>61000 g/mol</td>
</tr>
<tr>
<td>Graft copolymer MW (5%)</td>
<td>109000 g/mol</td>
</tr>
<tr>
<td>Graft copolymer MW (10%)</td>
<td>154000 g/mol</td>
</tr>
<tr>
<td>MW of graft (Jeffamine® M-2070)</td>
<td>2000 g/mol</td>
</tr>
</tbody>
</table>
As seen in Table 1, the molecular weight of LL-37 was relatively large compared to polymyxin B and tobramycin. Therefore, due to its size and amphiprotic nature to both accept and donate a proton, LL-37 was anticipated to exhibit weaker binding to the PSs.

The following calculations, utilizing the parameters in Table 1, were employed to determine either the amount of PS needed for a given antimicrobial drug amount and charge ratio, or the amount of antimicrobial drug needed for a given PS amount and charge ratio.42

\[
\text{Number of units of parent polymer} = \frac{\text{Parent polymer MW}}{\text{MW of parent monomer}}
\]

\[
= \text{Number of COOH groups in parent polymer}
\]

Number of assumed negative charges per mole parent polymer

\[
= \frac{\text{Number of COOH groups in parent polymer}}{3}
\]

Number of graft chains per parent

\[
= \frac{(\text{Graft copolymer MW} - \text{Parent polymer MW})}{\text{MW of graft}}
\]

Actual % grafting = \[
\frac{\text{Number of graft chains per parent}}{\text{Number of units of parent polymer}}
\]

Number of COOH groups in graft copolymer

\[
= \text{Number of COOH groups in parent polymer} - \text{Number of graft chains per parent}
\]
Number of assumed negative charges per mole graft copolymer

\[ \text{Number of assumed negative charges per mole graft copolymer} = \frac{\text{Number of COOH groups in graft copolymer}}{3} \]

For a given amount of antimicrobial drug and desired charge ratio:

\[ \text{Peptide microequivalent} = \frac{(1000)(\text{Drug mass})(\text{Number of positive charges in one mole of drug})}{\text{MW of drug}} \]

\[ \text{Polymer microequivalent} = \frac{\text{Peptide microequivalent}}{\text{Desired charge ratio}} \]

Amount of PS needed =

\[ \left( \frac{\text{Polymer microequivalent}}{\text{Number of assumed negative charges per mole graft copolymer}} \right) \left( \frac{1}{1000} \right) \]

For a given amount of PS and desired charge ratio:

\[ \text{Polymer microequivalent} = \frac{(1000)(\text{Polymer mass})(\text{Number of assumed negative charges per mole graft copolymer})}{\text{Graft copolymer MW}} \]

\[ \text{Peptide microequivalent} = (\text{Polymer microequivalent})(\text{Desired charge ratio}) \]

Amount of drug needed

\[ = \left( \frac{\text{Peptide microequivalent}}{\text{Number of positive charges in one mole of drug}} \right) \left( \frac{1}{1000} \right) \]

2.1.2 Characterization of Nanoparticle Hydrodynamic Diameter and Zeta Potential

Proper formation and stability of the drug-encapsulating PS complexes were determined by their hydrodynamic diameter and zeta potential. Nanoparticles with hydrodynamic diameters of 100-200 nm were considered stable and optimal for proper delivery. Nanoparticle hydrodynamic diameters were measured in a zeta cell, using dynamic light scattering (DLS) methods of the Malvern Zetasizer Nano ZS90. This
instrument utilized the theory of Brownian motion, or the random movement of fluid particles due to collisions, to estimate hydrodynamic diameters over the span of 100+ runs, via measured diffusion coefficients and the Stokes-Einstein equation. The particle dispersions were illuminated with a laser and a photon detector captured the scattered light. Fluctuations in the scattered light depended on the size of the molecules, as smaller particles moved faster and created larger fluctuations.\textsuperscript{43}

Zeta potentials provide a measure of the net charge of the surface-active, electrostatically self-assembling nanoparticles and how they may interact with other molecules in the body. Changes in zeta potential were indicative of PS adsorption as well as the drug to polymer charge ratio. Additionally, nanoparticles with zeta potentials, or overall charges, greater than $|5|$ mV were considered stable and, therefore, properly formed. Nanoparticle zeta potentials were measured in a zeta cell, using the Malvern Zetasizer Nano ZS90 over the span of 100+ runs. The zeta cell contained two electrodes and a capillary, which the solution was loaded into, and the zeta potential was measured via microelectrophoresis, or the movement of particles in an applied electric field.\textsuperscript{43}

\section*{2.2 Controlled Release of Antimicrobial Drugs}

\subsection*{2.2.1 Membrane Dialysis Assays}

The “GRAPLON” nanomedicines were designed to first aerosolize into the lungs of cystic fibrosis patients, then penetrate their mucus biofilms and finally release the encapsulated antimicrobial drug at a controlled rate to kill bacteria. Unbound antimicrobials were expected to release at a faster rate, while bound antimicrobials were expected to release at a slower rate. This controlled release of the bound drug is integral
to increase the bioavailability of the antimicrobial in vivo. The PS coating prevents drug degradation and, thus, lowers the dosage required for the drug to work, which lowers the chance of adverse side effects in other parts of the body. Therefore, high bioavailability due to controlled release increases the effectiveness of the antimicrobial drug.

Controlled release profiles for various formulations were obtained via membrane dialysis assays, in which the antimicrobial drug solution, within the semi-permeable membrane, diffused over time into the external solution, or dialysate. Each study tested the free antimicrobial drug as well as its complex with the PS, PMAA-g-10%J. Two dialysis cassettes were soaked in diH$_2$O for 10 minutes and then injected with two separate antimicrobial drug solutions, one with and the other without PS, at specific volumes. The dialysis cassettes were placed in two separate beakers, each filled with specific dialysate volumes of diH$_2$O. The beakers were covered with parafilm and then placed in a 37°C Isotemp 210 Water Bath (Fisher Scientific). The antimicrobial drug solution and dialysate volumes for each study can be seen in Table 2. Upon placement in the water bath, 3 mL of the dialysate were removed from each beaker and stored in two separate conical tubes as time 0h samples. 3 mL of diH$_2$O were added to each beaker to maintain the total dialysate volume, and the parafilm was secured back on the beakers. This removal of dialysate and replacement of diH$_2$O was repeated for several time points, as seen in Table 2, and the conical tubes were stored at 4°C until further HPLC analysis.

Table 2. Parameters for each controlled release study.

<table>
<thead>
<tr>
<th>Controlled Release Study #</th>
<th>Antimicrobial Drug Solution</th>
<th>Antimicrobial Drug Concentration (mg/mL)</th>
<th>Antimicrobial Drug Solution Volume (mL)</th>
<th>Dialysate Volume (mL)</th>
<th>Sample Time Points (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>PMAA-g-</td>
<td>1</td>
<td>2</td>
<td>150</td>
<td>0, 1, 3, 6,</td>
</tr>
<tr>
<td>Sample</td>
<td>Drug Name</td>
<td>Concentration</td>
<td>Time Points</td>
<td></td>
<td></td>
</tr>
<tr>
<td>--------</td>
<td>-----------</td>
<td>---------------</td>
<td>-------------</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>LL-37</td>
<td>1</td>
<td>0, 1, 3, 6, 24, 96</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>PMAA-g-10%J:LL-37</td>
<td>1</td>
<td>0, 1, 3, 6, 24, 72, 144</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>PMAA-g-10%J:TB</td>
<td>2.96</td>
<td>0, 1, 3, 6, 24, 48, 72, 96, 168</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Tobramycin</td>
<td>2.96</td>
<td>0, 1, 3, 6, 24, 48, 72, 96, 168</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

2.2.2 HPLC Analysis

In addition to the 3 mL samples stored in conical tubes, standard solutions of each antimicrobial drug were prepared to estimate the amount of antimicrobial drug, which diffused out of the dialysis cassettes and into the dialysates at each time point. A stock solution of 1 mg/mL of LL-37 was prepared and serially diluted 1:1 for a total of 12 standard solutions, with each proceeding solution obtaining half the antimicrobial drug concentration than the previous one. This serial dilution was carried out by adding the stock solution to the first collection tube and 0.5 mL of HPLC-grade water to the next 11 collection tubes. 0.5 mL of the stock solution was removed from the first collection tube.
and mixed into the second collection tube by pipetting up and down. 0.5 mL of the contents in the second collection tube was then removed and mixed into the third collection tube, and so on and so forth. The final 0.5 mL removed from the twelfth collection tube was discarded. The same serial dilution procedure was carried out for the tobramycin standard solutions, with an initial stock solution concentration of 2.96 mg/mL.

LL-37 HPLC analysis was conducted using the Waters 2695 Separations Module, Waters 2489 UV/Visible Detector and Empower 2 software. 1 mL of the membrane dialysis assay samples were transferred directly into labeled HPLC vials, and 0.5 mL of the standard solutions were transferred into shell vial glass inserts within labeled HPLC vials. The vials were then capped and loaded into the HPLC for analysis. A Phenomenex Jupiter 5u C18 300A, 250 x 4.6 mm Column was utilized and the HPLC ran at 40°C, with a 50 µL sample injection volume, a wavelength of 220 nm, a flow rate of 2 mL/min and a run time of 12 minutes. Mobile phase A was 100% HPLC-grade water and mobile phase B was 70% acetonitrile, both with 0.1% trifluoroacetic acid. LL-37 peak elutes were found between 8-9 minutes. Using these peak areas and the known concentrations of the standard solutions, a standard curve of LL-37 was created to determine the unknown concentrations of the membrane dialysis assay samples, whose peak areas were also measured by the HPLC.

Tobramycin HPLC analysis was conducted using the Waters 2695 Separations Module and Empower 2 software. The Sedere Sedex 75 Evaporative Light Scattering Detector was used, running nitrogen at 2.8 bar and 40°C, as tobramycin lacks a chromophore and is not visible by the UV/Visible Detector used for LL-37 without
attaching a fluorescent molecule to it first. 1 mL of the membrane dialysis assay samples were transferred directly into labeled HPLC vials, and 0.5 mL of the standard solutions were transferred into shell vial glass inserts within labeled HPLC vials. The vials were then capped and loaded into the HPLC for analysis. A Phenomenex Jupiter 5u C18 300A 250 x 2.6 mm 5 micron column was utilized, as it provides better retention for hydrophilic molecules like tobramycin. The HPLC ran an isocratic method of 45% acetonitrile and 55% HPLC-grade water at 40°C, with a 50 µL sample injection volume, a flow rate of 1 mL/min and a run time of 30 minutes. Tobramycin peak elutes were found between 2-5 minutes. Using these peak areas and the known concentrations of the standard solutions, a standard curve of tobramycin was created to determine the unknown concentrations of the membrane dialysis assay samples, whose peak areas were also measured by the HPLC.

2.3 Formulation and Characterization of Aerosol Droplets

2.3.1 Formation of Aerosols via Collision Nebulizer

The “GRAPLON” nanomedicines are to be inhaled by cystic fibrosis patients for various reasons. Systemic delivery increases potential cytotoxicity and involves barriers, which may degrade the antimicrobial drug and lower its absorption. On the contrary, direct and localized administration to the lungs via nebulization increases the antimicrobial drug concentration delivered to the site of infection, thereby increasing its overall effectiveness and clinical response. This allows for lower doses to achieve the same therapeutic effect, which, in turn, decreases adverse side effects. Moreover,
aerosolization extends the controlled release of the nanoparticles’ drug cargoes, allowing time for mucus penetration and bacterial biofilm eradication.

Nanoparticles were aerosolized into droplets via a 3-jet Collision nebulizer, at an airflow rate of 5 L/min and an operating pressure of 20 psig. The nebulizer utilized the pressurized air supply to generate energy for turbulent mixing. Initial aerosols were produced from a liquid reservoir, containing the antimicrobial drug. These aerosols then moved in the direction of the airflow to primary and secondary baffles, where they were broken up into smaller droplets. An airflow of 80 L/min diluted and desiccated the aerosol droplets. Flow rate meters regulated the pressurized airflow, and outlet tubes were attached to two size distribution particle counters, a Scanning Mobility Particle Sizer 3986 (SMPS) and an Aerodynamic Particle Sizer 3321 (APS), to span both nano- and micro-sized droplets. The size data, typically 14-20,000 nm, was combined using Merge software (TSI, Inc.). Surfactants, or substances that reduce surface tension, and viscosifiers, which increase viscosity, were added to improve the suspension’s stability. Polyvinyl alcohol (PVA) and hydroxypropylmethylcellulose (HPMC) were biocompatible with the nanocomplexes and enhanced their aerosol properties. Therefore, it was expected that PS surface tension and nanocomplex HLB and Hildebrand solubility parameters affected aerosol properties as well. Colleagues, Drs. Gediminas Mainelis and Leonardo Calderón, at the Rutgers University department of Environmental Sciences, aerosolized the nanoparticles and obtained a size distribution of the resulting droplets.

2.3.2 Aerosol Droplet Size Distribution

The SMPS is an electrical technique, which measures 10 nm – 1 µm particle sizes, and the APS uses light-scattering methods to measure 0.5 – 20 µm particle sizes. Most
pharmaceutical aerosols are polydisperse; therefore, two particle counters were necessary for obtaining a complete particle size distribution.\textsuperscript{44}

Particle size affects lung delivery and, therefore, antimicrobial drug effectiveness. Nanoparticle liquid suspensions, optimally 100-200 nm in size, were nebulized into clusters of aerosol droplets, ideally 1-5 µm in diameter for effective passive targeting and biodistribution to the sputum of the lungs. Aerosol particle sizes of 10-30 µm deposit in the oro-pharyngeal region via inertial impaction, sizes of 2-16 µm deposit in the conducting zone, including the bronchioles, via gravitational sedimentation, and sizes less than 2 µm deposit in the respiratory zone via Brownian diffusion.\textsuperscript{45,46} Therefore, the objective of the aerosolization experiment was to obtain aerosol particles of about 2 µm in size. The nebulizer created aerosol droplets, which then desiccated through a tube under low humidity conditions to form even smaller droplets. Small, single particles are more effective than agglomerated particles due to an increase in surface area. The key variables, which affected aerosol droplet size, include the concentration in the suspension, the type of liquid in which the particles were suspended in, the aerosolization pressure and the dilution airflow.

2.4 Antimicrobial Activity of “GRAPLON” Nanomedicines

2.4.1 Production of Tryptic Soy Agar Plates

15 g of Trypticase Soy Broth (Becton Dickinson) was weighed out into a 1000 mL flask (Pyrex) and dissolved in 500 mL of diH\textsubscript{2}O. 9 g of Bacto Agar (Becton Dickinson) was added to this mixture, which was then brought up to 600 mL with more diH\textsubscript{2}O. The flask was sealed with aluminum foil and autoclave tape was adhered to the
side of it. The flask was then placed in an autoclave (Getinge Model 533LS Vacuum Steam Sterilizer), running on program p13 (for liquids) at 121°C for a total time of 53 minutes. Upon removal from the autoclave, the solution cooled at room temperature for 30-60 minutes. A package of petri dishes (VWR International) were laid out on a sterile counter and the tryptic soy agar (TSA) solution was poured into each plate at a thickness of about ½ in. The petri dishes were covered, stacked and left to cool for about 30 minutes. Once the TSA solution within the plates solidified, the stack was flipped over and stored in the 4°C refrigerator in a sealed bag for later use.

2.4.2 Streaking Tryptic Soy Agar Plates

The TSA plates and clinical isolates from cystic fibrosis patient sputum samples, collected and isolated by the team of Dr. Kirn at the Rutgers Robert Wood Johnson Hospital, were removed from the 4°C fridge. The bacteria isolates were put on ice, and the TSA plates were labeled with the date and sample number. The Bunsen burner tube was connected to the blue gas line, the valve was turned on and the flame was ignited using a sparker. For each bacteria isolate, the inoculation loop was burned with the flame of the Bunsen burner for about 10 seconds and inserted into the layer of TSA at the edge of the plate. The icy bacteria isolate was then touched by the inoculation loop, which then streaked the TSA plate in a zigzag manner 3-4 times. The first zigzag streak obtained the highest concentration of bacteria, while the last zigzag streak obtained the lowest concentration, or single colonies of bacteria. Once all TSA plates were streaked with their respective bacteria isolates, they were placed in the Innova 42 Incubator Shaker (New Brunswick Scientific) at 37°C, with their covers on the bottom. After 24 hours, the plates
were removed from the incubator, wrapped with parafilm and stored in the 4°C fridge. Streaked TSA plates lasted about a month. Visuals of such plates can be seen in Table 3.

2.4.3 Bacteria Inoculation

15 g of tryptic soy broth (TSB) was weighed out into a 1000 mL flask and dissolved in 600 mL of diH₂O. The flask was sealed with aluminum foil and autoclave tape was adhered to the side of it. The flask was then placed in an autoclave, running on program p13 (for liquids) at 121°C for a total time of 53 minutes, in order to sterilize the TSB solution. This stock of sterile TSB was utilized in the remaining procedures. The Bunsen burner tube was connected to the blue gas line, the valve was turned on and the flame was ignited using a sparker. For each bacteria TSA plate, the inoculation loop was burned with the flame of the Bunsen burner for about 10 seconds and inserted into the layer of TSA at the edge of the plate. The inoculation loop then lightly glided across the surface of the plate to pick up one colony of bacteria. This colony was mixed into 1 mL of TSB in a cell culture tube (Fisherbrand), and its cover was placed on the top, without clicking shut so that the bacteria could aerate and grow. These steps were repeated for each bacteria inoculation, with each cell culture tube labeled respectively. The cell culture tubes were placed in the incubator shaker at 37°C and 150 rpm for about 24 hours.

2.4.4 Serial Broth Dilution Method for Minimum Inhibitory Concentrations

The minimum inhibitory concentrations (MICs) of the nanomedicines were determined, based upon the Clinical and Laboratory Standards Institute guidelines for the serial broth dilution method against a commercial strain of Pseudomonas aeruginosa.
(ATCC® 27853™) and clinical isolates from cystic fibrosis patient sputum samples collected and isolated by the team of Dr. Kirn at the Rutgers Robert Wood Johnson Hospital. These clinical isolates provided greater efficacy for the nanomedicines than the commercial strain.

1 – 4 mL of TSB was added to each inoculated bacteria cell culture tube, depending on the concentration of the solutions. 1 mL of TSB was transferred into a cuvette (Plastibrand), as a blank, and 1 mL of the diluted bacteria solutions were transferred into other cuvettes. The cuvettes were inserted into a Nicolet Evolution 100 UV-Visible Spectrophotometer (Thermo Scientific) and the optical density (OD) values of the solutions were measured at a wavelength of 600 nm. If the OD value was below 0.1, the sample was left in the incubator for another day or discarded, as seen in Table 3. If the OD value was above 1, more TSB was added to the solution to dilute it.

**Table 3.** Useful and discarded TSA plates, streaked with 20 clinical isolates from cystic fibrosis patient sputum samples collected at Rutgers Robert Wood Johnson Hospital.

<table>
<thead>
<tr>
<th>Clinical Isolate Sample #</th>
<th>Streaked TSA Plate</th>
<th>Useful or Discarded</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><img src="image" alt="Streaked TSA Plate" /></td>
<td>Useful to an extent- bacterial colonies did not proliferate after first plate</td>
</tr>
<tr>
<td>2</td>
<td><img src="image1.png" alt="Image" /></td>
<td>Useful</td>
</tr>
<tr>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>3</td>
<td><img src="image2.png" alt="Image" /></td>
<td>Discarded due to poor inoculation</td>
</tr>
<tr>
<td>4</td>
<td>Bacterial colonies did not proliferate</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td><img src="image3.png" alt="Image" /></td>
<td>Useful</td>
</tr>
<tr>
<td>6</td>
<td><img src="image4.png" alt="Image" /></td>
<td>Discarded due to poor inoculation</td>
</tr>
<tr>
<td>7</td>
<td>Bacterial colonies did not proliferate</td>
<td></td>
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<tr>
<td>8</td>
<td></td>
<td>Useful</td>
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<tr>
<td>9</td>
<td></td>
<td>Discarded due to poor inoculation</td>
</tr>
<tr>
<td>10</td>
<td>Bacterial colonies did not proliferate</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>Bacterial colonies did not proliferate</td>
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</tr>
<tr>
<td>12</td>
<td></td>
<td>Useful</td>
</tr>
<tr>
<td>13</td>
<td>Bacterial colonies did not proliferate</td>
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<tr>
<td>14</td>
<td></td>
<td>Useful</td>
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<tr>
<td></td>
<td><img src="image1" alt="Image" /></td>
<td><img src="image2" alt="Image" /></td>
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<tr>
<td>15</td>
<td><img src="image4" alt="Image" /></td>
<td>Useful</td>
</tr>
<tr>
<td>16</td>
<td><img src="image5" alt="Image" /></td>
<td>Useful</td>
</tr>
<tr>
<td>17</td>
<td><img src="image6" alt="Image" /></td>
<td>Discarded due to poor inoculation</td>
</tr>
<tr>
<td>18</td>
<td><img src="image7" alt="Image" /></td>
<td>Useful</td>
</tr>
</tbody>
</table>
As seen in Table 3, those samples whose OD values were consistently less than 0.1 were discarded and not tested upon further. Such bacteria include samples 3, 6, 9, 17, and 19-1. Samples 4, 7, 10, 11, and 13 were initially discarded, as bacterial colonies did not proliferate on the TSA plates. Therefore, samples 1, 2, 5, 8, 12, 14, 15, 16, 18 and 19-2 were investigated further.

Serial dilutions of the antimicrobial drug formulations were performed under a sterile Purifier Class II Biosafety Cabinet (Labconco) in 96-well cell culture plates (Greiner Cellstar). First, 100 µL of TSB were transferred from a 50 mL reservoir (VWR International) into each well shown in yellow in Figure 3, using a 50-300 µL Finnpipette Model 4510 Multichannel Digital Pipette (Thermo Fisher) with 200 µL pipette tips (Eppendorf). Then, 200 µL of the antimicrobial drug formulations, specified in Table 4, were transferred into the respective wells of column 2. After pipetting up and down a few
times to mix the two solutions, 100 µL of the contents in column 2 were drawn up, using
the multichannel pipette, and mixed into column 3. Again, 100 µL of the solution in
column 3 were removed and transferred into column 4. This serial dilution was repeated
until column 11. Ultimately, each successive column contained half the concentration of
antimicrobial drug than the previous one. This 96-well plate was then set-aside until
further use. The minimum amount of antimicrobial drug needed was determined by
multiplying the number of rows utilized by 200 µL. This number was then divided by
1000 and multiplied by the initial drug concentration that the serial dilution began with in
column 2 to obtain the amount of antimicrobial drug needed to fill the 96-well plate.

<table>
<thead>
<tr>
<th></th>
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<th>2</th>
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<th>4</th>
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</tr>
</tbody>
</table>

**Figure 3.** Antimicrobial drug serial dilution 96-well plate set-up for (a) 2 samples in
triplicate or 3 samples in duplicate (b) 4 conditions in duplicate.

As seen in Table 4, trials 1 and 2 examined bacteria samples against polymyxin B
(PB), trials 3 and 4 examined bacteria samples against PMAA-g-10%-PB, trials 5 and 6
examined bacteria samples against tobramycin (TB) and trial 7 examined bacteria
samples against PMAA-g-10%-TB, all using the set-up in Figure 3(a). Trial 8 examined
one bacteria sample per 96-well plate against all four antimicrobial drug formulations in
duplicate using the set-up in Figure 3(b). Trial 9 examined bacteria samples against new
polymyxin B and its complex with PMAA-g-10%-J using the set-up in Figure 3(b).
Finally, trial 10 examined a commercial strain of *Pseudomonas aeruginosa* (ATCC® 27853™), to serve as a comparison against the clinical isolates from cystic fibrosis patient sputum samples.

**Table 4.** Antimicrobial drug concentrations due to serial dilution for all trials.

<table>
<thead>
<tr>
<th>Trial #</th>
<th>Antimicrobial Drug Formulation</th>
<th>Initial Antimicrobial Drug Concentration</th>
<th>Calculation for Initial Antimicrobial Drug Concentration</th>
<th>Concentrations in Columns 2 – 11 (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Polymyxin B</td>
<td>40 µg/mL</td>
<td>( \frac{1000 \ µg \text{ PB}}{25 \ mL \ TSB} )</td>
<td>40 20 10 5 2.5 1.25 .625 .313 .156 .078</td>
</tr>
<tr>
<td>2</td>
<td>Polymyxin B</td>
<td>320 µg/mL</td>
<td>( \frac{1000 \ µg \text{ PB}}{3.125 \ mL \ TSB} )</td>
<td>320 160 80 40 20 10 5 2.5 1.25 .625</td>
</tr>
<tr>
<td>3</td>
<td>PMAA-g-10%J:PB (CR 1)</td>
<td>10 µg/mL</td>
<td>( \frac{1000 \ µg \text{ PB}}{2850 \ µL} ) ( V_{stock} = 57 \ µL \text{ PMAA} - g - 10\%j: \text{ PB,} 2000 - 57 = 1943 \ µL \ TSB )</td>
<td>445 320 160 80 40 20 10 5 2.5 1.25 .625</td>
</tr>
<tr>
<td>4</td>
<td>PMAA-g-10%J:PB (CR 1)</td>
<td>40 µg/mL</td>
<td>( \frac{1000 \ µg \text{ PB}}{2850 \ µL} ) ( V_{stock} = 228 \ µL \text{ PMAA} - g - 10\%j: \text{ PB,} 2000 - 228 = 1772 \ µL \ TSB )</td>
<td>445 320 160 80 40 20 10 5 2.5 1.25 .625</td>
</tr>
<tr>
<td>5</td>
<td>Tobramycin</td>
<td>200 µg/mL</td>
<td>( \frac{1000 \ µg \text{ TB}}{5 \ mL \ TSB} )</td>
<td>200 100 50 25 12.5 6.25 3.13 1.56 .781 .391</td>
</tr>
<tr>
<td>6</td>
<td>Tobramycin</td>
<td>50 µg/mL</td>
<td>( \frac{1000 \ µg \text{ TB}}{20 \ mL \ TSB} )</td>
<td>50 25 12.5 6.25 3.13 1.56 .781 .391 .195 .098</td>
</tr>
<tr>
<td>7</td>
<td>PMAA-g-10%J:TB (CR 1)</td>
<td>40 µg/mL</td>
<td>( \frac{2957 \ µg \text{ TB}}{2850 \ µL} ) ( V_{stock} = 77 \ µL \text{ PMAA} - g - )</td>
<td>445 320 160 80 40 20 10 5 2.5 1.25 .625</td>
</tr>
</tbody>
</table>
Another 96-well plate was seeded with bacteria at a density of 1x10^6 cells/well.

Using the spectrophotometer OD value, calculations were performed to solve for the total
volume of inoculated bacteria to be mixed in 5 or 10 mL of TSB, depending on how many rows of the 96-well plate needed to be filled, as seen in Table 5.

**Table 5.** Inoculated bacteria volume calculations for a density of 1x10^6 cells/well.

<table>
<thead>
<tr>
<th>Trial #</th>
<th>Clinical Isolate Sample #</th>
<th>OD Value</th>
<th>Calculation</th>
<th>Volume of Inoculated Bacteria (μL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>0.877</td>
<td>X = (0.877 \times 10^9 \text{ cells/mL} \times 5 \text{ mL} \times \frac{1000 \mu L}{1 \text{ mL}}) (\rightarrow) 5.7 μL</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>2</td>
<td>0.744</td>
<td>X = (0.744 \times 10^9 \text{ cells/mL} \times 5 \text{ mL} \times \frac{1000 \mu L}{1 \text{ mL}}) (\rightarrow) 6.72 μL</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>5</td>
<td>0.144</td>
<td>X = (0.144 \times 10^9 \text{ cells/mL} \times 5 \text{ mL} \times \frac{1000 \mu L}{1 \text{ mL}}) (\rightarrow) 34.7 μL</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>8</td>
<td>0.116</td>
<td>X = (0.116 \times 10^9 \text{ cells/mL} \times 5 \text{ mL} \times \frac{1000 \mu L}{1 \text{ mL}}) (\rightarrow) 43.1 μL</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>12</td>
<td>0.333</td>
<td>X = (0.333 \times 10^9 \text{ cells/mL} \times 5 \text{ mL} \times \frac{1000 \mu L}{1 \text{ mL}}) (\rightarrow) 15.0 μL</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>14</td>
<td>1.28</td>
<td>X = (1.28 \times 10^9 \text{ cells/mL} \times 5 \text{ mL} \times \frac{1000 \mu L}{1 \text{ mL}}) (\rightarrow) 3.91 μL</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>16</td>
<td>1.12</td>
<td>X = (1.12 \times 10^9 \text{ cells/mL} \times 5 \text{ mL} \times \frac{1000 \mu L}{1 \text{ mL}}) (\rightarrow) 4.46 μL</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>0.653</td>
<td>X = (0.653 \times 10^9 \text{ cells/mL} \times 5 \text{ mL} \times \frac{1000 \mu L}{1 \text{ mL}}) (\rightarrow) 7.66 μL</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>5</td>
<td>0.329</td>
<td>X = (0.329 \times 10^9 \text{ cells/mL} \times 5 \text{ mL} \times \frac{1000 \mu L}{1 \text{ mL}}) (\rightarrow) 15.2 μL</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>15</td>
<td>0.252</td>
<td>X = (0.252 \times 10^9 \text{ cells/mL} \times 5 \text{ mL} \times \frac{1000 \mu L}{1 \text{ mL}}) (\rightarrow) 19.8 μL</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>18</td>
<td>0.349</td>
<td>X = (0.349 \times 10^9 \text{ cells/mL} \times 5 \text{ mL} \times \frac{1000 \mu L}{1 \text{ mL}}) (\rightarrow) 14.3 μL</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>( X = 0.01433 \text{ mL} \times \frac{1000 \mu L}{1 \text{ mL}} )</td>
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<tr>
<td>2</td>
<td>19-2</td>
<td>0.305</td>
<td>( \frac{305 \times 10^9 \text{ cells}}{\text{mL}} \times (5 \text{ mL})(1 \times 10^6) )</td>
<td>16.4</td>
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<tr>
<td>3</td>
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<td>0.172</td>
<td>( \frac{172 \times 10^9 \text{ cells}}{\text{mL}} \times (5 \text{ mL})(1 \times 10^6) )</td>
<td>29.1</td>
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<td>3</td>
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<td>0.163</td>
<td>( \frac{163 \times 10^9 \text{ cells}}{\text{mL}} \times (5 \text{ mL})(1 \times 10^6) )</td>
<td>30.7</td>
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<td>3</td>
<td>16</td>
<td>0.131</td>
<td>( \frac{131 \times 10^9 \text{ cells}}{\text{mL}} \times (5 \text{ mL})(1 \times 10^6) )</td>
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<td>3</td>
<td>18</td>
<td>0.462</td>
<td>( \frac{462 \times 10^9 \text{ cells}}{\text{mL}} \times (5 \text{ mL})(1 \times 10^6) )</td>
<td>10.8</td>
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<tr>
<td>3</td>
<td>19-2</td>
<td>0.786</td>
<td>( \frac{786 \times 10^9 \text{ cells}}{\text{mL}} \times (5 \text{ mL})(1 \times 10^6) )</td>
<td>6.4</td>
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<tr>
<td>4</td>
<td>1</td>
<td>0.266</td>
<td>( \frac{266 \times 10^9 \text{ cells}}{\text{mL}} \times (5 \text{ mL})(1 \times 10^6) )</td>
<td>18.8</td>
</tr>
<tr>
<td>4</td>
<td>8</td>
<td>0.427</td>
<td>( \frac{427 \times 10^9 \text{ cells}}{\text{mL}} \times (5 \text{ mL})(1 \times 10^6) )</td>
<td>11.7</td>
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<tr>
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<td>0.699</td>
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<td>7.15</td>
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<tr>
<td>4</td>
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<td>0.723</td>
<td>( \frac{723 \times 10^9 \text{ cells}}{\text{mL}} \times (5 \text{ mL})(1 \times 10^6) )</td>
<td>6.92</td>
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<td>4</td>
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<td>0.798</td>
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<td>6.26</td>
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<td>4</td>
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<td>0.550</td>
<td>( \frac{550 \times 10^9 \text{ cells}}{\text{mL}} \times (5 \text{ mL})(1 \times 10^6) )</td>
<td>9.09</td>
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<tr>
<td>4</td>
<td>19-2</td>
<td>0.560</td>
<td>( \frac{560 \times 10^9 \text{ cells}}{\text{mL}} \times (5 \text{ mL})(1 \times 10^6) )</td>
<td>8.92</td>
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<td>5</td>
<td>12</td>
<td>0.350</td>
<td>( \frac{350 \times 10^9 \text{ cells}}{\text{mL}} \times (5 \text{ mL})(1 \times 10^6) )</td>
<td>14.3</td>
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\[
\begin{array}{|c|c|c|c|}
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\text{Tissue} & \text{Concentration} & \text{Calculation} & \text{Result} \\
\hline
5 & 14 & 0.321 & \frac{321 \times 10^9 \text{ cells}}{\text{mL}} (X) = (5 \text{ mL})(1 \times 10^6) \rightarrow X = 0.01429 \text{ mL} \times \frac{1000 \mu\text{L}}{1 \text{ mL}} \rightarrow 15.6 \\
5 & 16 & 0.419 & \frac{419 \times 10^9 \text{ cells}}{\text{mL}} (X) = (5 \text{ mL})(1 \times 10^6) \rightarrow X = 0.01558 \text{ mL} \times \frac{1000 \mu\text{L}}{1 \text{ mL}} \rightarrow 11.9 \\
5 & 18 & 0.582 & \frac{582 \times 10^9 \text{ cells}}{\text{mL}} (X) = (5 \text{ mL})(1 \times 10^6) \rightarrow X = 0.00859 \text{ mL} \times \frac{1000 \mu\text{L}}{1 \text{ mL}} \rightarrow 8.59 \\
6 & 8 & 0.312 & \frac{312 \times 10^9 \text{ cells}}{\text{mL}} (X) = (5 \text{ mL})(1 \times 10^6) \rightarrow X = 0.016025 \text{ mL} \times \frac{1000 \mu\text{L}}{1 \text{ mL}} \rightarrow 16.0 \\
6 & 15 & 0.187 & \frac{187 \times 10^9 \text{ cells}}{\text{mL}} (X) = (5 \text{ mL})(1 \times 10^6) \rightarrow X = 0.026737 \text{ mL} \times \frac{1000 \mu\text{L}}{1 \text{ mL}} \rightarrow 26.7 \\
6 & 19-2 & 0.436 & \frac{436 \times 10^9 \text{ cells}}{\text{mL}} (X) = (5 \text{ mL})(1 \times 10^6) \rightarrow X = 0.011467 \text{ mL} \times \frac{1000 \mu\text{L}}{1 \text{ mL}} \rightarrow 11.5 \\
7 & 1 & 0.274 & \frac{274 \times 10^9 \text{ cells}}{\text{mL}} (X) = (5 \text{ mL})(1 \times 10^6) \rightarrow X = 0.018248 \text{ mL} \times \frac{1000 \mu\text{L}}{1 \text{ mL}} \rightarrow 18.2 \\
7 & 5 & 0.143 & \frac{143 \times 10^9 \text{ cells}}{\text{mL}} (X) = (5 \text{ mL})(1 \times 10^6) \rightarrow X = 0.03496 \text{ mL} \times \frac{1000 \mu\text{L}}{1 \text{ mL}} \rightarrow 35.0 \\
7 & 8 & 0.139 & \frac{139 \times 10^9 \text{ cells}}{\text{mL}} (X) = (5 \text{ mL})(1 \times 10^6) \rightarrow X = 0.03597 \text{ mL} \times \frac{1000 \mu\text{L}}{1 \text{ mL}} \rightarrow 36.0 \\
7 & 12 & 0.183 & \frac{183 \times 10^9 \text{ cells}}{\text{mL}} (X) = (5 \text{ mL})(1 \times 10^6) \rightarrow X = 0.027322 \text{ mL} \times \frac{1000 \mu\text{L}}{1 \text{ mL}} \rightarrow 27.3 \\
7 & 16 & 0.109 & \frac{109 \times 10^9 \text{ cells}}{\text{mL}} (X) = (5 \text{ mL})(1 \times 10^6) \rightarrow X = 0.045871 \text{ mL} \times \frac{1000 \mu\text{L}}{1 \text{ mL}} \rightarrow 45.9 \\
7 & 18 & 0.224 & \frac{224 \times 10^9 \text{ cells}}{\text{mL}} (X) = (5 \text{ mL})(1 \times 10^6) \rightarrow X = 0.022321 \text{ mL} \times \frac{1000 \mu\text{L}}{1 \text{ mL}} \rightarrow 22.3 \\
7 & 19-2 & 0.926 & \frac{926 \times 10^9 \text{ cells}}{\text{mL}} (X) = (5 \text{ mL})(1 \times 10^6) \rightarrow X = 0.005399 \text{ mL} \times \frac{1000 \mu\text{L}}{1 \text{ mL}} \rightarrow 5.39 \\
8 & 2 & 0.211 & \frac{211 \times 10^9 \text{ cells}}{\text{mL}} (X) = (10 \text{ mL})(1 \times 10^6) \rightarrow X = 0.00501 \text{ mL} \times \frac{1000 \mu\text{L}}{1 \text{ mL}} \rightarrow 47.4 \\
\hline
\end{array}
\]
| 8 | 5 | 0.253 | \[
\frac{0.253 \times 10^9 \text{cells}}{\text{mL}} (X) = (10 \text{mL})(1 \times 10^6) \rightarrow 
X = 0.03953 \text{mL} \times \frac{1000 \mu \text{L}}{1 \text{mL}} 
\] | 39.5 |
| 8 | 8 | 0.274 | \[
\frac{0.274 \times 10^9 \text{cells}}{\text{mL}} (X) = (10 \text{mL})(1 \times 10^6) \rightarrow 
X = 0.036496 \text{mL} \times \frac{1000 \mu \text{L}}{1 \text{mL}} 
\] | 36.5 |
| 8 | 12 | 0.352 | \[
\frac{0.352 \times 10^9 \text{cells}}{\text{mL}} (X) = (10 \text{mL})(1 \times 10^6) \rightarrow 
X = 0.02841 \text{mL} \times \frac{1000 \mu \text{L}}{1 \text{mL}} 
\] | 28.4 |
| 8 | 14 | 0.364 | \[
\frac{0.364 \times 10^9 \text{cells}}{\text{mL}} (X) = (10 \text{mL})(1 \times 10^6) \rightarrow 
X = 0.02747 \text{mL} \times \frac{1000 \mu \text{L}}{1 \text{mL}} 
\] | 27.5 |
| 8 | 15 | 0.114 | \[
\frac{0.114 \times 10^9 \text{cells}}{\text{mL}} (X) = (10 \text{mL})(1 \times 10^6) \rightarrow 
X = 0.087719 \text{mL} \times \frac{1000 \mu \text{L}}{1 \text{mL}} 
\] | 87.7 |
| 8 | 16 | 0.403 | \[
\frac{0.403 \times 10^9 \text{cells}}{\text{mL}} (X) = (10 \text{mL})(1 \times 10^6) \rightarrow 
X = 0.02481 \text{mL} \times \frac{1000 \mu \text{L}}{1 \text{mL}} 
\] | 24.8 |
| 8 | 18 | 0.370 | \[
\frac{0.370 \times 10^9 \text{cells}}{\text{mL}} (X) = (10 \text{mL})(1 \times 10^6) \rightarrow 
X = 0.02703 \text{mL} \times \frac{1000 \mu \text{L}}{1 \text{mL}} 
\] | 27.0 |
| 8 | 19-2 | 0.245 | \[
\frac{0.245 \times 10^9 \text{cells}}{\text{mL}} (X) = (10 \text{mL})(1 \times 10^6) \rightarrow 
X = 0.040816 \text{mL} \times \frac{1000 \mu \text{L}}{1 \text{mL}} 
\] | 40.8 |
| 9 | 2 | 0.105 | \[
\frac{0.105 \times 10^9 \text{cells}}{\text{mL}} (X) = (5 \text{mL})(1 \times 10^6) \rightarrow 
X = 0.04762 \text{mL} \times \frac{1000 \mu \text{L}}{1 \text{mL}} 
\] | 47.6 |
| 9 | 5 | 0.287 | \[
\frac{0.287 \times 10^9 \text{cells}}{\text{mL}} (X) = (5 \text{mL})(1 \times 10^6) \rightarrow 
X = 0.01742 \text{mL} \times \frac{1000 \mu \text{L}}{1 \text{mL}} 
\] | 17.4 |
| 9 | 8 | 0.398 | \[
\frac{0.398 \times 10^9 \text{cells}}{\text{mL}} (X) = (5 \text{mL})(1 \times 10^6) \rightarrow 
X = 0.01256 \text{mL} \times \frac{1000 \mu \text{L}}{1 \text{mL}} 
\] | 12.6 |
| 9 | 12 | 0.423 | \[
\frac{0.423 \times 10^9 \text{cells}}{\text{mL}} (X) = (5 \text{mL})(1 \times 10^6) \rightarrow 
X = 0.01182 \text{mL} \times \frac{1000 \mu \text{L}}{1 \text{mL}} 
\] | 11.8 |
| 9 | 14 | 0.347 | \[
\frac{0.347 \times 10^9 \text{cells}}{\text{mL}} (X) = (5 \text{mL})(1 \times 10^6) \rightarrow 
X = 0.01441 \text{mL} \times \frac{1000 \mu \text{L}}{1 \text{mL}} 
\] | 14.4 |
| 9 | 15 | 0.399 | \[
\frac{0.399 \times 10^9 \text{cells}}{\text{mL}} (X) = (5 \text{mL})(1 \times 10^6) \rightarrow 
X = 0.01441 \text{mL} \times \frac{1000 \mu \text{L}}{1 \text{mL}} 
\] | 12.5 |
The calculated volume of inoculated bacteria was mixed into 5 or 10 mL of TSB, and 100 µL of this solution was multichannel pipetted into the wells, as seen in Figure 4. Finally, 100 µL of the respective rows of the antimicrobial drug serial dilution 96-well plate were transferred into this bacteria 96-well plate. Each row of the antimicrobial drug serial dilution 96-well plate was transferred using a multichannel pipette, ensuring that they matched up correctly with the rows of the bacteria 96-well plate.

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<td>X = 0.01253 mL x \frac{1000 \mu L}{1 \text{mL}}</td>
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<td>X = 0.01362 mL x \frac{1000 \mu L}{1 \text{mL}}</td>
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<tr>
<td>X = 0.00907 mL x \frac{1000 \mu L}{1 \text{mL}}</td>
<td>9.07</td>
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<td>X = 0.01126 mL x \frac{1000 \mu L}{1 \text{mL}}</td>
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<td>X = 0.01894 mL x \frac{1000 \mu L}{1 \text{mL}}</td>
<td>18.9</td>
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<td>9</td>
<td>16</td>
<td>0.367</td>
<td>\frac{0.367 \times 10^9 \text{cells}}{\text{mL}} (X) = (5 \text{mL})(1 \times 10^6) \Rightarrow X = 0.001253 \text{mL} x \frac{1000 \mu L}{1 \text{mL}}</td>
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<tr>
<td>9</td>
<td>18</td>
<td>0.551</td>
<td>\frac{0.551 \times 10^9 \text{cells}}{\text{mL}} (X) = (5 \text{mL})(1 \times 10^6) \Rightarrow X = 0.01362 \text{mL} x \frac{1000 \mu L}{1 \text{mL}}</td>
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<td>9</td>
<td>19-2</td>
<td>0.444</td>
<td>\frac{0.444 \times 10^9 \text{cells}}{\text{mL}} (X) = (5 \text{mL})(1 \times 10^6) \Rightarrow X = 0.01126 \text{mL} x \frac{1000 \mu L}{1 \text{mL}}</td>
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<tr>
<td>10</td>
<td>P. aeruginosa</td>
<td>0.264</td>
<td>\frac{0.264 \times 10^9 \text{cells}}{\text{mL}} (X) = (5 \text{mL})(1 \times 10^6) \Rightarrow X = 0.01894 \text{mL} x \frac{1000 \mu L}{1 \text{mL}}</td>
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**Figure 4.** Bacteria 96-well plate set-up for (a) 2 samples in triplicate (b) 3 samples in duplicate (c) 1 sample per plate (d) 2 samples per plate. Different colors specify different bacteria samples. Each column was treated with the antimicrobial drug concentrations specified in Table 4.

Trials 1-7 utilized the set-ups in Figures 4(a) and 4(b). Trial 8, which examined one bacteria sample per 96-well plate against all four antimicrobial drug formulations, used the set-up in Figure 4(c). Trial 9, which examined bacteria samples against new polymyxin B and its complex with PMAA-g-10%J, utilized the set-up in Figure 4(d).

### 2.5 Anti-Biofilm Activity of “GRAPLON” Nanomedicines

#### 2.5.1 Bacteria Inoculation

Tryptic soy agar (TSA) plates for biofilms were prepared, as described in section 2.4.1, and streaked, as described in section 2.4.2. The inoculation loop was burned with the flame of the Bunsen burner for about 10 seconds and inserted into the layer of TSA at
the edge of the plate, for each bacteria sample. The inoculation loop then lightly glided across the surface of the plate to pick up either one colony of bacteria, if the inoculation was overnight, or a few colonies of bacteria, if the inoculation was performed the day of seeding the 96-well plates. The bacteria was mixed into 1 mL of sterile TSB in a cell culture tube, and its cover was placed on the top, without clicking shut, so that the bacteria could aerate and grow. These steps were repeated for each bacteria inoculation, with each cell culture tube labeled respectively. The cell culture tubes were placed in an incubator shaker at 37°C and 150 rpm for about 24 hours, if the inoculations were overnight, or for about 4 hours, if the inoculations were performed the day of seeding the 96-well plates.

If the inoculations were overnight, a 1:100 dilution of the inoculated bacteria was performed the next day. Such a dilution was made by mixing 10 µL of inoculated bacteria in 990 µL of TSB. The cell culture tubes were placed in an incubator shaker at 37°C and 150 rpm for about 4 hours, in order for the bacteria to start growing in the log phase. Such a dilution was not necessary if the inoculations were performed the day of seeding the 96-well plates, as the bacteria were already growing in the log phase.

2.5.2 Seeding the 96-Well Plate at a Density of 1x10^6 cells/well

Nanomedicine dose-response activities, or minimum biofilm eradication concentrations (MBECs), were determined against bacteria biofilms via high-throughput microtiter 96-well plate assays. First, 1 mL of TSB was added to each inoculated bacteria sample. 1 mL of each solution was transferred into a cuvette, and 1 mL of TSB was transferred into another cuvette for a blank. The cuvettes were inserted into a UV spectrophotometer and the optical density (OD) values were measured at a wavelength of
600 nm. If the OD value was below 0.1, the sample was discarded. If the OD value was above 1, more TSB was added to the solution to dilute it. Using the measured OD value, the following calculations were performed to determine the amounts of bacteria and TSB for a 96-well plate seeding density of $\frac{1 \times 10^6}{100 \, \mu L}$ or $\frac{1 \times 10^7}{mL}$. The values determined by these calculations for each study can be seen in Table 6.

\[
\frac{\text{OD value} \times 10^9}{\text{mL}} = \frac{(\text{OD value two decimal places to the right}) \times 10^7}{\text{mL}}
\]

\[
\frac{\text{Total solution volume}}{(\text{OD value two decimal places to the right})} = \text{total volume of bacteria needed (\(\mu L\))}
\]

\[
\text{Total solution volume} - \text{total volume of bacteria needed} = \text{total volume of TSB needed (\(\mu L\))}
\]

**Table 6.** Bacteria solution volume data, based upon OD values of each clinical isolate sample, to seed 96-well plates at a density of $1 \times 10^6$ cells/well.

<table>
<thead>
<tr>
<th>Study #</th>
<th>Clinical Isolate Sample #</th>
<th>OD value</th>
<th>Dilution for $\frac{1 \times 10^7}{mL}$</th>
<th>Total Solution Volume ($\mu L$)</th>
<th>Bacteria Amount ($\mu L$)</th>
<th>TSB Amount ($\mu L$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2</td>
<td>0.075</td>
<td>7.5X</td>
<td>5000</td>
<td>667</td>
<td>4333</td>
</tr>
<tr>
<td>1</td>
<td>5</td>
<td>0.347</td>
<td>34.7X</td>
<td>5000</td>
<td>144</td>
<td>4856</td>
</tr>
<tr>
<td>1</td>
<td>8</td>
<td>0.279</td>
<td>27.9X</td>
<td>5000</td>
<td>179</td>
<td>4821</td>
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<tr>
<td>1</td>
<td>12</td>
<td>0.451</td>
<td>45.1X</td>
<td>5000</td>
<td>111</td>
<td>4889</td>
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<tr>
<td>1</td>
<td>14</td>
<td>0.487</td>
<td>48.7X</td>
<td>5000</td>
<td>103</td>
<td>4897</td>
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<tr>
<td>1</td>
<td>15</td>
<td>0.308</td>
<td>30.8X</td>
<td>5000</td>
<td>162</td>
<td>4838</td>
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<td>1</td>
<td>16</td>
<td>0.373</td>
<td>37.3X</td>
<td>5000</td>
<td>134</td>
<td>4866</td>
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<td>2</td>
<td>2</td>
<td>0.503</td>
<td>50.3X</td>
<td>10000</td>
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<td>9801</td>
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<tr>
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<td>10000</td>
<td>373</td>
<td>9627</td>
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<td>0.249</td>
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<td>10000</td>
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<td>9598</td>
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<td>2</td>
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<td>0.274</td>
<td>27.4X</td>
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<tr>
<td>2</td>
<td>16</td>
<td>0.239</td>
<td>23.9X</td>
<td>10000</td>
<td>418</td>
<td>9582</td>
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The calculated bacteria amount was mixed in a vortex with the calculated TSB amount and the solution was poured into a reservoir. Using a multichannel pipette, 100 µL of the bacteria solution was transferred into columns 2-12 of the 96-well plate, as seen in Figure 5. Column 1 served as blanks and remained untouched until the final alamarBlue reagent step. The 96-well plate was then incubated at 37°C for about 48 hours to allow the bacteria to grow biofilms.

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**Figure 5.** Bacteria 96-well plate set-up for (a) 2 samples in triplicate (b) 4 samples in duplicate. Different colors specify different bacteria samples.

### 2.5.3 Washing the 96-Well Plate and Adding the Serially Diluted Antimicrobial Drug

First, 1 L of 1X PBS was filtered for sterilization via two 500 mL vacuum suction bottle top filter devices (Corning Incorporated). This stock of sterile 1X PBS was poured in a reservoir and utilized in the remaining procedures. The 96-well plate was removed from the incubator after 48 hours, and a cup of nine parts water to one part bleach was prepared. Using a multichannel pipette, 100 µL of bacteria solution were removed from each well of the 96-well plate and disposed of in the cup of bleach solution. It was important to not touch the edges of the bleach cup with the pipette tips, as any bleach exposure would kill the bacteria biofilms in the 96-well plate. Using different multichannel pipette tips, 100 µL of 1X PBS were inserted into the wells that contained
the removed bacteria solution. The 100 µL of 1X PBS were then removed from each well using the same multichannel pipette tips and the solutions were released into the cup of bleach, without touching the edges of the cup. If the biofilms were fragile and started to break apart, washing with 1X PBS only occurred once. If not, the washing step of adding and removing 100 µL of 1X PBS was repeated again. At the end of washing, column 12 was used as a control by adding 100 µL of 1X PBS to it, without removal.

Next, a serial dilution of the antimicrobial drug formulation was performed in another 96-well plate. Biofilms are broken down by dosages 10-1,000 times greater than their MICs.\textsuperscript{17,18} Therefore, the initial antimicrobial drug concentration to be serially diluted from column 2 was calculated by multiplying the obtained MIC value for that specific drug formulation and bacteria sample by 500. The drug formulations examined were tobramycin and PMAA-g-10\%J:TB, both of which exhibited MIC values of about 1.25 \( \frac{\mu g}{mL} \) for most bacteria samples. Therefore, an initial tobramycin concentration of 625 \( \frac{\mu g}{mL} \) was utilized for the following studies.

In a sterile biosafety cabinet, 100 µL of TSB were transferred from a reservoir into columns 3-11 of a new 96-well plate, using a multichannel pipette. Upon testing the dose-response activity of tobramycin, 50 mg of tobramycin were mixed in a vortex with 1 mL of HPLC-grade water for a concentration of 50000 \( \frac{\mu g}{mL} \). This solution was then sterilized with a 0.45 µm pore size syringe filter (Whatman). In order to bring the 50000 \( \frac{\mu g}{mL} \) tobramycin stock solution to 625 \( \frac{\mu g}{mL} \), the following calculations were performed for an 80X dilution.

\[
\frac{200 \mu L \text{ in each well of column } 2}{\left(\frac{50000}{625}\right)} = 2.5 \mu L \text{ of } 50000 \frac{\mu g}{mL} \text{ tobramycin solution}
\]
200 µL − 2.5 µL = 197.5 µL of TSB

Therefore, 2.5 µL of sterile 50000 µg/mL tobramycin solution were added to the corners of each well of column 2 (in the same rows as those designated in Figure 5), and then 197.5 µL of TSB were added into the same wells. Upon testing the dose-response activity of PMAA-g-10%J:TB, the following calculations were performed to obtain the desired initial tobramycin concentration of 625 µg/mL from the complex’s tobramycin concentration of \( \frac{2.957 \text{ mg}}{2.85 \text{ mL}} \).

\[
\left( \frac{2957 \text{ µg TB}}{2850 \text{ µL}} \right)V_{\text{complex}} = \left( \frac{625 \text{ µg}}{1000 \text{ µL}} \right)(200 \text{ µL in each well of column 2})
\]

\[
V_{\text{complex}} = 120.5 \text{ µL of PMAA – g – 10%J: TB}
\]

\[
200 \text{ µL} − 120.5 \text{ µL} = 79.5 \text{ µL of TSB}
\]

Therefore, 79.5 µL of TSB were added to each well of column 2 (in the same rows as those designated in Figure 5), and then 120.5 µL of PMAA-g-10%J:TB, sterilized with a 0.45 micron syringe filter, were added into the same wells.

The antimicrobial drug solutions in column 2 were mixed, by pipetting up and down, and serially diluted, using a multichannel pipette. The serial dilution occurred by transferring 100 µL of the contents in column 2 into column 3. The contents in column 3 were mixed and 100 µL were pipetted and mixed into column 4. This method was repeated until column 11. Ultimately, each successive column obtained half the concentration of tobramycin than the previous one. Since column 2 began with 625 µg/mL, columns 3, 4, 5, 6, 7, 8, 9, 10 and 11 obtained concentrations of 312.5 µg/mL, 156.3 µg/mL, 78.1 µg/mL, 39.1 µg/mL, 19.5 µg/mL, 9.8 µg/mL, 4.9 µg/mL, 2.4 µg/mL, and 0 µg/mL, respectively. Finally, 100 µL of the
respective rows of the serial dilution 96-well plate were transferred into the original biofilm 96-well plate. Each row of the serial dilution 96-well plate was transferred using a multichannel pipette, ensuring that they matched up correctly with the rows of the biofilm 96-well plate. The transfer began from the rightmost column, or the column with the lowest tobramycin concentration, to the leftmost column, or the column with the highest tobramycin concentration, in order to use the same pipette tips throughout. The 96-well plate was then incubated at 37°C for 24 hours.

2.5.4 Applying alamarBlue Reagent and Reading the 96-Well Plate

The 96-well plate was removed from the incubator after 24 hours, and a cup of nine parts water to one part bleach was prepared. Using a multichannel pipette, the liquid in each well was removed and disposed of in the cup of bleach solution, without touching the edges of the cup. Using different multichannel pipette tips, 100 µL of 1X PBS were transferred from a reservoir into the wells that contained liquid. The 100 µL of 1X PBS were then removed and released into the cup of bleach, without touching the edges of the cup, using the same multichannel pipette tips by working from left to right. The leftmost column was treated with the highest concentration of tobramycin and, therefore, no longer contained bacteria biofilms, whereas the rightmost column was treated with the lowest concentration of tobramycin and, therefore, still contained bacteria biofilms.

A solution of 10% alamarBlue Cell Viability Reagent (Thermo Fisher Scientific), stored at 4°C, and 90% TSB was prepared in a reservoir using the following calculations.

\[(\text{# of rows})(\text{# of columns}) = \text{# of wells} + 5 \text{ for excess} = \#\]

\[(\#)(100 \, \mu\text{L}) = \text{total } \mu\text{L of alamarBlue solution} = A\]

\[A(0.1) = \mu\text{L of alamarBlue}, A(0.9) = \mu\text{L of TSB}\]
100 μL of the formulated alamarBlue solution were inserted into all of the wells being tested, including the blanks in column 1 and the controls in column 12, using a multichannel pipette. The 96-well plate was then incubated at 37°C for 15-30 minutes, depending on how soon the color of the wells containing biofilms changed from blue to pink. After the expected wells underwent the colorimetric change, the 96-well plate was inserted into the Tecan Spark 10M Plate Reader. The cells to be read, or those which contained alamarBlue, were selected in the Spark Control program, along with the input of 60% optimal gain. After taking the readings, the program automatically exported the data into an excel file, where further analysis took place. The blank fluorescence data values in column 1 were subtracted from the fluorescence data values in columns 2-11. The averages of the triplicate or duplicate results were calculated and superimposed against the corresponding tobramycin concentrations to form scatter plots and bar graphs of relative fluorescence units vs. tobramycin concentration. The MBECs were the minimum concentrations where the biofilms were eradicated, or where the values were down to noise levels. Noise levels were interpreted as less than 5% of the maximum fluorescence.

CHAPTER 3: RESULTS & DISCUSSION

3.1 Average Hydrodynamic Diameter and Zeta Potential of Nanoparticles

The average hydrodynamic diameter and zeta potential results for various nanoparticle formulations can be seen in Table 7. The PSs utilized include PMAA-g-10%J, PMAA-g-5%J, PMAA-g-1%J, and PPAA-g-1%J. Such PSs were dialyzed in
either HPLC-grade water or 1X PBS and complexed with polymyxin B, tobramycin or LL-37 at specific charge ratios (CRs). Nanoparticles with sizes less than 200 nm and zeta potentials greater than |5| mV were considered stable. In theory, nanoparticles with hydrodynamic diameters less than 200 nm have longer circulation times due to decreased uptake by the reticuloendothelial system. Anionic surfaces close to neutral charge exhibit faster mucus penetration, while cationic particles are more mucoadhesive and, therefore, less penetrative. Still, PEGylation with various graft percentages of Jeffamine® accounted for these properties, increasing circulation time and mucus penetration; therefore, it was most important for the charge to be greater than |5| mV for increased stability. Whether the charge was negative or positive did not matter, as stability occurs with opposite attractive forces of the anionic PS and the cationic antimicrobial drug. Hence, the absolute value of this product indicated stability.

Table 7. Nanoparticle formation data table. *Indicates use for controlled release study, **indicates use for antimicrobial study, ***indicates use for aerosolization, and ****indicates use for anti-biofilm study. Polydispersity values closer to 1 signify less accurate sizing and quality factor values less than 1 indicate low quality data.

<table>
<thead>
<tr>
<th>Sample #</th>
<th>Polyelectrolyte Surfactant</th>
<th>Dialyzed in</th>
<th>Antimicrobial Drug</th>
<th>CR</th>
<th>Average Particle Size</th>
<th>Average Zeta Potential</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>20 mg PMAA-g-10%J</td>
<td>HPLC-grade water</td>
<td>8.5 mg polymyxin B</td>
<td>1</td>
<td>130 nm (polydispersity = 0.2)</td>
<td>3.01 mV (quality factor = 1.03)</td>
</tr>
<tr>
<td>2</td>
<td>20 mg PMAA-g-10%J</td>
<td>1X PBS</td>
<td>8.5 mg polymyxin B</td>
<td>1</td>
<td>76 nm (polydispersity = 0.132)</td>
<td>-10.3 mV (quality factor = 1.56)</td>
</tr>
<tr>
<td>3</td>
<td>11.1 mg PMAA-g-5%J</td>
<td>HPLC-grade water</td>
<td>8.5 mg polymyxin B</td>
<td>1</td>
<td>224 nm (polydispersity = 0.175)</td>
<td>4.74 mV (quality factor = 1.15)</td>
</tr>
<tr>
<td>4</td>
<td>11.1 mg</td>
<td>1X PBS</td>
<td>8.5 mg</td>
<td>1</td>
<td>129 nm</td>
<td>-2.7 mV</td>
</tr>
<tr>
<td></td>
<td>PMAA-g-5%J</td>
<td>polymyxin B</td>
<td>(polydispersity = 0.0562)</td>
<td>(quality factor = 1.01)</td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>5.78 mg PMAA-g-1%J</td>
<td>HPLC-grade water</td>
<td>8.5 mg polymyxin B</td>
<td>1</td>
<td>624 nm (polydispersity = 0.386)</td>
<td>13 mV (quality factor = 2.29)</td>
</tr>
<tr>
<td>6</td>
<td>5.78 mg PMAA-g-1%J</td>
<td>1X PBS</td>
<td>8.5 mg polymyxin B</td>
<td>1</td>
<td>284 nm (polydispersity = 0.881)</td>
<td>6.4 mV (quality factor = 1.13)</td>
</tr>
<tr>
<td>7</td>
<td>20 mg PMAA-g-10%J</td>
<td>1X PBS</td>
<td>8.5 mg polymyxin B</td>
<td>1</td>
<td>77.3 nm (polydispersity = 0.0991)</td>
<td>-3.22 mV (quality factor = 1.01)</td>
</tr>
<tr>
<td>8</td>
<td>5.78 mg PMAA-g-1%J</td>
<td>1X PBS</td>
<td>4.25 mg polymyxin B</td>
<td>0.5</td>
<td>672 nm (polydispersity = 0.859)</td>
<td>-4.67 mV (quality factor = 1.26)</td>
</tr>
<tr>
<td>9</td>
<td>10 mg PPAA-g-1%J</td>
<td>1X PBS</td>
<td>3.2 mg tobramycin</td>
<td>1</td>
<td>2.98E+03 nm (polydispersity = 0.338)</td>
<td>-7.61 mV (quality factor = 1.04)</td>
</tr>
<tr>
<td>10</td>
<td>10 mg PPAA-g-1%J</td>
<td>1X PBS</td>
<td>2.957 mg tobramycin</td>
<td>1</td>
<td>267 nm (polydispersity = 0.338)</td>
<td>-33.8 mV (quality factor = 5.38)</td>
</tr>
<tr>
<td>11</td>
<td>20 mg PMAA-g-10%J</td>
<td>1X PBS</td>
<td>2.957 mg tobramycin</td>
<td>1</td>
<td>83.8 nm (polydispersity = 0.0899)</td>
<td>-12.4 mV (quality factor = 1.2)</td>
</tr>
<tr>
<td>12</td>
<td>20 mg PMAA-g-10%J</td>
<td>1X PBS</td>
<td>284 nm (polydispersity = 0.453)</td>
<td>-33.8 mV (quality factor = 3.3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>11.1 mg PMAA-g-5%J</td>
<td>1X PBS</td>
<td>8.5 mg polymyxin B</td>
<td>1</td>
<td>179 nm (polydispersity = 0.123)</td>
<td>2.06 mV (quality factor = 1.06)</td>
</tr>
<tr>
<td>14</td>
<td>11.1 mg PMAA-g-5%J</td>
<td>1X PBS</td>
<td>358 nm (polydispersity = 0.457)</td>
<td>-13.6 mV (quality factor = 1.25)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Experiment</td>
<td>PMAA-g-%</td>
<td>PBS</td>
<td>Mixture %</td>
<td>N</td>
<td>Diameter (nm)</td>
<td>Polydispersity Factor</td>
</tr>
<tr>
<td>------------</td>
<td>---------</td>
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</tr>
<tr>
<td>15</td>
<td>11.1 mg</td>
<td>1X PBS</td>
<td>1.795 mg tobramycin</td>
<td>1</td>
<td>71.2 nm (polydispersity = 0.275)</td>
<td>-9.13 mV (quality factor = 1.47)</td>
</tr>
<tr>
<td>16</td>
<td>1.54 mg</td>
<td>1X PBS</td>
<td>2 mg LL-37</td>
<td>1</td>
<td>985 nm (polydispersity = 0.637)</td>
<td>-24.3 mV (quality factor = 4.74)</td>
</tr>
<tr>
<td>17</td>
<td>1.54 mg</td>
<td>1X PBS</td>
<td>2 mg LL-37</td>
<td>1</td>
<td>457 nm (polydispersity = 0.670)</td>
<td>-21.2 mV (quality factor = 2.49)</td>
</tr>
<tr>
<td>18</td>
<td>1.69 mg</td>
<td>1X PBS</td>
<td>2 mg LL-37</td>
<td>1</td>
<td>1457 nm (polydispersity = 0.609)</td>
<td>-12.6 mV (quality factor = 2.21)</td>
</tr>
<tr>
<td>19</td>
<td>1.69 mg</td>
<td>1X PBS</td>
<td>2 mg LL-37</td>
<td>1</td>
<td>496 nm (polydispersity = 0.725)</td>
<td>-16 mV (quality factor = 2.35)</td>
</tr>
<tr>
<td>20</td>
<td>1.69 mg</td>
<td>1X PBS</td>
<td>2 mg LL-37</td>
<td>1</td>
<td>104.2 nm (polydispersity = 0.660)</td>
<td>-16.7 mV (quality factor = 2.17)</td>
</tr>
<tr>
<td>21</td>
<td>1.69 mg</td>
<td>1X PBS</td>
<td>2 mg LL-37</td>
<td>1</td>
<td>330 nm (polydispersity = 0.428)</td>
<td>-18.2 mV (quality factor = 2.55)</td>
</tr>
<tr>
<td>22*</td>
<td>1.69 mg</td>
<td>1X PBS</td>
<td>2 mg LL-37</td>
<td>0.5</td>
<td>198 nm (polydispersity = 0.807)</td>
<td>-23.9 mV (quality factor = 2.52)</td>
</tr>
<tr>
<td>23</td>
<td>1.69 mg</td>
<td>1X PBS</td>
<td>2 mg LL-37</td>
<td>1</td>
<td>396 nm (polydispersity = 0.467)</td>
<td>-26.1 mV (quality factor = 3.15)</td>
</tr>
<tr>
<td>24</td>
<td>1.69 mg</td>
<td>1X PBS</td>
<td>2 mg LL-37</td>
<td>1</td>
<td>339 nm (polydispersity = 0.519)</td>
<td>15.4 mV (quality factor = 1.53)</td>
</tr>
<tr>
<td>25*</td>
<td>1.69 mg</td>
<td>1X PBS</td>
<td>2 mg LL-37</td>
<td>0.5</td>
<td>350 nm (polydispersity = 0.447)</td>
<td>-28.8 mV (quality factor = 2.49)</td>
</tr>
<tr>
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<td></td>
</tr>
<tr>
<td>26</td>
<td>1.69 mg PMAA-g-10%J</td>
<td>1X PBS</td>
<td>509 nm (polydispersity = 0.755)</td>
<td>-21.9 mV (quality factor = 2.39)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>27**</td>
<td>1.947 mg PMAA-g-10%J</td>
<td>1X PBS</td>
<td>1 mg polymyxin B</td>
<td>144 nm (polydispersity = 0.315)</td>
<td>-9.52 mV (quality factor = 1.3)</td>
<td></td>
</tr>
<tr>
<td>28</td>
<td>1.947 mg PMAA-g-10%J</td>
<td>1X PBS</td>
<td>596 nm (polydispersity = 0.553)</td>
<td>-22.5 mV (quality factor = 2.33)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>29***</td>
<td>11.1 mg PMAA-g-5%J</td>
<td>1X PBS</td>
<td>8.5 x 4 = 34 mg polymyxin B</td>
<td>157 nm (polydispersity = 0.106)</td>
<td>1.2 mV (quality factor = 0.747)</td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>11.1 mg PMAA-g-5%J</td>
<td>1X PBS</td>
<td>415 nm (polydispersity = 0.860)</td>
<td>-20.2 mV (quality factor = 2.34)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>31***</td>
<td>20 mg PMAA-g-10%J</td>
<td>1X PBS</td>
<td>2.96 x 4 = 11.8 mg tobramycin</td>
<td>232 nm (polydispersity = 0.258)</td>
<td>-11.4 mV (quality factor = 1.85)</td>
<td></td>
</tr>
<tr>
<td>32</td>
<td>20 mg PMAA-g-10%J</td>
<td>1X PBS</td>
<td>257 nm (polydispersity = 0.577)</td>
<td>-29.8 mV (quality factor = 3.66)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>33***</td>
<td>11.1 mg PMAA-g-5%J</td>
<td>1X PBS</td>
<td>1.795 x 4 = 7.18 mg tobramycin</td>
<td>46 nm (polydispersity = 0.338)</td>
<td>-14.13 mV (quality factor = 1.47)</td>
<td></td>
</tr>
<tr>
<td>34</td>
<td>11.1 mg PMAA-g-5%J</td>
<td>1X PBS</td>
<td>1257 nm (polydispersity = 0.704)</td>
<td>-21.7 mV (quality factor = 1.74)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>35**</td>
<td>1.947 mg PMAA-g-10%J</td>
<td>1X PBS</td>
<td>1 mg polymyxin B</td>
<td>270 nm (polydispersity = 0.332)</td>
<td>-5.69 mV (quality factor = 1.26)</td>
<td></td>
</tr>
<tr>
<td>36</td>
<td>1.947 mg</td>
<td>1X PBS</td>
<td>26.5 nm</td>
<td>-24.8 mV</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>PMAA-g-10%J</td>
<td></td>
<td>(polydispersity = 0.793)</td>
<td>(quality factor = 2.73)</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>37</td>
<td>20 mg PMAA-g-10%J</td>
<td>1X PBS</td>
<td>2.957 mg tobramycin</td>
<td>1</td>
<td>576 nm (polydispersity = 0.523)</td>
<td>-7.8 mV (quality factor = 1.25)</td>
</tr>
<tr>
<td>38</td>
<td>20 mg PMAA-g-10%J</td>
<td>1X PBS</td>
<td></td>
<td></td>
<td>975.67 nm (polydispersity = 0.485)</td>
<td>-14.6 mV (quality factor = 3.18)</td>
</tr>
<tr>
<td>39**</td>
<td>1.947 mg PMAA-g-10%J</td>
<td>1X PBS</td>
<td>1 mg polymyxin B</td>
<td>1</td>
<td>222 nm (polydispersity = 0.232)</td>
<td>-6.4 mV (quality factor = 1.08)</td>
</tr>
<tr>
<td>40</td>
<td>1.947 mg PMAA-g-10%J</td>
<td>1X PBS</td>
<td></td>
<td></td>
<td>496 nm (polydispersity = 0.381)</td>
<td>-15.7 mV (quality factor = 1.3)</td>
</tr>
<tr>
<td>41**</td>
<td>20 mg PMAA-g-10%J</td>
<td>1X PBS</td>
<td>2.957 mg tobramycin</td>
<td>1</td>
<td>495 nm (polydispersity = 0.421)</td>
<td>-10.05 mV (quality factor = 1.18)</td>
</tr>
<tr>
<td>42</td>
<td>20 mg PMAA-g-10%J</td>
<td>1X PBS</td>
<td></td>
<td></td>
<td>439 nm (polydispersity = 0.549)</td>
<td>-15.3 mV (quality factor = 2.3)</td>
</tr>
<tr>
<td>43***</td>
<td>11.1 mg PMAA-g-5%J</td>
<td>HPLC-grade water</td>
<td>8.5 x 4 = 34 mg polymyxin B</td>
<td>1</td>
<td>194 nm (polydispersity = 0.351)</td>
<td>5.36 mV (quality factor = 1.04)</td>
</tr>
<tr>
<td>44</td>
<td>11.1 mg PMAA-g-5%J</td>
<td>HPLC-grade water</td>
<td></td>
<td></td>
<td>424 nm (polydispersity = 0.544)</td>
<td>3.24 mV (quality factor = 1.56)</td>
</tr>
<tr>
<td>45***</td>
<td>11.1 mg PMAA-g-5%J</td>
<td>HPLC-grade water</td>
<td>1.795 x 4 = 7.18 mg tobramycin</td>
<td>1</td>
<td>195 nm (polydispersity = 0.561)</td>
<td>3.92 mV (quality factor = 1.07)</td>
</tr>
<tr>
<td>46***</td>
<td>20 mg PMAA-g-10%J</td>
<td>HPLC-grade water</td>
<td>2.96 x 4 = 11.8 mg tobramycin</td>
<td>1</td>
<td>125 nm (polydispersity = 0.355)</td>
<td>9.66 mV (quality factor = 2.29)</td>
</tr>
<tr>
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<td></td>
<td></td>
</tr>
<tr>
<td><strong>47</strong></td>
<td>20 mg PMAA-g-10%J</td>
<td>HPLC-grade water</td>
<td>152 nm (polydispersity = 0.395)</td>
<td>11.16 mV (quality factor = 1.78)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>48</strong>*</td>
<td>20 mg PMAA-g-10%J</td>
<td>HPLC-grade water</td>
<td>8.5 x 4 = 34 mg polymyxin B</td>
<td>141 nm (polydispersity = 0.422)</td>
<td>9.57 mV (quality factor = 1.6)</td>
<td></td>
</tr>
<tr>
<td><strong>49</strong></td>
<td>20 mg PMAA-g-10%J</td>
<td>1X PBS</td>
<td>2.957 mg tobramycin</td>
<td>250 nm (polydispersity = 0.380)</td>
<td>10.3 mV (quality factor = 0.983)</td>
<td></td>
</tr>
<tr>
<td><strong>50</strong></td>
<td>20 mg PMAA-g-10%J</td>
<td>1X PBS</td>
<td></td>
<td>374 nm (polydispersity = 0.679)</td>
<td>5 mV (quality factor = 0.859)</td>
<td></td>
</tr>
<tr>
<td><strong>51</strong></td>
<td>1.947 mg PMAA-g-10%J</td>
<td>1X PBS</td>
<td>1 mg polymyxin B</td>
<td>302 nm (polydispersity = 0.585)</td>
<td>9.2 mV (quality factor = 0.713)</td>
<td></td>
</tr>
<tr>
<td><strong>52</strong></td>
<td>1.947 mg PMAA-g-10%J</td>
<td>1X PBS</td>
<td></td>
<td>313 nm (polydispersity = 0.278)</td>
<td>2.94 mV (quality factor = 0.423)</td>
<td></td>
</tr>
<tr>
<td><strong>53</strong>**</td>
<td>20 mg PMAA-g-10%J</td>
<td>HPLC-grade water</td>
<td>2.957 mg tobramycin</td>
<td>153 nm (polydispersity = 0.221)</td>
<td>6.17 mV (quality factor = 1.15)</td>
<td></td>
</tr>
<tr>
<td><strong>54</strong></td>
<td>20 mg PMAA-g-10%J</td>
<td>1X PBS</td>
<td>2.957 mg tobramycin</td>
<td>251 nm (polydispersity = 0.555)</td>
<td>-1.054 mV (quality factor = 0.504)</td>
<td></td>
</tr>
</tbody>
</table>

Plots, depicting average hydrodynamic diameters, correlograms and average zeta potentials, according to the sample numbers labeled in Table 7, can be found in Figures A1 and A2 in the Appendix. A correlogram with a high y-intercept indicated a large signal-to-noise ratio. In other words, there were enough detected photons scattered off the
particles moving in Brownian motion. Meanwhile, a low y-intercept indicated either a low concentration of particles or a high buffer concentration that was clouding the analysis. The gradient of the correlogram indicated the polydispersity of the sample, or an estimate of the width of the size distribution. The polydispersity index ranged from 0 to 1 in the Zetasizer software, and values closer to 1 indicated high polydispersity. High polydispersity signified that the nanoparticles were non-uniform and that the size measurements were less accurate. Accurate results were monodisperse and displayed low polydispersity index values. High polydispersity can be observed by the size distribution graphs with multiple peaks in Figure A1. Meanwhile, quality factor values greater than 1, which were observed for most nanoparticles, indicate good quality zeta potential data.

As seen in Table 7, samples 1-6 exhibited smaller and more negatively charged nanoparticles via PS dialysis with 1X PBS, as opposed to HPLC-grade water. This phenomenon was explained by polyelectrolyte theory. The sodium and potassium ions in 1X PBS took away protons and inhibited hydrogen bonds with the PS, causing a more negative zeta potential. Therefore, 1X PBS was utilized for the remainder of the formulations, with the exception of those used for aerosolization, as salts were seen as particles in the airborne phase.

Complexes with PMAA-g-1%J and PPAA-g-1%J appeared to aggregate, as exhibited by their large hydrodynamic diameters. An attempt to resolve this issue involved lowering the charge ratio from 1:1 to 0.5:1, or using half the amount of positively charged polymyxin B, as depicted by sample 8. Still, this formulation resulted in a large hydrodynamic diameter. Therefore, graft 1% ceased to be utilized after sample
10, and its sizing results were attributed to the nature of having less graft on the complex. The heavy ethylene oxide groups in the graft typically resisted aggregation; however, 1% graft had fewer groups and, thus, less aggregation resistance than 5% and 10% graft.

The possibility of polymyxin B bridging the polymer chains was also explored, hence the implementation of sizing and charging the PSs without antimicrobial drug. As expected, most PSs exhibited larger sizes and more negative charges than their self-assembled complexes with cationic antimicrobial drug. The PSs exhibited high polydispersity, as seen by multiple peaks in their size distribution plots in Figure A1, which was not entirely expected. Without an oppositely charged antimicrobial, the PSs self-associated based on weak, non-specific forces, such as hydrogen bonding and some hydrophobic interactions. This led to multiple populations instead of one equilibrium state.

Nanoparticle formulations encapsulating LL-37 at a charge ratio of 1 exhibited large sizes, as seen by samples 16 and 18. Implementing a charge ratio of 0.5, as seen by samples 20, 22 and 25, lowered the average hydrodynamic diameters, yet produced polydispersed solutions, displaying many peaks in the size distribution plots, as seen in Figure A1. LL-37, whose primary amino acid sequence is LLGDFFRKSK–EKIGKEFKRI–VQRKDFLR–LVPRTES, has a net charge of +6 (-2 from D, -3 from E, +5 from R and +6 from K). The zeta potential values did not change drastically between the free PS and its complex with LL-37. Due to the negative charge of the PS and the positive charge of LL-37, a shift in zeta potential was expected. Therefore, LL-37 and the PS could have each been self-associating, rather than forming complexes. This could have also been due in part by the large molecular size and amphiprotic nature of
LL-37, causing weaker binding to the PSs. Sample 24 tested the size and zeta potential of free LL-37. It exhibited a small, nano-scale size, which may have proven that the peptide itself self-associated, as it is a polyelectrolyte and contains both positive and negative groups. As expected, the zeta potential was highly positive. Though the shift in charge from PS to its LL-37 complex was small, the zeta potential distribution plots did not display positive peaks, as seen with free LL-37; therefore, some interaction must have been taking place.

As seen in Table 7, the samples had a certain level of variability to them. Hydrodynamic diameters of complexes with PMAA-g-10%J grew larger and zeta potentials became positively charged. Samples 35-54 utilized PMAA-g-10%J produced in lab, whereas samples 1-34 utilized PMAA-g-10%J synthesized by the Polysciences company. Therefore, the differences in hydrodynamic diameter and zeta potential may have been attributed to this change. New solvents and zeta cells were ordered in case there were any contaminants, and Malvern zeta potential standards were run to confirm that the Zetasizer was working properly. All of these attempts to explain the changes checked out; therefore, it was concluded that the new PMAA-g-10%J was slightly impure and caused the variability.

In theory, nanoparticles coated with low molecular weight, high density PEG exhibit faster mucus penetration, due to their increased hydrophilicity and biocompatibility. Therefore, nanoparticles formulated with PMAA-g-10%J, or the highest density PEG, were utilized in further experiments along with antimicrobials polymyxin B and tobramycin, which exhibited hydrodynamic diameter and zeta potential values of greatest consistency.
3.2 Controlled Antimicrobial Drug Release

3.2.1 Controlled LL-37 Release

As seen in Figure 6, a linear standard curve of LL-37 was created using the known concentrations of its standards along with their corresponding HPLC peak areas. As LL-37 concentrations decreased, peak areas decreased as well.

![Figure 6. LL-37 standard curve, used to obtain unknown LL-37 concentration values from membrane dialysis assay sample peak areas via the equation \( \frac{(\text{Peak area} - \text{y-intercept})}{\text{Slope}} \), where the y-intercept was -76478 and the slope was 8582.]

The peak areas used to create the standard curve in Figure 6 indicated that LL-37 was undetectable below the concentration of about 15.6 µg/mL, where the peak area was 85981. Unfortunately, all LL-37 membrane dialysis assay samples had peaks and, therefore, concentrations below this value, as seen in Table 8. This was expected, as the maximum concentration possible, if all LL-37 was released from the dialysis cassette into the dialysate, was \( \frac{2 \text{ mL in dialysis cassette}}{150 \text{ mL dialysate}} = 13.3 \text{ µg/mL} \). Therefore, another LL-37 controlled release study was conducted using lower antimicrobial drug solution and dialysate volumes, as seen in Table 2, in attempt to obtain higher, detectable concentrations.
Table 8. (A) LL-37 standard solution peak areas, displaying inaccurate detection of concentrations below 15.6 µg/mL. (B) LL-37 controlled release study 1 sample peak areas, used to calculate membrane dialysis assay sample concentrations via the standard curve in Figure 6. All membrane dialysis assay samples were below the limit of detection achieved with this setup.

<table>
<thead>
<tr>
<th>Sample Name</th>
<th>Concentration (µg/mL)</th>
<th>Peak Area</th>
</tr>
</thead>
<tbody>
<tr>
<td>LL-37 Standard 1 mg/mL</td>
<td>1000</td>
<td>8759010</td>
</tr>
<tr>
<td>LL-37 Standard 0.5 mg/mL</td>
<td>500</td>
<td>3758213</td>
</tr>
<tr>
<td>LL-37 Standard 0.25 mg/mL</td>
<td>250</td>
<td>2009626</td>
</tr>
<tr>
<td>LL-37 Standard 0.125 mg/ml</td>
<td>125</td>
<td>906994</td>
</tr>
<tr>
<td>LL-37 Standard 0.063 mg/mL</td>
<td>62.5</td>
<td>446658</td>
</tr>
<tr>
<td>LL-37 Standard 0.031 mg/mL</td>
<td>31.3</td>
<td>200725</td>
</tr>
<tr>
<td>LL-37 Standard 0.015 mg/mL</td>
<td>15.6</td>
<td>85981</td>
</tr>
<tr>
<td>LL-37 Standard 0.0075 mg/mL</td>
<td>7.81</td>
<td>29583</td>
</tr>
<tr>
<td>LL-37 Standard 0.0037 mg/mL</td>
<td>3.91</td>
<td>12432</td>
</tr>
<tr>
<td>LL-37 Standard 0.0018 mg/mL</td>
<td>1.95</td>
<td>10567</td>
</tr>
<tr>
<td>LL-37 Standard 0.0009 mg/mL</td>
<td>0.977</td>
<td>10245</td>
</tr>
<tr>
<td>LL-37 Standard 0.0005 mg/mL</td>
<td>0.488</td>
<td>12036</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Sample Name</th>
<th>Peak Area</th>
<th>Concentration (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LL-37 Release 0h</td>
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</tr>
<tr>
<td>LL-37 Release 1h</td>
<td>20831</td>
<td>11.3</td>
</tr>
<tr>
<td>LL-37 Release 3h</td>
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<td>LL-37 Release 24h</td>
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<td>11.0</td>
</tr>
<tr>
<td>LL-37 Release 96h</td>
<td>15512</td>
<td>10.7</td>
</tr>
<tr>
<td>PMAA-g-10%J:LL-37 Release 0h</td>
<td>11437</td>
<td>10.2</td>
</tr>
<tr>
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<td>12247</td>
<td>10.3</td>
</tr>
<tr>
<td>PMAA-g-10%J:LL-37 Release 3h</td>
<td>17239</td>
<td>10.9</td>
</tr>
<tr>
<td>PMAA-g-10%J:LL-37 Release 6h</td>
<td>14014</td>
<td>10.5</td>
</tr>
<tr>
<td>PMAA-g-10%J:LL-37 Release 24h</td>
<td>24016</td>
<td>11.7</td>
</tr>
<tr>
<td>PMAA-g-10%J:LL-37 Release 96h</td>
<td>19266</td>
<td>11.2</td>
</tr>
</tbody>
</table>

The second LL-37 controlled release study was, unfortunately, unsuccessful as well, despite the lower antimicrobial drug solution and dialysate volumes. Though larger
than the previous study, most LL-37 membrane dialysis assay sample peak areas remained below the limit of detection, as seen in Table 9. The peak values of the blanks were similar to the small membrane dialysis assay sample peaks; therefore, these measurements were attributed to noise. The self-association of LL-37 may have caused it to come off the column in an unexpected way. Due to these controlled release study issues, the high cost of LL-37, as well as its problematic hydrodynamic diameter and zeta potential values, this peptide was excluded from further studies.

Table 9. LL-37 controlled release study 2 sample peak areas, used to calculate membrane dialysis assay sample concentrations via the standard curve in Figure 6. Most membrane dialysis assay samples were below the limit of detection achieved with this setup, and their values were attributed to noise, as seen by the similar peak areas of the blanks.

<table>
<thead>
<tr>
<th>Sample Name</th>
<th>Peak Area</th>
<th>Concentration (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LL-37 Release 0h</td>
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</tr>
<tr>
<td>LL-37 Release 1h</td>
<td>63727</td>
<td>16.3</td>
</tr>
<tr>
<td>LL-37 Release 3h</td>
<td>68436</td>
<td>16.9</td>
</tr>
<tr>
<td>LL-37 Release 6h</td>
<td>68137</td>
<td>16.9</td>
</tr>
<tr>
<td>LL-37 Release 24h</td>
<td>86909</td>
<td>19.0</td>
</tr>
<tr>
<td>LL-37 Release 72h</td>
<td>66007</td>
<td>16.6</td>
</tr>
<tr>
<td>LL-37 Release 144h</td>
<td>56445</td>
<td>15.5</td>
</tr>
<tr>
<td>PMAA-g-10%J:LL-37 Release 0h</td>
<td>78509</td>
<td>18.1</td>
</tr>
<tr>
<td>PMAA-g-10%J:LL-37 Release 1h</td>
<td>58480</td>
<td>15.7</td>
</tr>
<tr>
<td>PMAA-g-10%J:LL-37 Release 3h</td>
<td>53558</td>
<td>15.2</td>
</tr>
<tr>
<td>PMAA-g-10%J:LL-37 Release 6h</td>
<td>62283</td>
<td>16.2</td>
</tr>
<tr>
<td>PMAA-g-10%J:LL-37 Release 24h</td>
<td>61269</td>
<td>16.1</td>
</tr>
<tr>
<td>PMAA-g-10%J:LL-37 Release 72h</td>
<td>62736</td>
<td>16.2</td>
</tr>
<tr>
<td>PMAA-g-10%J:LL-37 Release 144h</td>
<td>70440</td>
<td>17.1</td>
</tr>
<tr>
<td>Blank</td>
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<td></td>
</tr>
<tr>
<td>Blank</td>
<td>60274</td>
<td></td>
</tr>
</tbody>
</table>
3.2.2 Controlled Tobramycin Release

As seen in Figure 7, a non-linear standard curve of tobramycin was created using the known concentrations of its standards along with their corresponding HPLC peak areas. As tobramycin concentrations decreased, peak areas decreased as well; however, the decrease in peak area was not linear, but quadratic.

\[
y = -8.87x^2 + 52395x + 2E+07
\]

\[R^2 = 0.970\]

**Figure 7.** Tobramycin standard curve, used to obtain unknown tobramycin concentration values (x) from membrane dialysis assay sample peak areas (y) via the equation \(y = -8.87x^2 + 52395x + (2x10^7)\).

Figure 7 revealed that the Evaporative Light Scattering Detector was very sensitive, as there were relatively large peaks with even low concentration standards. As seen in Table 10, the tobramycin solution did not appear to diffuse across the dialysis membrane, as all of the peak areas were below that of the lowest tobramycin standard. The peak areas increased with increasing time, as expected for a controlled release, but the values were a significant figure too small. The explanation for this phenomenon was unknown, as free tobramycin should have released faster than bound tobramycin. It is possible that tobramycin degraded without the PS’s protection, thus leading to low HPLC detection. It is also possible that tobramycin was aggregating or sticking to the membrane.
during the membrane dialysis assay, causing the low dialysate concentrations. Lastly, it is possible that the HPLC column or solvent ratio were not optimal; therefore, further method development, including mobile-phase variation, should be explored.

The time scale at which the tobramycin release occurred for PMAA-g-10%J:TB was approximately 0-6 hours. Rather than plateau, the measured peak areas and respective measured concentrations decreased after the release had ceased. This was due to the procedure of removing 3 mL of dialysate and replacing it with 3 mL of diH₂O at each time point. Once all of the tobramycin had been released from the dialysis cassettes, collecting further samples only diluted the concentration of tobramycin in the dialysate by a fraction of \( \frac{3 \text{ mL}}{20 \text{ mL}} \). Therefore, the following calculations were implemented to correct this inaccuracy, as seen by the actual concentration values, \( C_{\text{actual},n} \), in Table 10.

\[
m_{\text{measured},n} = (C_{\text{measured},n})(V)
\]

\[
m_{\text{actual},n} = m_{\text{measured},n} + f \sum_{j=1}^{n-1} m_{\text{measured},j}
\]

\[
C_{\text{actual},n} = \frac{m_{\text{actual},n}}{V}
\]

\( C_{\text{measured},n} \) values were obtained by plugging the measured peak area values into the tobramycin standard curve equation and solving the quadratic for \( x \), as seen by the measured concentration values in Table 10. \( V \), or the total dialysate volume, was 20 mL and \( f \), or the dilution fraction, was \( \frac{3}{20} \) or 0.15.
Table 10. (A) Tobramycin (TB) standard solution peak areas, used to create the standard curve in Figure 7. (B) Tobramycin (TB) controlled release study sample peak areas, used to calculate membrane dialysis assay concentrations via the standard curve in Figure 7. Correction calculations for the 3 mL dialysate dilutions were performed to obtain actual concentration values for PMAA-g-10%J:TB release.

### A. Tobramycin Standards

<table>
<thead>
<tr>
<th>Sample Name</th>
<th>Concentration (µg/mL)</th>
<th>Peak Area</th>
</tr>
</thead>
<tbody>
<tr>
<td>TB Standard 2.96 mg/mL</td>
<td>2957</td>
<td>96421094</td>
</tr>
<tr>
<td>TB Standard 1.48 mg/mL</td>
<td>1479</td>
<td>67630951</td>
</tr>
<tr>
<td>TB Standard 0.739 mg/mL</td>
<td>739</td>
<td>56884512</td>
</tr>
<tr>
<td>TB Standard 0.370 mg/mL</td>
<td>370</td>
<td>40267238</td>
</tr>
<tr>
<td>TB Standard 0.185 mg/mL</td>
<td>185</td>
<td>32666483</td>
</tr>
<tr>
<td>TB Standard 0.0924 mg/mL</td>
<td>92.4</td>
<td>24863330</td>
</tr>
<tr>
<td>TB Standard 0.046 mg/mL</td>
<td>46.2</td>
<td>22874146</td>
</tr>
<tr>
<td>TB Standard 0.023 mg/mL</td>
<td>23.1</td>
<td>18347454</td>
</tr>
<tr>
<td>TB Standard 0.0115 mg/mL</td>
<td>11.6</td>
<td>16273480</td>
</tr>
<tr>
<td>TB Standard 0.0058 mg/mL</td>
<td>5.78</td>
<td>14305643</td>
</tr>
<tr>
<td>TB Standard 0.00289 mg/mL</td>
<td>2.89</td>
<td>12859438</td>
</tr>
<tr>
<td>TB Standard 0.00144 mg/mL</td>
<td>1.44</td>
<td>12781108</td>
</tr>
</tbody>
</table>

### B. Tobramycin Membrane Dialysis Assay Samples

<table>
<thead>
<tr>
<th>Sample Name</th>
<th>Measured Peak Area</th>
<th>Measured Concentration (µg/mL)</th>
<th>Actual Concentration (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TB Release 0h</td>
<td>214421</td>
<td>~ 0</td>
<td>~ 0</td>
</tr>
<tr>
<td>TB Release 1h</td>
<td>554680</td>
<td>~ 0</td>
<td>~ 0</td>
</tr>
<tr>
<td>TB Release 3h</td>
<td>811930</td>
<td>~ 0</td>
<td>~ 0</td>
</tr>
<tr>
<td>TB Release 6h</td>
<td>1063779</td>
<td>~ 0</td>
<td>~ 0</td>
</tr>
<tr>
<td>TB Release 24h</td>
<td>1749400</td>
<td>~ 0</td>
<td>~ 0</td>
</tr>
<tr>
<td>TB Release 48h</td>
<td>3667688</td>
<td>~ 0</td>
<td>~ 0</td>
</tr>
<tr>
<td>TB Release 72h</td>
<td>3695126</td>
<td>~ 0</td>
<td>~ 0</td>
</tr>
<tr>
<td>TB Release 96h</td>
<td>3407362</td>
<td>~ 0</td>
<td>~ 0</td>
</tr>
<tr>
<td>TB Release 168h</td>
<td>3110408</td>
<td>~ 0</td>
<td>~ 0</td>
</tr>
<tr>
<td>PMAA-g-10%J:TB Release 0h</td>
<td>468848</td>
<td>~ 0</td>
<td>~ 0</td>
</tr>
<tr>
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<td>32470606</td>
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<td>596</td>
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<td>PMAA-g-10%J:TB Release 48h</td>
<td>27991586</td>
<td>157</td>
<td>416</td>
</tr>
</tbody>
</table>
After correcting for the dilutions, concentrations still decreased after about 6 hours. This could have been due to tobramycin degrading over time; however, further studies must be performed to investigate this hypothesis. Due to this issue, the concentration data was only plotted over hours 0-6. As seen in Figure 8, the Higuchi kinetic model mathematically described this controlled tobramycin release. Korsmeyer-Peppas, zero-order and first-order models were taken into consideration as well, but the Higuchi model produced the highest $R^2$ value, as expected for most drug delivery systems. The model expression is given by the equation $Q = A\sqrt{D(2C - C_S)C_S t}$, where $Q$ is the amount of drug released, $A$ is the unit area, $D$ is the diffusion coefficient, $C$ is the initial drug concentration, $C_S$ is the drug solubility and $t$ is the time.\(^{47}\)

![Figure 8. Tobramycin release from PMAA-g-10%J:TB, described by the Higuchi model, in which the amount released was proportional to $t^{1/2}$.](image)
3.3 Aerosol Droplet Size Distribution

The nanoparticle formulation of PMAA-g-10%J:PB, containing PS dialyzed in HPLC-grade water and polymyxin B at a concentration of 2.2 mg/mL, was aerosolized and analyzed first. Both single nanoparticles and clusters of nanoparticles were analyzed via varying humidity conditions during the drying process. The nanoparticles aerosolized easily, without mechanical damage due to shear force, using both a desiccation chamber and a nebulizer only. Despite the high shear forces, the nanoparticles proved to be strong, multi-molecular aggregates. Figure 9 depicts the size distribution graph for the two aerosolizations of this antimicrobial drug delivery suspension, using the combined measurements of both Scanning Mobility Particle Sizer (SMPS) and Aerodynamic Particle Sizer (APS) instruments. The distribution of droplet sizes was less than 5 µm in diameter, which is appropriate for cystic fibrosis lung delivery. The background air circulated inside a Type II bio-hood with HEPA filters, and Mili Q water was used as a reference.
Figure 9. PMAA-g-10%J:PB aerosolization log-normal size distribution, displaying a mean size of about 100 nm. Electrical mobility diameters (10^1 – 10^3 nm) were computed via SMPS and aerodynamical diameters (10^3 – 10^4 nm) were computed via APS. DDS(1), in black, was aerosolized using a desiccation chamber, and DDS(2), in red, was aerosolized using a nebulizer only.

3.4 Minimum Inhibitory Concentrations

Planktonic cultures of the clinical *Pseudomonas aeruginosa* strains were treated with varying concentrations of polymyxin B, PMAA-g-10%J:PB, tobramycin and PMAA-g-10%J:TB in microbroth dilution assays. The key results are shown in Table 11. After overnight incubation in a 96-well plate, minimum inhibitory concentrations (MICs) were identified as the concentrations of the clear wells next to the leftmost cloudy wells on that particular row of a 96-well plate. MIC values of 10 µg/mL or greater indicated that the strain was resistant to that particular drug formulation.
Table 11. Minimum inhibitory concentrations for various bacteria samples and antimicrobial drug formulations.

<table>
<thead>
<tr>
<th>Clinical Isolate Sample #</th>
<th>Trial #</th>
<th>Antimicrobial Drug Formulation</th>
<th>Initial (leftmost) Antimicrobial Drug Concentration ($\mu g/mL$)</th>
<th>MIC (µg/mL)</th>
<th>96-Well Plate Visual</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2</td>
<td>Polymyxin B</td>
<td>320</td>
<td>2.5</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>2</td>
<td>Polymyxin B</td>
<td>320</td>
<td>2.5</td>
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</tr>
<tr>
<td>8</td>
<td>2</td>
<td>Polymyxin B</td>
<td>320</td>
<td>2.5</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>2</td>
<td>Polymyxin B</td>
<td>320</td>
<td>2.5</td>
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</tr>
<tr>
<td>14</td>
<td>2</td>
<td>Polymyxin B</td>
<td>320</td>
<td>2.5</td>
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</tr>
<tr>
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</tr>
<tr>
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</tr>
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<td>3.13</td>
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<tr>
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<td>5</td>
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</tr>
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<td>Tobramycin</td>
<td>50</td>
<td>0.781</td>
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<tr>
<td>19-2</td>
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</tr>
<tr>
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</tr>
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<td>7</td>
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<td>3.13</td>
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</tr>
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</tr>
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<td>8</td>
<td>Tobramycin</td>
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<td>8</td>
<td>PMAA-g-10%J:TB</td>
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</tr>
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<td>8</td>
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<td>---</td>
<td>-------------------------</td>
<td>-------------</td>
<td>------------</td>
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</tr>
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<tr>
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<td>Tobramycin</td>
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<td>1.25</td>
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</tr>
<tr>
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<td>8</td>
<td>PMAA-g-10%:TB</td>
<td>40</td>
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<td></td>
</tr>
<tr>
<td>15</td>
<td>8</td>
<td>Tobramycin</td>
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<td>1.25</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>8</td>
<td>PMAA-g-10%:TB</td>
<td>40</td>
<td>1.25</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>8</td>
<td>Tobramycin</td>
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</tr>
<tr>
<td>18</td>
<td>8</td>
<td>PMAA-g-10%:TB</td>
<td>40</td>
<td>1.25</td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>8</td>
<td>Tobramycin</td>
<td>40</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>19-2</td>
<td>8</td>
<td>Tobramycin</td>
<td>40</td>
<td>1.25</td>
<td></td>
</tr>
<tr>
<td>19-2</td>
<td>8</td>
<td>PMAA-g-10%:TB</td>
<td>40</td>
<td>2.5</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>9</td>
<td>Polymyxin B</td>
<td>40</td>
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</tr>
<tr>
<td>2</td>
<td>9</td>
<td>PMAA-g-10%:PB</td>
<td>40</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>9</td>
<td>Polymyxin B</td>
<td>40</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>9</td>
<td>PMAA-g-10%:PB</td>
<td>40</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>9</td>
<td>Polymyxin B</td>
<td>40</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>9</td>
<td>PMAA-g-10%:PB</td>
<td>40</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>9</td>
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<td>10</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>9</td>
<td>PMAA-g-10%:PB</td>
<td>40</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>9</td>
<td>Polymyxin B</td>
<td>40</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>9</td>
<td>PMAA-g-10%:PB</td>
<td>40</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>9</td>
<td>Polymyxin B</td>
<td>40</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>9</td>
<td>PMAA-g-10%:PB</td>
<td>40</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>9</td>
<td>Polymyxin B</td>
<td>40</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>9</td>
<td>PMAA-g-10%:PB</td>
<td>40</td>
<td>2.5</td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>9</td>
<td>Polymyxin B</td>
<td>40</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>9</td>
<td>PMAA-g-10%:PB</td>
<td>40</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>19-2</td>
<td>9</td>
<td>Polymyxin B</td>
<td>40</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>19-2</td>
<td>9</td>
<td>PMAA-g-10%:PB</td>
<td>40</td>
<td>5</td>
<td></td>
</tr>
</tbody>
</table>

*P. aeruginosa* 10 Polymyxin B 40 5

*P. aeruginosa* 10 Tobramycin 40 2.5
The first trial investigated the antimicrobial activity of polymyxin B against clinical isolate samples 1, 2 and 5, utilizing an initial concentration of $40 \frac{\mu g\,PB}{mL\,TSB}$ in the leftmost column. The resultant MICs were unclear and unreasonably high; therefore, these results were excluded from Table 11. Trial 2 began with a concentration of $320 \frac{\mu g\,PB}{mL\,TSB}$ in the leftmost column. All MICs were well below $40 \mu g/mL$, as MICs of $2.5 \mu g/mL$ were determined for samples 1, 5, 8, 12 and 14, and MICs of $5 \mu g/mL$ were determined for samples 15, 16, 18 and 19-2. Other than the initial polymyxin B concentration, the methodology did not change between trials 1 and 2; therefore, it was odd that the first trial exhibited such high MICs.

Trial 3, which investigated the antimicrobial activity of PMAA-g-10%J:PB (sample # 27 in Table 7) against clinical isolate samples 12, 14, 16, 18 and 19-2, began with a concentration of $10 \frac{\mu g\,PB}{mL\,TSB}$ in the leftmost column. Once again, unclear and unreasonably high MICs were observed; therefore, these results were excluded from Table 11. Trial 4 utilized PMAA-g-10%J:PB (sample # 35 in Table 7) at a higher initial concentration of $40 \frac{\mu g\,PB}{mL\,TSB}$. MICs of $2.5 \mu g/mL$ and $5 \mu g/mL$ were determined for samples 1 and 8, respectively. Sample 14 exhibited an unreasonably high MIC of $20 \mu g/mL$, and samples 15, 16, 18 and 19-2 exhibited high MICs of $10 \mu g/mL$; therefore, these samples were excluded from Table 11.

Trial 5 began the investigation of the antimicrobial activity of tobramycin, utilizing an initial concentration of $200 \frac{\mu g\,TB}{mL\,TSB}$ in the leftmost column. MICs of $3.13 \mu g/mL$ were determined for clinical isolate samples 12, 14, 16 and 18. Trial 6 utilized a
concentration of $50 \frac{\mu g_{TB}}{mL_{TSB}}$ in the leftmost column. MICs of 0.781 µg/mL were determined for samples 8, 15 and 19-2.

Trial 7 investigated the antimicrobial activity of PMAA-g-10%J:TB (sample # 41 in Table 7), utilizing an initial concentration of $40 \frac{\mu g_{TB}}{mL_{TSB}}$ in the leftmost column. Samples 1, 5 and 16 exhibited MICs of 1.25 µg/mL. Samples 8 and 12 exhibited MICs of 2.5 µg/mL, and samples 18 and 19-2 exhibited MICs of 3.13 µg/mL.

Due to the variability of previous MICs, trial 8 examined one clinical isolate sample per 96-well plate, testing the antimicrobial activities of all four drug formulations at once. Each formulation began with a concentration of $40 \frac{\mu g \text{ drug}}{mL_{TSB}}$ in the leftmost column, and clinical isolate samples 2, 5, 8, 12, 14, 15, 16, 18 and 19-2 were utilized. Polymyxin B and its complex (sample # 39 in Table 7) mainly exhibited MICs of 10 and 20 µg/mL, and tobramycin and its complex (sample # 41 in Table 7) mainly exhibited MICs of 1.25 and 2.5 µg/mL.

Due to the unreasonably high polymyxin B and PMAA-g-10%J:PB MIC values in previous trials, trial 9 reexamined the antimicrobial activity of these formulations using brand new peptide (sample # 51 in Table 7). It was hypothesized that the previously used polymyxin B was either old or degraded, and that a fresh stock of peptide was required to achieve potency. Again, an initial concentration of $40 \frac{\mu g_{PB}}{mL_{TSB}}$ was utilized in the leftmost column. The results of this trial supported the degradation hypothesis, as the antimicrobial activities exhibited lower MIC values, predominantly around 5 µg/mL; therefore, the MIC results for polymyxin B and PMAA-g-10%J:PB from trial 8 were excluded from Table 11. It is important to note that MICs remained about the same for
free antimicrobials and their complexes with PMAA-g-10%J. This corroborated the notion that the antimicrobial activity of drugs should be retained following PS encapsulation into nanoparticles. Moreover, though the median MIC value of 5 µg/mL was lower than previous polymyxin B MICs, it was not as low as the MIC values obtained for tobramycin. It is possible that polymyxin B was absorbing onto the 96-well plates, as it has a high affinity for plastic. Further studies must be performed with glassware to investigate this theory.

Trial 10 examined the antimicrobial activities of polymyxin B and tobramycin against a commercial strain of *Pseudomonas aeruginosa* (ATCC® 27853™). The purpose of this trial was to check if the clinical isolate strains acquired multi-drug resistance against polymyxin B, as the patients they derived from were already undergoing treatment. This trial utilized an initial concentration of $40 \frac{\text{µg drug}}{\text{mL TSB}}$ in the leftmost column, and obtained a polymyxin B MIC of 5 µg/mL and a tobramycin MIC of 2.5 µg/mL. Therefore, the clinical isolate sample results were representative of the drugs’ antimicrobial activities, and it was concluded that tobramycin was more effective than polymyxin B in these studies. As a result, tobramycin and its complex were examined further in the following anti-biofilm activity studies. Due to the variability of the ten trials, Table 12 provides a summary of the most reliable MIC results, displaying the typical preservation of antimicrobial activity following PS encapsulation into nanoparticles. It is evident in this table that the clinical strains were representative of *P. aeruginosa*, as the MIC values were similar to those of the commercial strain. It is also apparent that tobramycin exhibited higher antimicrobial activity, as compared to polymyxin B. MICs of 10 µg/mL were considered resistant and, therefore, unreliable.
Table 12. Summary of minimum inhibitory concentration results.

<table>
<thead>
<tr>
<th>Clinical Isolate Sample #</th>
<th>Polymyxin B MIC (µg/mL)</th>
<th>PMAA-g-10%J:PB MIC (µg/mL)</th>
<th>Tobramycin MIC (µg/mL)</th>
<th>PMAA-g-10%J:TB MIC (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2.5</td>
<td>2.5</td>
<td>Not tested</td>
<td>1.25</td>
</tr>
<tr>
<td>2</td>
<td>10</td>
<td>5</td>
<td>2.5</td>
<td>5</td>
</tr>
<tr>
<td>5</td>
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<td>1.25</td>
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<tr>
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<td>5</td>
<td>2.5</td>
<td>1.25</td>
<td>1.25</td>
</tr>
<tr>
<td>18</td>
<td>5</td>
<td>5</td>
<td>3.13</td>
<td>3.13</td>
</tr>
<tr>
<td>19-2</td>
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<td>Not tested</td>
</tr>
<tr>
<td><em>P. aeruginosa</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

3.5 Minimum Biofilm Eradication Concentrations

Anti-biofilm activity studies were performed to determine the minimum biofilm eradication concentrations (MBECs), or the lowest tobramycin concentrations, which eliminate biofilms, for various clinical isolate samples. The results of these experiments were indicative of the dosages necessary to break down biofilms. The higher the drug dosage, the greater the chance of adverse side effects in vivo; therefore, these MBEC results were suggestive of the effectiveness of PMAA-g-10%J:TB.

Visual results of the remaining biofilms after 24 hour incubation with the serially diluted drug can be seen in Table 13. Bacterial biofilms remained on the right side of the plate, where the tobramycin concentrations were lowest. Biofilms were seen as both cloudy wells and purple/pink fluorescence. The alamarBlue phase-contrast microscopy indicated greater bacterial proliferation with greater fluorescence intensity. Similar
biofilm conservation was observed for clinical isolate samples treated with tobramycin and its complex with PMAA-g-10%J (sample # 53 in Table 7), upon visual inspection.

**Table 13.** Visual results of the remaining biofilms after 24 hour incubation with the serially diluted drug, washing the plate with 1X PBS and 30 minute incubation with alamarBlue. Similar biofilm conservation was observed for tobramycin and PMAA-g-10%J:TB.

<table>
<thead>
<tr>
<th>Clinical Isolate Sample # / Drug Formulation</th>
<th>Biofilms After 24 Hour Incubation</th>
<th>Biofilms After Washing with 1X PBS</th>
<th>Biofilms After 30 Minute Incubation with alamarBlue</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 / Tobramycin</td>
<td><img src="image1.png" alt="Image" /></td>
<td><img src="image2.png" alt="Image" /></td>
<td><img src="image3.png" alt="Image" /></td>
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<tr>
<td>2 / Tobramycin</td>
<td><img src="image4.png" alt="Image" /></td>
<td><img src="image5.png" alt="Image" /></td>
<td><img src="image6.png" alt="Image" /></td>
</tr>
<tr>
<td>2 / PMAA-g-10%J:TB</td>
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<td><img src="image8.png" alt="Image" /></td>
<td><img src="image9.png" alt="Image" /></td>
</tr>
<tr>
<td>5 / Tobramycin</td>
<td><img src="image10.png" alt="Image" /></td>
<td><img src="image11.png" alt="Image" /></td>
<td><img src="image12.png" alt="Image" /></td>
</tr>
<tr>
<td>5 / Tobramycin</td>
<td><img src="image13.png" alt="Image" /></td>
<td><img src="image14.png" alt="Image" /></td>
<td><img src="image15.png" alt="Image" /></td>
</tr>
<tr>
<td>5 / PMAA-g-10%J:TB</td>
<td><img src="image16.png" alt="Image" /></td>
<td><img src="image17.png" alt="Image" /></td>
<td><img src="image18.png" alt="Image" /></td>
</tr>
<tr>
<td>8 / Tobramycin</td>
<td><img src="image19.png" alt="Image" /></td>
<td><img src="image20.png" alt="Image" /></td>
<td><img src="image21.png" alt="Image" /></td>
</tr>
<tr>
<td>8 / PMAA-g-10%J:TB</td>
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<td><img src="image23.png" alt="Image" /></td>
<td><img src="image24.png" alt="Image" /></td>
</tr>
<tr>
<td>12 / Tobramycin</td>
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<td><img src="image26.png" alt="Image" /></td>
<td><img src="image27.png" alt="Image" /></td>
</tr>
<tr>
<td>12 / PMAA-g-10%J:TB</td>
<td><img src="image28.png" alt="Image" /></td>
<td><img src="image29.png" alt="Image" /></td>
<td><img src="image30.png" alt="Image" /></td>
</tr>
<tr>
<td>14 / Tobramycin</td>
<td><img src="image31.png" alt="Image" /></td>
<td><img src="image32.png" alt="Image" /></td>
<td><img src="image33.png" alt="Image" /></td>
</tr>
<tr>
<td>14 / PMAA-g-10%J:TB</td>
<td><img src="image34.png" alt="Image" /></td>
<td><img src="image35.png" alt="Image" /></td>
<td><img src="image36.png" alt="Image" /></td>
</tr>
<tr>
<td>15 / Tobramycin</td>
<td><img src="image37.png" alt="Image" /></td>
<td><img src="image38.png" alt="Image" /></td>
<td><img src="image39.png" alt="Image" /></td>
</tr>
</tbody>
</table>
Relative fluorescence units were plotted against the corresponding tobramycin concentrations to form bar graphs, as seen in Figure 10. MBECs were determined as the concentrations at which the fluorescence unit values were down to noise levels, or less than 5% of the maximum fluorescence. It was hypothesized that MBECs would be lower for samples treated with PMAA-g-10%J:TB than free tobramycin, as the PS enhances mucus penetration and reduces antibiotic degradation. Table 14 summarizes the MBEC results for each bacteria sample and antimicrobial drug formulation.
Figure 10. Bar graphs of relative fluorescence units vs. tobramycin concentration, displaying the quantitative proliferation of bacteria for various clinical isolate samples and tobramycin formulations. Minimum biofilm eradication concentrations were exhibited at concentration values less than 5% of the maximum fluorescence.

Table 14. Results summary of minimum biofilm eradication concentrations (MBECs) for various bacteria samples using tobramycin and PMAA-g-10%J:TB.

<table>
<thead>
<tr>
<th>Clinical Isolate Sample #</th>
<th>Antimicrobial Drug Formulation</th>
<th>MBECs (μg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>Tobramycin</td>
<td>19.5 R</td>
</tr>
<tr>
<td>2</td>
<td>PMAA-g-10%J:TB</td>
<td>9.8 R</td>
</tr>
<tr>
<td>5</td>
<td>Tobramycin</td>
<td>39.1</td>
</tr>
<tr>
<td>5</td>
<td>PMAA-g-10%J:TB</td>
<td>39.1</td>
</tr>
<tr>
<td>8</td>
<td>Tobramycin</td>
<td>39.1</td>
</tr>
<tr>
<td>8</td>
<td>PMAA-g-10%J:TB</td>
<td>39.1</td>
</tr>
<tr>
<td>12</td>
<td>Tobramycin</td>
<td>9.8</td>
</tr>
<tr>
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<td>PMAA-g-10%J:TB</td>
<td>4.9</td>
</tr>
<tr>
<td>14</td>
<td>Tobramycin</td>
<td>9.8</td>
</tr>
<tr>
<td>14</td>
<td>PMAA-g-10%J:TB</td>
<td>4.9</td>
</tr>
<tr>
<td>15</td>
<td>Tobramycin</td>
<td>19.5</td>
</tr>
<tr>
<td>16</td>
<td>Tobramycin</td>
<td>39.1</td>
</tr>
</tbody>
</table>
As seen in Table 14, the MBECs of PMAA-g-10%J:TB were less than or equal to the MBECs of free tobramycin against the same clinical isolate samples, as expected due to the higher detergency and membrane permeability of the PS. Clinical isolate samples 2, 12, 14 and 16 exhibited lower MBECs with PMAA-g-10%J:TB than with free tobramycin, and samples 5 and 8 exhibited the same MBECs with each antimicrobial formulation. The R designation on the MBECs of clinical isolate sample 2 indicates that there was some residual fluorescence, as if the strain was resistant. This can be seen by the increase in fluorescence units for high tobramycin concentrations in Figure 10.

CHAPTER 4: CONCLUSIONS & FUTURE WORK

In conclusion, the “GRAPLON” nanomedicine, PMAA-g-10%J:TB, exhibited the most potential as an effective antimicrobial agent to eradicate cystic fibrosis lung infections. Various experiments were conducted on different nanoparticle formulations in order to draw this conclusion.

Hydrodynamic diameters less than 200 nm and zeta potential values greater than $|5| \text{ mV}$ were indicative of the proper formation and stability of the nanoparticles. Smaller and more negatively charged nanoparticles were determined via PS dialysis with 1X PBS, rather than with HPLC-grade water; therefore, 1X PBS was utilized for most formulations. Complexes with PMAA-g-1%J and PPAA-g-1%J appeared to aggregate, as exhibited by their large hydrodynamic diameters. This aggregation was due to the nature of having less graft, or lower density PEG, on the complex. 1% graft had fewer ethylene
oxide groups than 5% and 10% graft, which resisted aggregation. Therefore, 1% graft was not utilized in further experiments. PSs encapsulating LL-37 produced large and polydispersed nanoparticles. These observations may have been attributed to the high molecular weight and amphiprotic nature of LL-37, which may have caused weaker binding to the PSs. It is possible that the PSs and LL-37 were in part self-associating, rather than fully complexing. Small and stable complexes were observed for PMAA-g-10%J:PB, PMAA-g-5%J:PB, PMAA-g-10%J:TB and PMAA-g-5%J:TB; however, the PMAA-g-10%J complexes exhibited a certain level of variability to them over time. After substantial investigation, it was concluded that the most probable cause of this was the introduction of new PMAA-g-10%J, utilized in later studies. This PS was produced in lab, rather than by the Polysciences chemical manufacturing company, and may have had a certain level of impurity, which caused the variability.

Controlled release studies with LL-37 and PMAA-g-10%J:LL37 were unsuccessful. Standard solutions of LL-37 exhibited that the peptide was undetectable below the concentration of about 15.6 µg/mL, and the concentrations of the membrane dialysis assay samples were all below this limit of detection. The anionic functionality of LL-37 may have caused it to stick to the column, or the self-association of LL-37 may have caused it to come off the column in an unexpected way. Due to the problematic hydrodynamic diameter and zeta potential values, these controlled release study issues and the high cost of LL-37, the peptide was excluded from further studies. The tobramycin controlled release study exhibited no release of free tobramycin across the dialysis membrane, as all of the HPLC peak areas were below that of the lowest tobramycin standard. Meanwhile, PMAA-g-10%J:TB exhibited a controlled release of
tobramycin over the first 6 hours, demonstrating the release kinetics of the Higuchi model.

PMAA-g-10%J:PB nanoparticles, formulated with PMAA-g-10%J dialyzed in HPLC-grade water, aerosolized easily using both a desiccation chamber and a nebulizer only. The nanoparticles did not undergo mechanical damage upon aerosolization, and the size distribution plots exhibited droplet sizes under 5 µm in diameter, which is appropriate for cystic fibrosis lung delivery.

PMAA-g-10%J formed the most consistently sized particles and obtained the highest density PEG, which exhibits faster mucus penetration. Therefore, this PS, encapsulating polymyxin B and tobramycin, was utilized in the antimicrobial studies. As expected, MICs were retained for drugs encapsulated in PMAA-g-10%J. Tobramycin and its complex consistently exhibited higher antimicrobial activity against the clinical isolate and commercial P. aeruginosa strains, as compared to polymyxin B and its complex. This may be attributed to tobramycin being a more effective drug, or to polymyxin B’s high affinity for plastic. All 10 MIC trials were performed with plastic 96-well plates and, due to this affinity, it is possible that polymyxin B was absorbed onto the plates, exhibiting lower antimicrobial activities. Regardless, tobramycin exhibited lower MIC values than the seemingly resistant polymyxin B, and was utilized in further anti-biofilm activity studies.

As hypothesized, the MBECs of clinical isolate samples treated with PMAA-g-10%J:TB were less than or equal to the MBECs of analogous samples treated with free tobramycin. This was due to the PS’s high detergency and membrane permeability, which enhanced mucus penetration. Therefore, due to its consistently high anti-biofilm and
antimicrobial activities, its Higuchi-like release kinetics and its consistently small and stable nanoparticle formations, PMAA-g-10%J:TB was concluded as the most optimal “GRAPLON” nanomedicine for cystic fibrosis treatment.

Many experiments could be performed in future studies to substantiate these results. If time allotted, more nanoparticles would be formulated with newly formulated, quality-controlled PMAA-g-10%J. Particles should, ideally, be less than 200 nm and more negatively charged for enhanced mucus penetration and lung delivery, though cationic particles greater than |5| mV are considered stable. Therefore, it would be beneficial to directly confirm that the latter, variable results were due to using impure, lab-produced PS. Additionally, another controlled release study investigating free tobramycin would be advantageous to confirm that unbound antimicrobials release faster than bound ones. Free tobramycin did not appear to release at all, which does not corroborate theory. Finally, another antimicrobial activity study could be performed using glass 96-well plates, rather than plastic, to validate whether polymyxin B’s high MIC results were due to its affinity for plastic or not. This quality-controlled glassware study would also involve HPLC testing to determine how much polymyxin B was absorbing onto the plastic in previous trials.

In addition to further investigating existing issues, supplementary studies could be performed in the future to corroborate the conclusions that were drawn. PS, PS-PB and PS-TB surface activities may be determined in 1X PBS as a function of concentration to obtain the critical aggregation concentrations (CACs) via the DuNouy tensiometer. Cone-and-plate viscometry may determine the effects of the PSs, the PS-PBs and the PS-TBs on the shear-dependent viscosities of mucus-imitating hydrogels, CMC, HEC and mucin.
The binding levels of the antimicrobials may be determined via ultracentrifugation and HPLC analysis of supernatant drug concentrations. Biodegradation of the drugs in human serum may be determined via HPLC analysis as well. Moreover, the PSs could be conjugated with the far-red dye Cyanine5.5 (Cy5.5) via carbodiimide coupling reactions to fluorescently label the polymer. The labeled PSs could then form complexes with polymyxin B and tobramycin, which would be used to quantify penetration through an artificially formulated mucus barrier via a fluorescence plate reader and HPLC analysis. This could corroborate the fact that the anti-biofilm activities were enhanced due to increased nanoparticle mucus penetration. Even more, the cytotoxicity of the nanoparticle formulations may be tested via MTS assays against eukaryotic cells, such as fibroblasts and hepatocytes. In addition to MIC and MBEC assays, MBICs, or minimum biofilm inhibitory concentrations could be determined by incorporating the serially diluted drug before biofilm formation. MICs, MBECs and MBICs could be examined further by using cascade impactors to collect and deposit the aerosolized drug formulations on 96-well plates. Finally, the in vivo systemic and regional pharmacokinetic biodistributions of the PS-PB and PS-TB nanoparticles may be evaluated in healthy and cystic fibrosis mice. Cy5.5 fluorescently labeled nanoparticles would be delivered to the mice via intravenous injection and nebulizer inhalation delivery, and the efficacies of the two administrations may be compared via fluorescence detection using a plate reader for blood distribution and IVIS imaging for lung distribution.
Figure A1. Hydrodynamic diameter distribution plots and their corresponding correlograms.
Figure A2. Zeta potential distribution plots.
REFERENCES


