Polymeric Antibacterial Agents:
Cytotoxicity and Antimicrobial Properties of Amphiphilic Polymers

Honors Thesis

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Introduction

In recent years, antibiotic-resistance bacterial strains have become a growing concern. As of 2004, approximately 90,000 deaths per year in hospitals were attributed to bacterial infections, 70% of which were drug-resistant [1]. With the threat of traditional antibiotics and antimicrobials becoming obsolete, new classes of antibiotics have become an important subject of current research. Certain types of polymers have shown promise as potential antimicrobial agents [2]. An especially attractive property of polymeric antibiotics is that their proposed antimicrobial mechanism would decrease the ability of bacteria to develop resistances [2]. Here, the amphiphilic polymers branched and linear polyethylenimine (BPEI, LPEI), and poly-L-lysine (PLL) are characterized according to their antimicrobial properties. On-going research in this lab has focused on modifying synthesized polymers' structures and observing the effect on antimicrobial activity; the research presented here differed from prior projects in that it chose to analyze known bioactive polymers to determine if they constitute feasible antibiotic compounds.

These polymers display a wide range of structures and molecular weights in order to better relate structure to function (fig. 1). They were chosen for these varying characteristics as well as their availability, ease of use and familiarity in research and industry. Linear polyethylenimine has shown potential as a possible non-viral vector system for drug transport across cell membranes [3-5]. ε-poly-L-lysine is a commonly used food preservative and antimicrobial agent in Japan and other nations [6]. The FDA has deemed this form of poly-L-lysine non-toxic to humans in high doses [7]. It is the purpose of this paper to gain more insight into these polymers' antimicrobial activity with the hope that their membrane-active mechanism and the relationship between their structure and activity can at some point be elucidated. As it is extremely difficult

to correlate these polymers' structures to their interactions with biological systems, this project mainly focuses on characterizing these interactions. In a novel aspect in this field of research, the polymers' antibacterial properties are compared to their cytotoxic effects [8].

To achieve a viable antimicrobial compound, the agent's cytotoxic activity must be low while still having a sufficient effect on bacteria. This balance is necessary to ensure that the potential antibiotic is safe for human use at therapeutic levels but still effective against bacteria [9]. These target polymers were subjected to antimicrobial, hemolysis, LDH and XTT assays to determine their impact on the human physiology. The minimum polymer concentration needed to inhibit bacteria growth for 24 hrs (minimum inhibitory concentration – MIC) was determined by measuring both Gram-positive and Gram-negative bacteria growth in the presence of varying concentrations of polymers [10]. Another important property of an antimicrobial agent is the minimum bactericidal concentration (MBC), or the minimum polymer concentration required to kill the bacteria as opposed to merely inhibiting growth [11]. A polymer's ability to inhibit growth is only applicable for as long as the bacteria is exposed to the polymer. Its bactericidal property refers to the polymer's long-range effect on the system after it has been diluted or removed [10,11]. The spectroscopic analysis of hemoglobin release from erythrocytes after polymer exposure was used to determine the polymers' compatibility with blood cells. Tissue cell compatibility was ascertained by the cell viability of human epithelial HEp-2 cells after their exposure to the polymers as measured by the LDH and XTT assays [12]. The release of lactate dehydrogenase (LDH) as observed by the conversion of the tetrazolium salt INT to formazan quantified cell membrane rupture in the tissue cells by the polymers. Decreased cell metabolic activity in the epithelial cells due to polymer exposure was determined by spectroscopically

measuring the amount of metabolized formazan in the XTT assay [12]. To gain a better understanding of the necrotic or apoptotic features of cell death due to polymer exposure, dye-filled synthetic liposomes modeled tissue and bacterial cells. Fluorescence spectroscopy was used to determine the dye leakage, and thus the degree of membrane damage to the liposomes, due to polymer exposure. Greater dye leakage correlates to a higher degree of membrane damage, which would suggest necrosis by membrane rupture in cells.

Experimental Procedures

Materials. Branched polyethylenimines (BPEIs; 600, 1800, 10000 Da), linear polyethylenimines (LPEIs; 2500, 25000 Da) and poly-L-lysine hydrobromide (PLLs; 1500-8000, 40000-60000, 100000-140000 Da) were purchased from Polysciences, Inc. (Warrington, PA). Varying concentrations of BPEI and PLL stock solutions were prepared in TBS buffer pH 7.3 (10 mM Tris buffer, 150 mM NaCl). A LPEI stock solution was prepared in ethanol then serially diluted in sterile water.

Hemolysis assay. A solution of fresh erythrocytes in TBS was centrifuged at 2000 rpm for 5 minutes. The separated red blood cells were then washed in TBS three times until the supernatant was clear. A 3.33% red blood cell stock solution in the TBS buffer was prepared and used immediately. Polymer solutions prepared in the TBS buffer (BPEIs and PLLs) or ethanol with water (PLLs) and the RBC stock solution were added to a 96-well microplate to achieve a final red blood cell concentration of $15x10^6$ cells per well. After an incubation period of 1 hr at 37º C, the microplate was centrifuged at 1000 rpm for 5 minutes. Hemoglobin release in the supernatant was measured at 415 nm. Percent hemolysis was determined by comparison

to the 100% hemoglobin release obtained by 0.1% (v/v) Triton X-100. The HC_{50} value was calculated as the polymer concentration which lyses 50% of red blood cells. Hemolytic activity less than 10% was considered non-toxic as the variability of the assay did not exceed 10%. The assay was performed in triplicate and repeated independently at least three times.

Antimicrobial assay. *E. coli* ATCC 25922 and *S. aureus* ATCC 25923 were incubated in Mueller-Hinton broth (pH 7.3) and diluted to concentrations of $5x10^5$ CFU/mL. The final inoculum for each bacteria strain and the polymer solutions (prepared by the same procedure as in the hemolysis assay) were added to a 96-well microplate and incubated for 18 hrs at 37º. Bacteria growth was measured at 595 nm and the minimum inhibitory concentration (MIC) was determined as the polymer concentration that resulted in an absorbance less than 0.2. To determine the minimum bactericidal concentration (MBC), the contents of the cells in the MIC plate were transferred to a new subculture, where they were serially diluted in M-H broth to obtain 10^{-1}, 10^{-2} and 10^{-3} dilutions of the original polymer concentrations and bacteria. The plate was incubated again for 18 hrs at 37º. The minimum bactericidal concentration for each polymer was determined as the polymer concentration that resulted in an absorbance of less than 0.2 at 595 nm. These assays were performed in triplicate and repeated at least three times.

Lactate dehydrogenase (LDH) assay. Human epithelial HEp-2 cells isolated from a larynx carcinoma were grown in MEM with 10% heat-inactivated fetal bovine serum, 0.1% nonessential amino acids and 1 mM sodium pyruvate. Cells at a concentration of 10^4 per well of MEM/FBS were seeded into the wells of a sterile 96-well microplate. After a 24 hr incubation period at 37 º C and 5% CO_2, the MEM/FBS solution was removed from the wells and the cells

were rinsed once with PBS (phosphate buffered saline, pH 7.4). Polymer stock solutions were prepared by 2-fold serial dilutions to give 16 different concentrations in TBS or water/ethanol. TBS or water/ethanol, and 0.9% v/v Triton X-100 were used as the negative and positive controls, respectively. The polymer solutions were added to the wells and the cells were incubated for 1 hr at 37º C and 5% CO_2. The LDH release assay was performed following the protocol given by a commercial kit (Promega, CytoTox 96 Non-Radioactive Cytotoxicity Assay, cat. # G1780). The absorbance at 490 nm was measured as a tetrazolium salt (INT) was converted into a red formazan product by LDH. The EC_{50} value for each polymer was determined as the polymer concentration that resulted in 50% cell viability. LDH release of less than 10% was considered non-toxic to the cells because the variability of the assay did not exceed 10%. The assay was performed in triplicate and repeated three times. Comparison of pure LDH from the test kit and LDH extracted from HEp-2 cells in the presence of the polymers showed that the polymers had no effect on LDH activity.

XTT assay. Polymer stock solutions and HEp-2 cells were prepared in the same manner as in the LDH assay. The cytotoxicity of polymers in the LDH and XTT assays was simultaneously evaluated for 1hr exposure time – the supernatant was used for the LDH assay and the cells for the XTT assay. Another set of samples was prepared as above and allowed to incubate for 24 hrs. After the exposure time of 1 or 24 hr to the polymers, cells were washed with PBS. Following the protocol from a commercial test kit (Roche Applied Sciences, Cell Proliferation Kit II, cat. # 11465015001), the cells' viability after exposure to the polymers was determined by measuring the amount of the metabolized formazan at 450 nm. TBS or water was used as non-treated control (100% cell viability). The EC_{50} value for each polymer was determined. Less than 10%

decrease in cell viability was regarded as non-toxic because the variability of the assay did not exceed 10 %. The assay was performed in triplicate and repeated three times.

Liposome Experiment

Liposome Preparation

Palmitoyl-2-oleoyl-sn-glycero-3-phosphatidylcholine (POPC), 1-palmitoyl-2-oleoyl-phosphatidylethanolamine (POPE) and 1-palmitoyl-2-oleoyl-phosphatidylglycerol (POPG) were purchased from Avanti Polar Lipids, Inc. Solutions of POPE and POPG were prepared in chloroform and methanol respectively at concentrations of 7.5 mM. A mixture of the two lipids (PE/PG) was formed by combining 8:2 POPE:POPG (266 μL:67μL). The organic solvents were removed by rotary evaporation and the resulting waxy residue was dried for 3 hrs under reduced pressure. To the lipid residue was added 175 μM aqueous sulforhodamine B (50 mM) to form liposomes. The solution was then vortexed and subjected to 5 freeze/thaw cycles in dry ice/acetone and a 37º C water bath to adequately hydrate the PE/PG liposomes. The resulting liposome suspension was passed through an extruder (Avanti Polar Lipids, Inc.; polycarbonate membrane, 400 nm pore size) 21 times. Excess dye from the extruded solution was removed by gel filtration (Sepharose CL-4B column) in TBS with adjusted osmolarity. The final concentration of liposomes was determined by phosphorus assay [13,14]. A 10 μM PE/PG liposome stock solution in TBS was then prepared. The POPC lipid in methanol was prepared as above to achieve a 10 μM POPC liposome stock solution in TBS. A fresh stock solution in buffer was prepared prior to each fluorescence measurement. The excess liposomes were stored at 4º C and used within a week of preparation.

Dye Leakage

Polymer (10 μL) was added to 1mL aliquots of the liposome stocks solutions from above to achieve thirteen samples ranging in polymer concentration from 0 to 2.5 μM. The liposome concentrations remained constant at approximately 10 μM. Each well-mixed sample was incubated at 37º C for 30 minutes. The sample's fluorescence intensity was then measured at λ_{Ex} = 565 nm and λ_{Em} = 577 nm. Triton X-100 (10 μL, 1%: POPC; 12.5 μL, 1%: PE/PG) was immediately added to the sample and the fluorescence was measured again. Dye leakage was calculated as $(I - I_{blank})/(I_{Triton\ X\text{-}100} - I_{blank})$ and reported as a percentage. This experiment was conducted for the BPEIs, LPEIs and PLLs with both PE/PG and POPC liposomes. Each polymer was tested twice independently using this procedure.

Results and Discussion

Note: The results from the biological assays are expressed in μg/mL to account for the polymers' weight ranges. Molar concentrations are used for the liposome experiment due to the preparation method.

Hemolytic Activity of BPEIs and LPEIs

The branched polyethylenimines showed extremely low hemolytic activity (fig. 2). For each molecular weight, the HC_{50} values were above 4000 μg/mL (table 1). Hemolysis for all BPEIs remained below 10% for concentrations from 16 – 2000 μg/mL. As the variability of this assay is ±10%, these BPEIs were classified as compatible with red blood cells. The LPEIs, on the other hand, showed considerable hemolytic activity (fig. 3). Increasing the polymer concentration from 8 – 1000 μg/mL resulted in greater hemolysis. When plotting percent hemolysis vs.

concentration for LPEI MW 2500 Da and LPEI MW 25000, the curves approximated the sigmoidal shape that is typically seen with surfactants and other bioactive, cationic substances [14]. The phase transitions for the LPEIs, however, were much less steep than those observed for known hemolytic compounds [15]. This observed difference may imply that these polymers employ a different hemolytic mechanism than other surfactants. The higher molecular weight LPEI was more hemolytic than its lower molecular weight analogue (HC_{50} = 576.5 μg/mL vs. 195.3 μg/mL). These results imply that hemolysis is dependent on molecular weight for LPEI: greater molecular weights increase the polymer's hemolytic activity.

Linear polyethylenimines showed hemolytic activity dependent on molecular weight while the BPEIs were compatible with erythrocytes. The different solvents were determined to have no effect on hemolysis in these assays, therefore it was hypothesized that the polymers' structural differences resulted in their varying hemolytic activity. Structural geometry may play a role in the discrepancy between their bioactivity. For instance, branched PEIs have a globular structure. It is possible that the polymer is too large to interact effectively with the erythrocyte membrane. Additionally, a molecule's branching side chains may entangle other polymer molecules to form aggregates, which could decrease their membrane activity. The LPEIs, however, as 'stream-lined' molecules, could more easily damage the membrane due to their ability to move more freely throughout the solvent. Alternatively, the polymers' chemical functionality could mediate their interactions with the erythrocyte membranes. It is known that the initial interaction between charged polymeric compounds and cellular membranes is due to the positively charged polymer's attraction to the negatively charged membrane [16,17]. Hydrophobic functionality is responsible for compromising the membrane [16]. BPEIs, LPEIs and PLLS are all amphiphilic

polymers, meaning they contain both hydrophobic and hydrophilic functional groups. In the case of these polymers, a cationic primary amine group contains the hydrophilic functionality. Linear PEIs are composed of a string of secondary amines terminating in a cationic primary amine. On the other hand, BPEIs contain tangles of primary, secondary and tertiary amines. It may be the difference in the electrostatic charge distribution or the distribution and type of amine groups between BPEIs and LPEIs that is responsible for their unique interactions with the erythrocyte membranes.

Hemolytic Activity of PLLs

Poly-L-lysine showed a more complex relationship between molecular weight and membrane activity. Like the BPEIs, PLL MW 1500 – 8000 Da and PLL MW 100000 – 140000 Da resulted in hemolysis less than 10% at polymer concentrations ranging from 31 – 4000 μg/mL, making them essentially non-hemolytic (fig. 4). PLL MW 40000 – 60000 Da, however, showed the characteristic hemolytic sigmoidal curve when percent hemolysis vs. concentration for the same concentration range was plotted. HC_{50} for the mid-weight PLL was found to be 3000 μg/mL. While PLL showed some membrane activity at a certain molecular weight, its hemolytic activity was still well below that of the LPEIs. Like BPEI, PLL contains both primary and secondary amines. Unlike either BPEI or LPEI, however, PLL also contains a carboxyl group in the lysine residue. Based on molecular structure alone, one would expect PLL to behave differently than polyethylenimine. While PLL's interactions with the erythrocyte membrane obviously differ from those of BPEI or LPEI based on the hemolysis data, the nature of the incongruity between these interactions is unknown.

MICs against *E. coli* and *S. aureus*

Branched PEI's MICs for *E. coli* showed a perplexing trend (fig. 5). The MIC decreased from 500 to 250 μg/mL as the polymer's molecular weight increased from 600 to 1800 Da. The highest molecular weight BPEI, however, resulted in MIC = 1000 μg/mL. For both LPEIs MIC = 31.25 μg/mL, which was lower than MIC = 250 μg/mL for BPEI. LPEIs, therefore, are more effective at inhibiting Gram-negative bacteria growth. PLLs showed another strange phenomenon: the MIC decreased from 125 to 31.25 μg/mL as molecular weight increased from 1500 to 40000 Da; however, MIC = 62.5 μg/mL for the highest molecular weight PLL. In general, PLLs and LPEIs showed comparable Gram-negative antibacterial activity across their molecular weight ranges based on their MICs.

The MICs against *S. aureus* were significantly smaller for all polymers when compared to those against *E. coli* (fig. 7). All molecular weights of PLL and LPEI resulted in the same MIC value: 15.63 μg/ML. BPEIs exhibited the same molecular weight-dependent phenomenon as with *E. coli*, and again were less effective antibacterial agents than PLL or LPEI. The discrepancies between the MICs against the different bacteria strains may be due to the bacterial cell membranes. *E. coli* is Gram-negative while *S. aureus* is Gram-positive, meaning their cellular membranes contain mosaics of different phospholipids [16]. The membrane compositions may interact with the polymers differently so that *S. aureus* is more susceptible to the polymers' cell death mechanism [16].

MBCs against *E. coli* and *S. aureus*

Traditional antibiotics typically inhibit bacteria growth. As such, their MBCs are significantly higher than their MICs [11]. With these polymers, however, the MBCs are comparable to, if not the same as, the MICs. For *E. coli,* only PLL MW 40000-60000 has a greater MBC than MIC (table 2). For *S. aureus*, the polymers' MBCs are consistently twice as large as their MICs (table 3). Due to the polymer preparation, these MIC and MBC values may still be within error of one another. This data implies that the polymers employ a mechanism that kills bacteria as opposed to merely inhibiting growth.

Cytotoxicity of PLLs

The LDH assay measures the amount of enzyme that leaks from the cell after exposure to a polymer. Not only is it a measure of cell death, therefore, but also an indicator of membrane integrity; a mechanism for cell death can thus be inferred from this assay [17]. A low EC_{50} value for a polymer indicates that the polymer is not only toxic to human cells, but also that the cell death is necrotic in nature and results from a ruptured cellular membrane. The XTT assay is another measure of cell viability; however, as it cannot differentiate between apoptotic and necrotic features in cell death, it is less useful as a means of studying polymer interactions [17]. According to the LDH and XTT assays, the PLLs are the most toxic of three general classes of polymers with EC_{50} values between 10 and 40 μg/mL (table 4). The PPLs' EC_{50} values for both the LDH and XTT assays are similar and only deviate by approximately 15 μg/mL (table 5). This result implies that PPLs cause cell death by perforating the cell membrane. There is little difference between PLLs of different molecular weights; the highest molecular weight PLL appears to be slightly more toxic than its lower weight analogues in both assays (fig. 9). The

PLLs show almost the same cytotoxicity after 1 hr as they do after 24 hrs in the XTT assay (fig. 10). It can then be concluded that their mechanism for cell death occurs quickly and is time-independent for this time scale. These results seem somewhat anomalous when compared to the hemolysis assay. Only the mid-weight PPL was hemolytic, meaning it caused the erythrocytes to lyse. All three PLLs are substantially toxic by the LDH assay, however, which implies that all the PLLs cause necrosis by membrane perforation. It is possible that the PLLs interact differently with tissue cells than with erythrocytes, however further experimentation into the mechanics of cell death caused by PLLs would be needed to provide a definitive reason for the observed results.

Cytotoxicity of BPEIs and LPEIs

In general, the LPEIs are more toxic than the BPEIs, although both are less toxic than PLLs. From the LDH assay, EC_{50} = 250 μg/mL for both LPEIs while EC_{50} = 4000 μg/mL for all BPEIs. In this assay, molecular weight did not affect cytotoxicity. These results mirror those from the hemolysis assay, where LPEIs were found to be hemolytic but BPEIs were not. Interestingly, even though LPEIs exhibited the greatest hemolytic activity, PLLs are the most toxic by the LDH assay. Again, this may be due to differences in the epithelial and erythrocyte cell compositions. The XTT assay showed a strong cytotoxic dependence on weight. As molecular weight increased for BPEIs and LPEIs, EC_{50} decreased from 4000 μg/mL to 26 μg/mL (BPEIs) and 153μg/mL to 70 μg/mL (LPEIs). After 24 hrs, the polymers' EC_{50} values decreased, indicating that they are more toxic as time increases. In contrast to the PLLs, these polymers employ a time-dependent cell death mechanism.

Liposome Experiment

Biological assays characterize polymers' antimicrobial properties. These results are vital to determine how a polymer will interact in a biological system and if its antibacterial properties outweigh its adverse effects on a living organism. In some cases, the assays can imply mechanisms by which the polymers interact with other cells, but other experimental methods are needed to truly explain the observed results from the assays. PEIs and PLLs had been shown to cause pore formation in supported DMPC bilayers, but further experimentation was needed to extend this model of polymer-lipid interaction to biological structures [18]. To determine these polymers' antibacterial mechanisms, POPC and PE/PG liposomes were employed to model both human and bacterial cells.

In the liposome experiment, POPC liposomes mimic eukaryotic cell membranes while PE/PG liposomes mimic Gram-negative (*E. coli*) bacterial membranes [16]. Gram-positive bacteria (*S. aureus*) were not modeled in this experiment. When filled with a dye (in this case, the fluorescent sulforhodamine B), the liposomes provide a crude approximation of living cells. If the antibacterial polymers employ a mechanism by which they rupture or perforate the cell membrane, then the model liposome membrane will break, allowing dye to leak into the buffer solution. Fluorescence spectroscopy can then measure the dye leakage. The surfactant Triton X-100 was used to obtain 100% dye leakage so that the percentage of the dye leakage caused by the polymers could be calculated.

This experiment had several problems to correct before valid data could be collected. In the original procedure, 50 μL of 1% TX-100 was added to the samples to achieve 100% dye leakage.

The amount of TX-100 had been optimized in previous experiments. When the experiment was first attempted, however, it was observed that TX-100 at that concentration quenched fluorescence. The surfactant concentration then had to be optimized again for this experiment, which used a buffer with a slightly higher salt concentration. To function correctly, the surfactant concentration had to be high enough to rupture all the liposomes, yet low enough to not interfere with the fluorescence measurements. It was found that 1% TX-100 (POPC) or 1.25% TX-100 (PE/PG) was the optimal concentration, yet subsequent experimentation still showed some reduced quenching effects. Eliminating the fluorescence quenching in this experiment has not yet been accomplished. Possibly other factors are responsible for the quenching, such as the lipid membranes or the polymers themselves, but these causes were not identified before the end of the project.

Reproducibility was another problem in this experiment. It was extremely difficult to get several sets of data with comparable results. To determine if the polymers themselves were responsible for quenching at the newly optimized surfactant concentration, a solution of sulforhodamine B in TBS at the same concentration as in the liposome stock solution was prepared. This stock solution was then used to conduct the liposome experiment as before. To determine if liposome aggregation was influencing the fluorescence measurements, the liposome experiment was repeated for POPC and PE/PG stock solutions without the polymers. An additional experiment was performed where POPC and PE/PG stock solutions were frozen to destroy the liposomes and release the dye before the addition of the polymers. It was hoped that this procedure would determine the magnitude of fluorescence quenching (if any) by the TX-100 in the presence of the liposomes. Ultimately, the results from these experiments did not show agreement between

separate trials, nor within the same trial. Repeating the liposome experiment twice with the optimized TX-100 concentration also did not show acceptable agreement in results. The reagent preparation or the procedure itself could be at fault, but as before, these problems could not be resolved by the end of the project.

Polymer-POPC Interaction

Although the collected data may not be completely accurate, it still gave some insight into the polymers' behavior. Most polymers caused little to no dye leakage in the POPC liposomes (fig. 11). PLL MW 40000-60000 Da appears to cause significant dye leakage, which could explain its hemolytic properties, but the same trend is not seen in the LPEIs. As eukaryotic cellular membranes contain POPC lipids, these results indicate that the polymers do not cause human tissue cell death by breaking the cellular membrane. The cell death as observed by the XTT assays, therefore, may be necrosis caused by internal damage to the cell or may in fact be apoptotic in nature due to inference with the cell's DNA. These results must be reconciled with the data from the LDH and hemolysis assays which imply that these polymers, specifically the PLLs, induce necrosis by membrane damage.

Polymer-PE/PG Interaction

It was found that the polymers caused significant dye leakage in the PE/PG liposomes (fig. 12). Although the lack of reproducibility limits the conclusions that can be made from this experiment, several characteristics should be noted about the features of the leakage profiles. In all trials, the leakage curves align based on molecular weight, regardless of the polymer. Phase transitions for polymers with molecular weights in the same order of magnitude occur at similar

concentrations. This phenomenon was seen with other bioactive polymers used in the lab as well. The sigmoidal portion of the leakage profile for PLL MW 100000 – 140000 Da (the highest molecular weight among all polymers) occurs at the lowest concentration (0.001 μM). Polymers in the 10000 – 60000 Da range show activity in the next lowest concentration range (0.01 μM) followed by those in the 1500 – 8000 Da range (1.0 μM) and then BPEI MW 600 (1.0 μM). The dye leakage for high molecular weight PLLs and BPEI curiously decreases at high concentrations. The decreased membrane rupture may be due to polymer aggregation at high concentrations, which limits the available number of molecules that can interact with the membrane, however this theory is untested. Quenching may also be responsible for the observed decrease in dye leakage at high polymer concentrations.

In relation to antimicrobial activity, the liposome experiment implies that necrosis in Gram-negative bacteria after polymer exposure is the result of perforation in the cellular membrane. Unfortunately, it was not possible to accurately correlate molecular weight or polymer concentration to the magnitude of membrane damage for the reasons stated above. Regardless, being able to observe membrane activity in the PE/PG liposomes and not the POPC liposomes was an important step in determining a definitive mechanism for cell death in both bacteria and tissue cells.

Prospective Experiments

Further research in this project would focus on optimizing the liposome experiments. As a simplified model for human and bacterial cells, liposomes are a convenient method for studying polymer-membrane interactions. Accurate biophysical data is therefore important in determining

mechanisms. Imaging was largely ignored in these experiments due to time constraints and equipment. Observing the polymers as they interact with cells, however, would be immensely helpful in modeling antimicrobial polymers. If the polymer were only found on the surface of the membrane, necrosis would be the most likely cause of cell death. On the other hand, if the polymer were seen inside the cell, cell death may result from an apoptotic mechanism. Thus labeling polymers and obtaining high-resolution images of their exposure to cellular membranes would be a sensible next step.

Summary

The amphiphilic polymers branched polyethylenimine, linear polyethylenimine and poly-L-lysine were selected as possible antimicrobial compounds. Their hemolytic activity, cytotoxicity, and antibacterial properties were determined to characterize the polymers' viability as novel antibiotics. Hypotheses were formed about the relationship between the polymers' structures and their observed antimicrobial properties, however there was not enough data to definitively relate polymer structure to function.

All polymers showed some antibacterial activity against both Gram-positive and Gram-negative bacteria; LPEIs and PLLs were more potent antibacterial agents than BPEIs. *S. aureus* (Gram-positive) showed a greater sensitivity towards the polymers than *E. coli*, which may be related to the differences in the bacterial cell membranes or the bacteria strains' growth rates. Only LPEIs were hemolytic, which implies that they are capable of rupturing cellular membranes. PLLs were appreciably toxic to human epithelial cells in both the LDH and XTT assays, and caused

necrosis quickly and completely. Their toxicity increased with increasing molecular weight. Cytotoxicity in the BPEIs and LPEIs was not related to molecular weight in the LDH assay, but was positively dependent on molecular weight in the XTT assay. Additionally, cytotoxicity increased for these polymers after 24 hrs. LPEIs were in general more toxic to tissue cells than the BPEIs.

These assays present conflicting theories as to the polymers' antimicrobial mechanisms. The hemolysis assay implies that only LPEIs perforate cell membranes, which would therefore cause necrotic cell death. PLLs, however, were the most toxic in the LDH assay – a measure of membrane integrity – therefore they should also rupture cell membranes. Based on the MICs and EC_{50} in the LDH and XTT assays for BPEIs, these types of polymers employ an apoptotic mechanism that results in bacterial cell death.

Liposomes of POPC and PE/PG phospholipids were synthesized to clarify the mechanics of the polymers' cell death mechanisms. Liposome membrane integrity after exposure to a polymer was determined by fluorescence spectroscopy. Very little membrane perforation was observed in the POPC liposomes. As these liposomes model eukaryotic cells, the results imply that cell death in human tissue cells is more apoptotic in nature and not the result of a ruptured membrane. All polymers caused significant dye leakage in the PE/PE liposomes, however. According to this model, necrosis in bacterial cells is caused by burst membranes.

Compiling the results from several biological assays and preliminary biophysical experimentation could not provide a definitive relationship between a polymer's bioactivity and

structure, nor could it define the interaction between the polymers and the cell membranes. The polymers' interactions with biological structures are complex and not easily ascertained. With subsequent experimentation in this field, hopefully a coherent model for antibacterial activity will be constructed so as to utilize amphiphilic polymers' potential as novel bioactive compounds.

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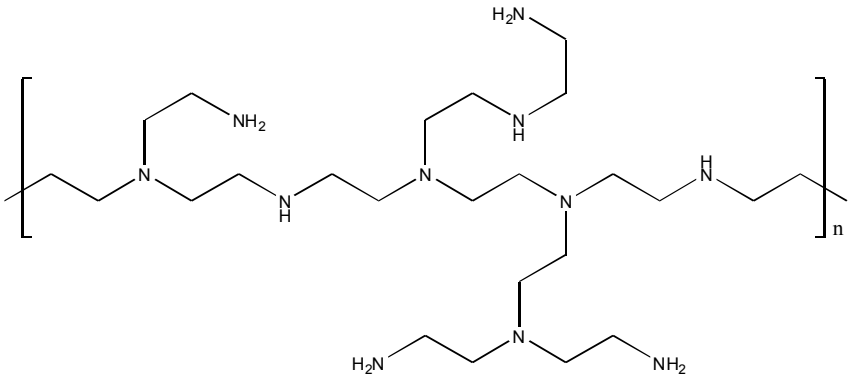
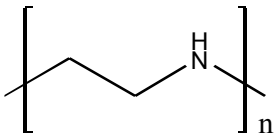
Appendix I

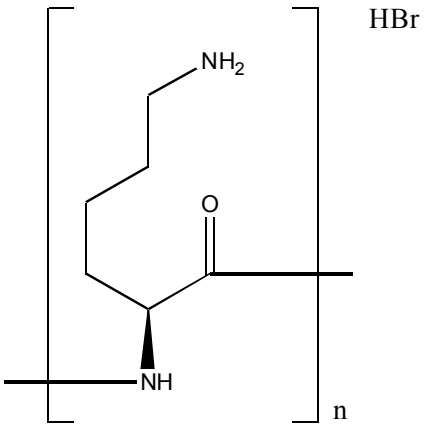
Structures



(a)



(b)



(c)

Figure 1. Amphiphilic polymer structures: **(a)** branched polyethylenimine, 600-10000 Da; **(b)** linear polyethylenimine, 2500-25000 Da; **(c)** poly-L-lysine hydrobromide, 1500-140000 Da.

Appendix II

Hemolysis Assay

Table 1. HC_{50} of BPEIs, LPEIs, and PLLs.

Polymer	Molecular Weight [Da]	HC_{50} [µg/mL]
BPEI	600	>4000
BPEI	1800	>4000
BPEI	10000	>4000
LPEI	2500	576.5
LPEI	25000	195.3
PLL	1500-8000	>4000
PLL	40000-60000	3000
PLL	100000-140000	>4000

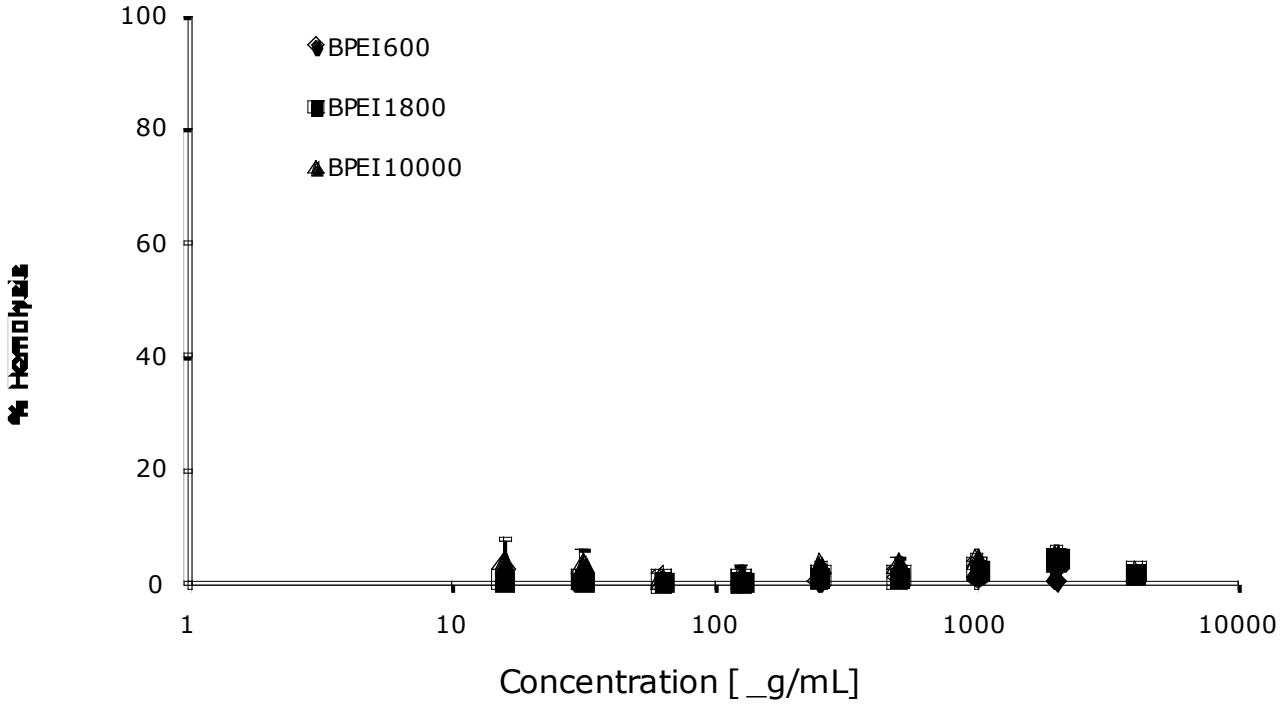


Figure 2. Percent hemolysis of erythrocytes induced by BPEIs.

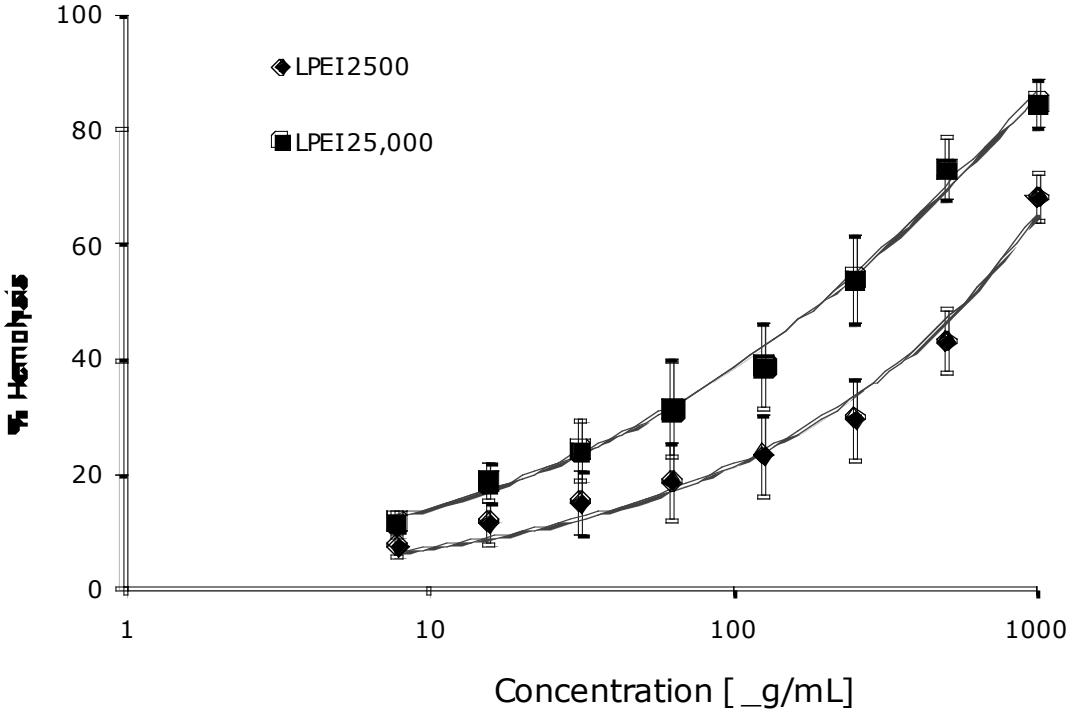


Figure 3. Percent hemolysis of erythrocytes induced by LPEIs.

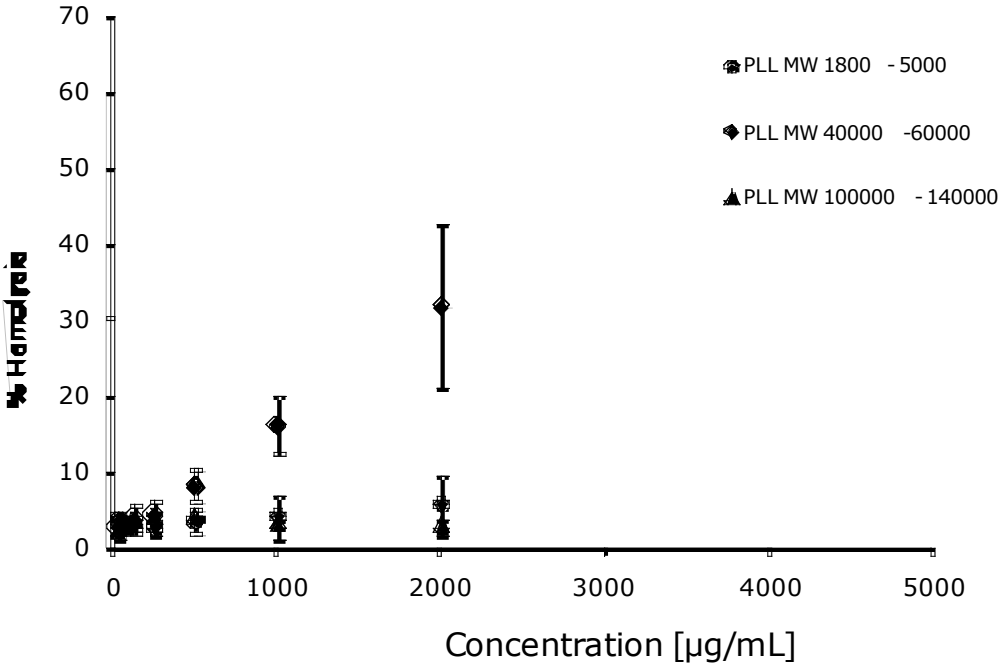


Figure 4. Percent hemolysis of erythrocytes induced by PLLs.

Appendix III

Antimicrobial Assays: Minimum Inhibitory Concentration; Minimum Bactericidal Concentration (MIC; MBC)

A) *E. coli*

Table 2. MICs and MBCs of BPEIs, LPEIs, and PLLs against *E. coli* ATCC 25922.

Polymer	Molecular Weight [Da]	MIC [µg/mL]	MBC [µg/mL]
BPEI	600	500	500
BPEI	1800	250	250
BPEI	10000	>1000	>1000
LPEI	2500	31.25	31.25
LPEI	25000	31.25	31.25
PLL	1500-8000	125	125
PLL	40000-60000	31.25	250
PLL	100000-140000	62.5	62.5

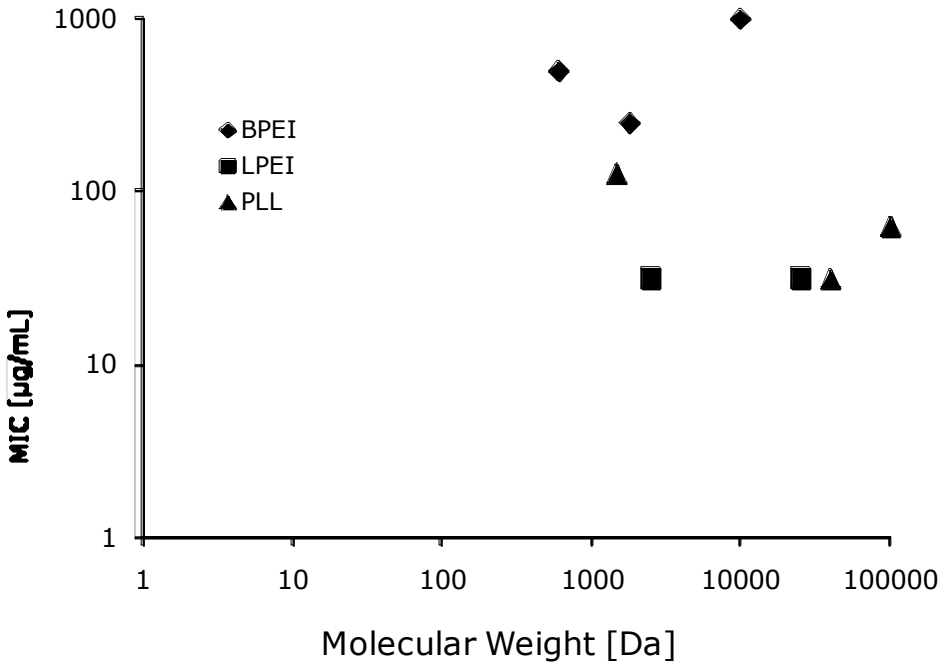


Figure 5. MICs of BPEIs, LPEIs, and PLLs against Gram-negative *E. coli*.

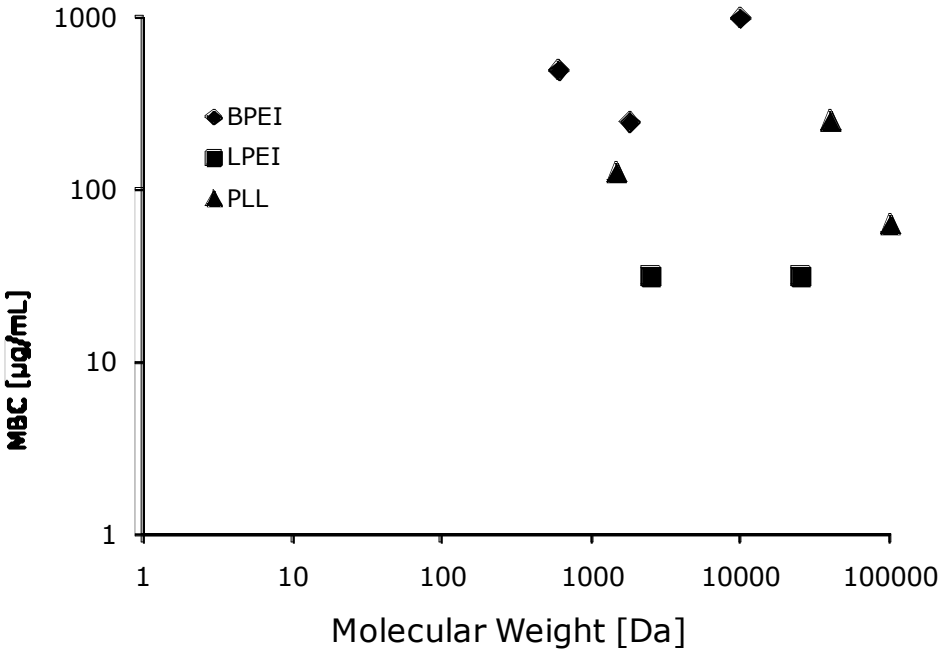


Figure 6. MBCs of BPEIs, LPEIs, and PLLs against Gram-negative *E. coli.*

B) *S. aureus*

Table 3. MICs and MBCs of BPEIs, LPEIs, and PLLs against *S. aureus* ATCC 25923.

Polymer	Molecular Weight (Da)	MIC [µg/mL]	MBC [µg/mL]
BPEI	600	15.63-31.25	62.5
BPEI	1800	<7.81	31.25
BPEI	10000	15.63	31.25
LPEI	2500	7.81-15.63	7.81
LPEI	25000	7.81-15.63	7.81
PLL	1500-8000	15.63	31.25
PLL	40000-60000	15.63	31.25
PLL	100000-140000	15.63	31.25

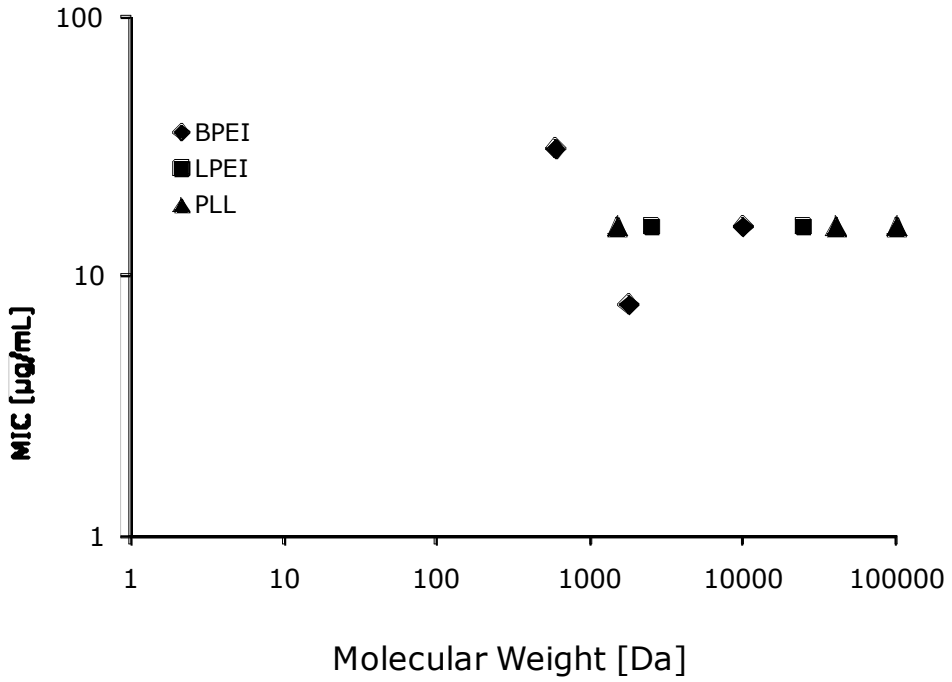


Figure 7. MICs of BPEIs, LPEIs, and PLLs against Gram-positive *S. aureus.*

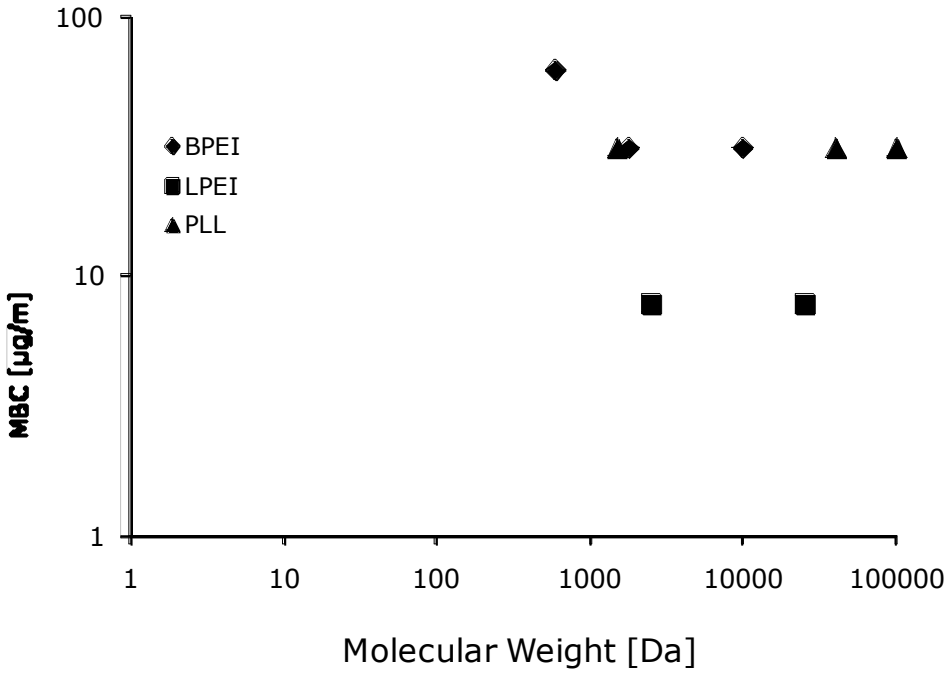


Figure 8. MBCs of BPEIs, LPEIs, and PLLs against Gram-positive *S. aureus.*

Appendix IV

Cytotoxicity Assays: Lactate Dehydrogenase (LDH), XTT

A) LDH

Table 4. EC_{50} of BPEIs, LPEIs and PLLs for human epithelial HEp-2 cells by the LDH assay.

Polymer	Molecular Weight [Da]	EC_{50} [µg/mL]
BPEI	600	>4000
BPEI	1800	>4000
BPEI	10000	>4000
LPEI	2500	>250
LPEI	25000	>250
PLL	1500-8000	37
PLL	40000-60000	36
PLL	100000-140000	20

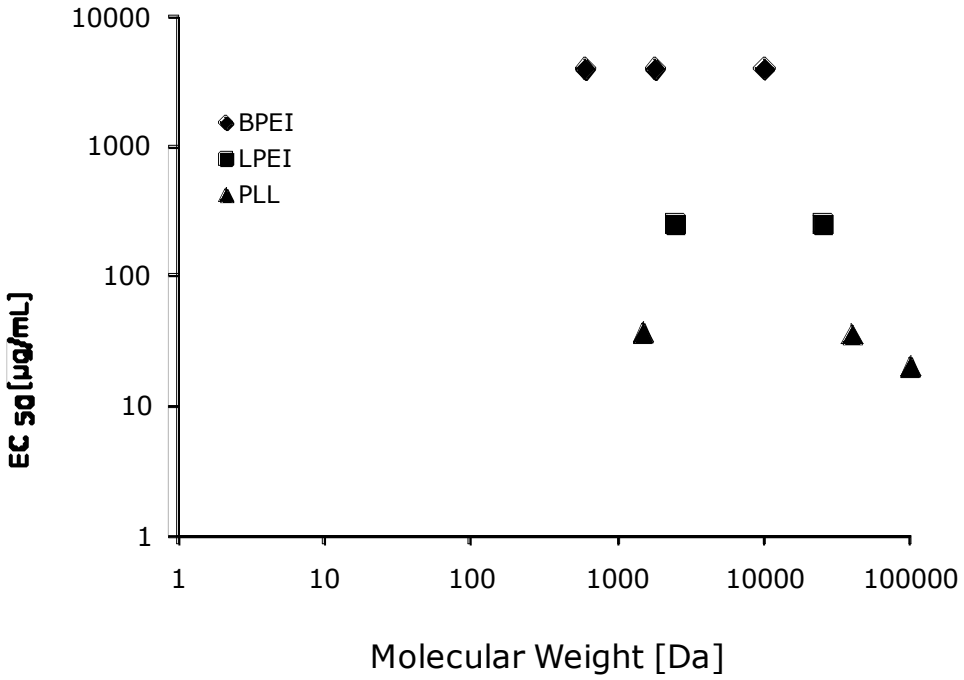


Figure 9. EC_{50} vs. molecular weight for BPEIs, LPEIs, and PLLs as determined by the conversion of tetrazolium salt (INT) into formazan by extracelluar LDH.

B) XTT

Table 5. EC_{50} of BPEIs, LPEIs and PLLs for human epithelial HEp-2 cells by the XTT assay.

Polymer	Molecular Weight (Da)	EC_{50} [µg/mL] (1 hr)	EC_{50} [µg/mL] (24 h)
BPEI	600	>4000	2293
BPEI	1800	1010	116
BPEI	10000	26	7
LPEI	2500	153	13
LPEI	25000	70	8
PLL	1500-8000	19	17
PLL	40000-60000	14	14
PLL	100000-140000	7	10

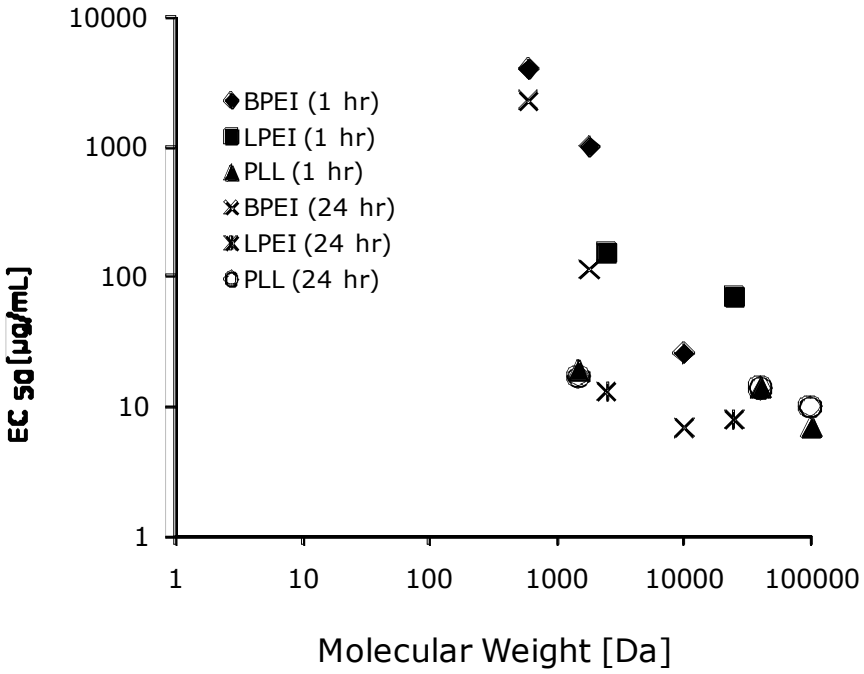


Figure 10. EC_{50} vs. molecular weight for BPEIs, LPEIs, and PLLs as determined by the amount of the metabolized formazan.

Appendix V

Liposome Experiment

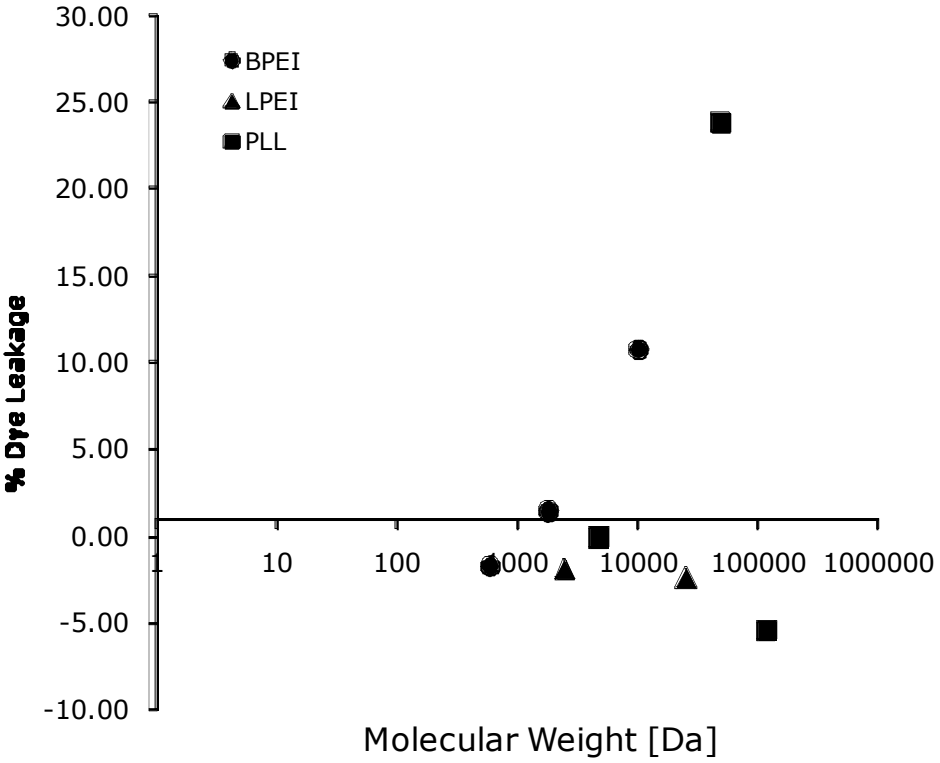


Figure 11. Polymer-induced dye leakage in POPC liposomes.

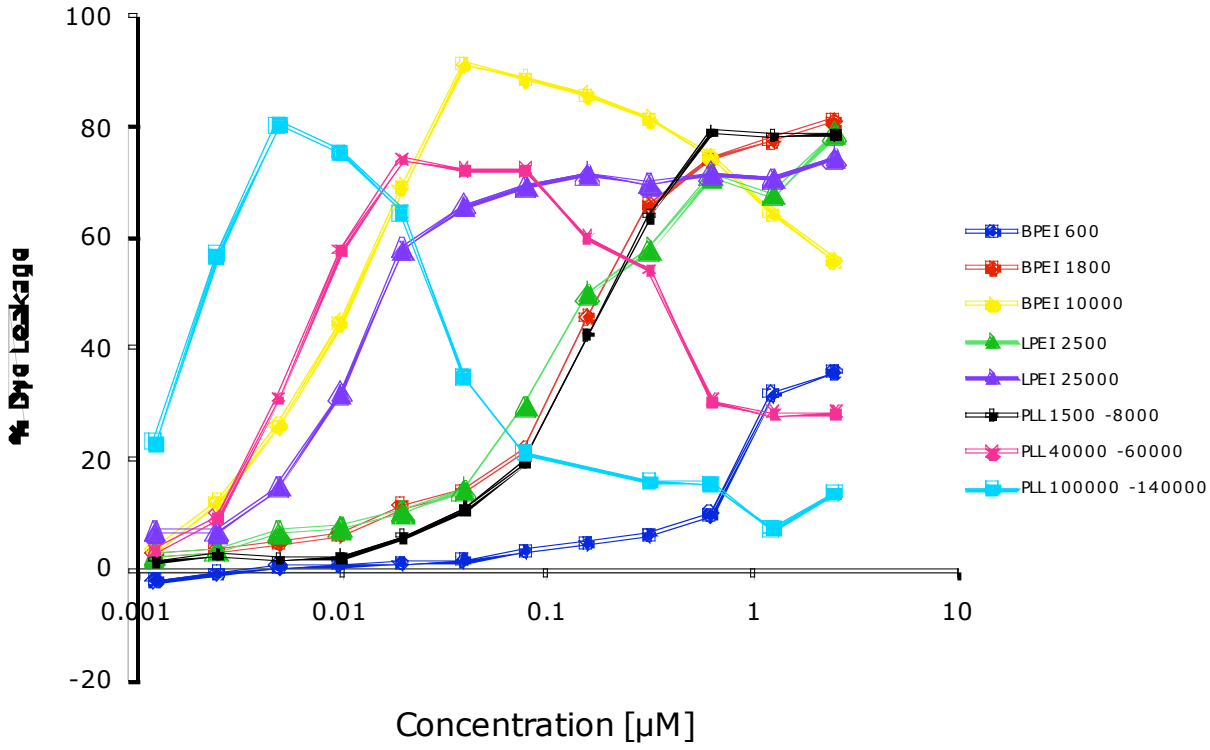


Figure 12. Polymer-induced dye leakage in PE/PG liposomes.