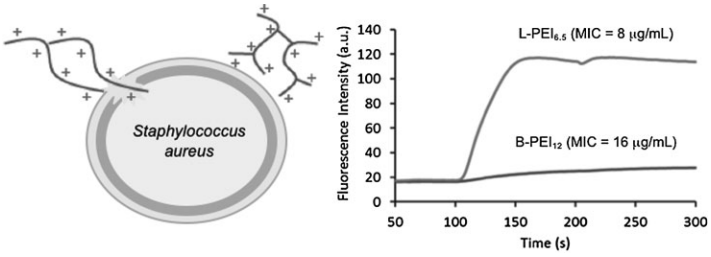
Poly(ethylene imine)s as Antimicrobial Agents with Selective Activity[a]

Katherine A. Gibney, Iva Sovadinova, Analette I. Lopez, Michael Urban, Zachary Ridgway, Gregory A. Caputo,* Kenichi Kuroda*

We report the structure–activity relationship in the antimicrobial activity of linear and branched poly(ethylene imine)s (L- and B-PEIs) with a range of molecular weights (MWs) (500–12 000). Both L- and B-PEIs displayed enhanced activity against *Staphylococcus aureus* over *Escherichia coli*. Both B- and L-PEIs did not cause any significant permeabilization of *E. coli* cytoplasmic membrane. L-PEIs induced depolarization of *S. aureus* membrane although B-PEIs did not. The low MW B-PEIs caused little or no hemolysis while L-PEIs are hemolytic. The low MW B-PEIs are less cytotoxic to human HEp-2 cells than other PEIs. However, they induced significant cell viability reduction after 24 h incubation. The results presented here highlight the interplay between polymer size and structure on activity.



1. Introduction

In recent years, antibiotic-resistant bacterial infections have become a growing concern.[1–3] With the threat of traditional antibiotics and antimicrobial agents becoming obsolete, the development of new classes of antibiotics has become an important subject of current research. Naturally occurring host defense peptides have shown potential as alternatives to the currently available antibiotics, but bioavailability complications and the high cost of production have limited their translation into the clinic. The cationic amphiphilic structure of natural antibacterial peptides has been recognized as the key properties of the peptide antibacterial mechanism of action.[4–6] The cationic groups of peptides facilitate the electrostatic binding of peptides to bacterial cell surfaces with high net negative charge, which leads to the selective attraction of the more negatively charged bacteria over human cells. The amphiphilic structure of the peptides drives the insertion of their hydrophobic side-chains into the lipid membrane, which disrupts the membrane integrity, causing leakage of cellular components, breakdown of membrane potential, and cell death. These naturally occurring amphiphilic biopolymers serve as models for the design, behavior, and mechanism of action of synthetic, cationic amphiphilic polymers.[7–9]

Traditional cationic poly(ethylene imine) polymers (PEIs) have been utilized as drug carriers in biomedical application because of their ability to enter cells or permeabilize cell membranes.[10–13] Linear and branched PEIs (L-PEIs and

Prof. K. Kuroda, K. A. Gibney
Department of Chemistry, University of Michigan, Michigan, 48109 USA
E-mail: kkuroda@umich.edu
Prof. G. A. Caputo, M. Urban, Z. Ridgway
Department of Chemistry and Biochemistry, Rowan University, New Jersey, 08028 USA
E-mail: caputo@rowan.edu
Prof. K. Kuroda, Dr. I. Sovadinova, Dr. A. I. Lopez
Department of Biologic and Materials Sciences, University of Michigan School of Dentistry, Michigan, 48109 USA

[a] **Supporting Information** for this article is available from the Wiley Online Library or from the author.

DOI: 10.1002/mabi.201200052

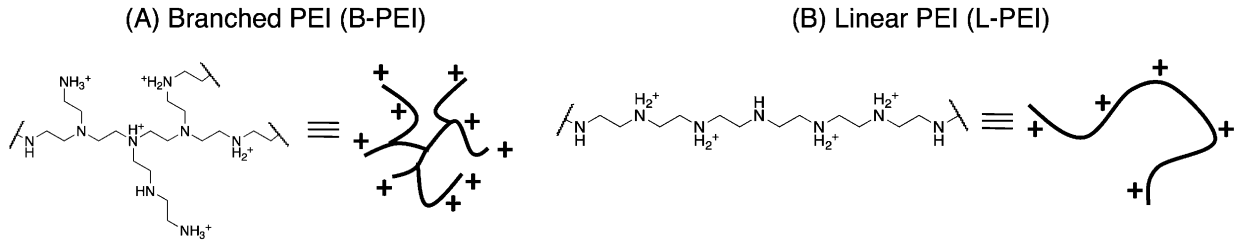


Figure 1. Cationic amphiphilic structures and schematic presentations of PEIs: (A) B-PEI and (B) L-PEI.

B-PEIs) (Figure 1) have shown potential as possible non-viral vector systems for drug transport across cell membranes.[14–16] In addition, a large number of studies have focused on the antibacterial activity of water-soluble PEI derivatives containing quaternized ammonium salt groups with long alkyl or aromatic groups[17,18] and applications for water-insoluble hydrophobic PEIs including nanoparticles,[19] and antibacterial coatings.[20,21] While the properties of PEI derivatives have been extensively studied, to the best of our knowledge, a systematic investigation of the antimicrobial activity and cytotoxicity of conventional unmodified PEIs is still limited.

The design strategy of modeling polymers on natural cationic peptides has been projected onto numerous synthetic polymers with cationic amphiphilic properties. The protonated ammonium groups of the PEIs are cationic, and the non-protonated amine groups and ethylene backbone serve as hydrophobic groups, which create repeating cationic amphiphilic structures along the polymer backbone at neutral pH without any further chemical modification by hydrophobic groups (Figure 1). Accordingly, we hypothesized that unmodified PEI provided the necessary cationic amphiphilic structures needed to induce membrane disruption or permeabilization, leading to antibacterial activity. To test this hypothesis, we investigated a series of L-PEIs and B-PEIs, which differ in their amine components as well as molecular weights, for antibacterial activity and antimicrobial mechanism. Herein, we report the structure–activity relationship for antimicrobial activity as well as toxicity to human red blood cells (RBCs hemolysis) and human epithelial carcinoma HEp-2 cells for these PEI molecules. In addition, we investigated the polymer-induced permeabilization of bacterial cell membranes of *Escherichia coli* and *Staphylococcus aureus* to gain insights into the mechanism of action.

2. Experimental Section

2.1. Materials and General Methods

Branched PEIs (B-PEIs); MW = 600 (cat.: 02371, lot: 558322), 1 800 (cat.: 06089, lot: 559792), 10 000 (cat.: 19850, lot 579738), L-PEIs; MW = 2 500 (cat.: 24313, lot: 587821), 25 000 (cat.: 23966, lot: 590965) were purchased from Polysciences, Inc. (Warrington, PA, USA). ^{1}H NMR and ^{13}C NMR spectra were recorded in a Varian MR400 NMR spectrometer at 400 and 100 MHz, respectively. See Supporting Information for detailed procedures of gel permeation chromatography (GPC), reverse-phase HPLC, dynamic light scattering, potentiometric titration, and biological assays.

3. Results and Discussion

3.1. GPC Characterization

As an initial step in our investigation into the biological activities of PEIs, we first characterized their molecular structures and properties. We chose commercially available PEIs because of their availability in a large quantities and widespread use in a broad range of applications ranging from biological transfection reagents to wet-strength resins in the paper industry.[16,22,23] Despite the broad application and plentiful availability of these molecules, these commercially available preparations lacked thorough investigation into both their chemical properties and, more importantly, their potential as antimicrobial agents. In order to fully understand any relationship between the molecular composition, structural architecture, and antimicrobial activity, an important benchmark was to completely characterize the PEI molecule preparations for testing. The molecular weight of polymers was determined by GPC (Table 1 and Figure S1 of Supporting Information for GPC elution curves). The PEIs are denoted as L/B-PEI$_x$ (L: linear, B: branched, x: number-averaged molecular weight determined by GPC in kDa). The B-PEIs used in this study have a range of molecular weights ($\overline{M}_n$ = 470–12 000). While B-PEI$_{0.5}$ has a broad MW distribution ($\overline{M}_w/\overline{M}_n = 4.5$), the higher MW B-PEIs have relatively low polydispersity ($\overline{M}_n/\overline{M}_w < 1.6$). Regarding L-PEIs, the both L-PEIs have similar $\overline{M}_n$, although the MWs reported by a supplier have tenfold difference (MW = 2 500 and 25 000), and the higher MW L-PEI$_{6.5}$ has a broader MW distribution. We used relatively low MW PEIs in this study since high MW cationic polymers have been shown to cause undesired cytotoxicity.[24,25] As such, we expected that low MW PEIs were likely to be less toxic while still exhibiting antibacterial behavior.

Table 1. Characterization of PEIs.

PEIs[a)]	Polymer structure	MW[b)]	$\overline{M}_n$ (GPC)	$\overline{M}_w$ (GPC)	$\overline{M}_w/\overline{M}_n$	RT [min][c)]
B-PEI$_{0.5}$	Branched	600	470	2 100	4.5	–[d)]
B-PEI$_{1.1}$	Branched	1 800	1 100	1 400	1.3	7.0
B-PEI$_{12}$	Branched	10 000	12 000	19 000	1.6	8.0
L-PEI$_{4.4}$	Linear	2 500	4 400	7 900	1.8	7.9
L-PEI$_{6.5}$	Linear	25 000	6 500	13 000	2.0	8.0

[a)]See the text for denotation; [b)]MW reported by a supplier; [c)]RT in reverse-phase HPLC; [d)]no distinctive peak was observed.

3.2. NMR Characterization

^{1}H and ^{13}C NMR confirmed that B-PEIs contain primary, secondary, and tertiary amine groups (Figure S2 and S3 of Supporting Information). The NMR spectra showed distinctive peaks from protons and carbons of methylene groups depending on adjacent different amine groups. The peaks of the ^{13}C NMR spectra appear to be sufficiently separated for integration to determine the ratio of amine groups. Integrated peaks of ^{13}C NMR spectra of B-PEIs have been used to determine the amine ratio of B-PEIs in literature.[26,27] However, the ^{13}C NMR spectra in Figure S3 of Supporting Information may not be sufficiently accurate for comparing integrated areas because the data acquisition and NMR parameters were not optimized. Quantitative analysis would require more time-intensive data acquisition and further optimization of NMR parameters, which would be beyond the scope of this study. Therefore, we carried out deconvolution analysis of overlapped peaks in the ^{1}H NMR spectra (Supporting Information) to estimate the ratio of amine groups (Table 2). Although the ratios of different amines do not exactly match the theoretical ratio (primary, secondary, and tertiary amine groups = 25/50/25% of each group per chain), the similar trend was found. The ratio of secondary amines (linear chain) to tertiary amines (branching point) increased as the PEI molecular weight increased. This indicates that the PEIs with higher MWs have more branching structures.

On the other hand, the ^{1}H NMR spectra of L-PEIs showed a single peak from the ethyleneimine unit with secondary amines,[28] indicating a linear polymer structure (Figure S2 of Supporting Information). The spectra also showed peaks from residual *N*-propionyl groups (Figure 2),[28,29] and analysis of integrated areas indicated that L-PEI$_{4.4}$ and L-PEI$_{6.5}$ contain 10.6 and 3.8 mol% of *N*-propionyl groups relative to the total number of repeating units, respectively (Supporting Information). In general, L-PEIs are synthesized by ring-opening isomerization polymerization of 2-ethyl-2-oxazoline (Figure 2) and the subsequent acid hydrolysis to remove *N*-propionyl groups. The removal of *N*-propionyl groups was evidently not complete for these commercially available L-PEIs.

Table 2. Amine ratio and apparent pK_a of B-PEIs.

PEIs	Amine [%]				pK_{a1}[a)]	pK_{a2}[a)]
	1°	2°	3°	2°/3°		
B-PEI$_{0.5}$	33	42	24	1.75	9.4	6.2
B-PEI$_{1.1}$	25	46	29	1.58	9.6	6.2
B-PEI$_{12}$	28	40	32	1.25	9.0	5.8
Ethanolamine	100	–	–	–	9.8	–

[a)]pK_a is reported as the pH to give the maximum buffer capacity. The pK_a value is an average from two experiments. The range of two data points was smaller than 4% relative to the average values (Supporting Information).

3.3. Potentiometric Titration

To assess the cationic functionality of the PEIs, their ionization behavior was examined by acid titration (Figure 3). All the B-PEIs tested in this study (oily liquid) are completely miscible with water or saline (150 mm NaCl). The titration curves showed multiple shoulders, indicating the different buffer effects depending on the amine structures (Figure 3A). To examine the buffer effect of B-PEIs, their buffer capacity [β = dc(HCl)/dpH, where c(HCl) is added HCl concentration], was approximated by the inverse slope of the adjacent two data points in the titration curves. The plots showed two peaks around pH 9–10 and 6–7 (Figure 3B), indicating that the B-PEIs have buffer

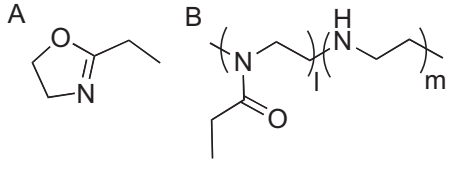


Figure 2. Chemical structure of L-PEIs: (A) 2-ethyl-2-oxazoline and (B) L-PEI with *N*-propionyl groups.

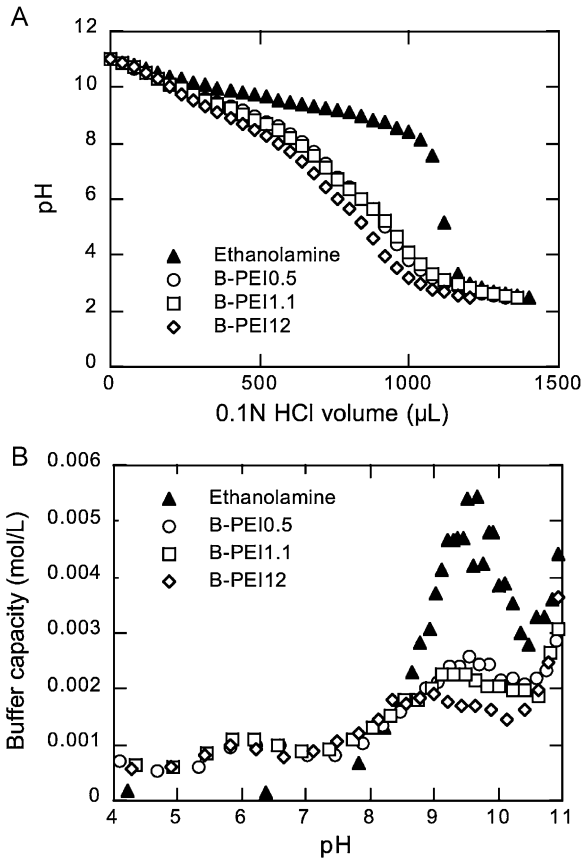


Figure 3. Potentiometric titration of B-PEIs: (A) representative pH titration curves and (B) buffer capacity.

effects in these pH regions. The former peak is more distinctive, likely corresponding to primary/secondary amines with strong basicity, and the latter is due to tertiary amines.[16,26,30] The apparent pK_a was determined as the pH value to give the peak top value of β (Table 2). As a control, the pK_a of ethanolamine was 9.8, which is in close agreement with the value (9.5) reported in literature.[31] Because the pK_{a1} is greater than 9, most primary and secondary amine groups are protonated under the assay conditions of pH 7. The pK_{a2} values for all B-PEIs are around 6–6.4, suggesting that a large fraction of tertiary amine groups are not protonated at pH 7. Combining the results of NMR analysis, approximately 72, 71, and 68 mol% of total amine groups of B-PEI$_{0.5}$, B-PEI$_{1.1}$, and B-PEI$_{12}$ are cationic at the assay pH, respectively. On the other hand, L-PEIs are not readily soluble in water at mg · mL^{-1} concentrations and caused turbidity in solution during titration. Therefore, titration curves and buffer capacities could not be determined; otherwise, the analysis would not be quantitative.

3.4. Reverse-phase HPLC

To further assess the amphiphilic properties of PEIs, the hydrophobicity of PEIs was examined by reverse-phase HPLC (Figure S5 of Supporting Information). The retention time (RT) measured by HPLC reflects the inherent hydrophobicity of the polymers at low pH where all amines are presumably protonated. The overall hydrophobicity of PEIs would therefore be dependent on the degree of protonation of amine groups and may be different under neutral pH assay conditions. Nonetheless, differences in RTs are a good gauge of inherent hydrophobicity differences under the same set of sample conditions. The RT of B-PEI$_{12}$ (7.7 min) is greater than that of B-PEI$_{1.1}$ (7.0 min) (Table 1), indicating B-PEI$_{12}$ is somewhat more hydrophobic than B-PEI$_{1.1}$. The greater relative hydrophobicity of B-PEI$_{12}$ corroborates the low basicity found in the titration, that is, the molecule has a greater fraction of uncharged groups at the neutral pH or lower pK_a value than other B-PEIs. The hydrophobic environment of B-PEI$_{12}$ likely disfavors the protonation of amine groups, contributing to factors for the lower pK_a value of B-PEI$_{12}$ (Table 2). Combining with the titration results, this indicates that the B-PEIs have cationic amphiphilic structures in water. Both L-PEIs showed similar RT of ≈8.0 min at low pH where L-PEIs are soluble although L-PEI$_{4.4}$ showed a broader peak in the elution curve compared to others.

3.5. Antimicrobial Activity

To examine the antimicrobial activities of these PEIs, we measured the minimum inhibitory concentration (MIC), which is the minimum polymer concentration necessary for completely inhibiting bacterial growth under standard assay conditions (Table 3). The B-PEIs are completely miscible with water; however, the L-PEIs are not readily soluble in water at mg · mL^{-1} concentrations. Instead, the L-PEIs were found to be readily soluble in ethanol. Therefore, different procedures were used when preparing the B-PEI and L-PEI solutions for biological assays. The L-PEIs were first dissolved in ethanol and serially diluted twofold with 0.01% acetic acid to give assay stock solutions with a range of polymer concentrations while B-PEI stock solutions were prepared by only tris-buffered saline (TBS, 10 mM Tris buffer, 150 mM NaCl, pH = 7.3). These stock solutions were mixed with bacterial solution in Mueller-Hinton (MH) broth for MIC determination. The highest ethanol concentration in the assay solution was 5%, and the control experiments using solvents (TBS, 0.01% acetic acid, and acetic acid/ethanol mixtures) showed no difference in the bacterial growth after the 18-h incubation as determined by the turbidity or OD$_{600}$.

Among the B-PEIs examined, B-PEI$_{1.1}$ displayed the lowest MIC value (250 μg · mL^{-1}) against *E. coli* (Gram-negative), while B-PEI$_{12}$ displayed no activity (MIC > 1 000 μg · mL^{-1}), indicating that increasing MW does not enhance antibacterial activity. For comparison, the MIC of the natural antimicrobial peptide magainin 2 (MW = 2 300)

Table 3. Antimicrobial and hemolytic activities of PEIs and peptides.

Polymers	MIC [$\mu g \cdot mL^{-1}$]		$MIC_{E.\ c.}/MIC_{S.\ a.}$ [a)]	HC_{50} [$\mu g \cdot mL^{-1}$] [b)]	HC_{50}/MIC	
	E. c.	*S. a.*			*E. c.*	*S. a.*
B-$PEI_{0.5}$	500	16	32	>4 000 (1%) [c)]	>4	>64
B-$PEI_{1.1}$	250	32	8	>4 000 (2%) [c)]	>8	>256
B-PEI_{12}	>1 000	16	>64	>4 000 (2%) [c)]	–	128
L-$PEI_{4.4}$	31	8	4	565 ± 104	18	37
L-$PEI_{6.5}$	31	8	4	163 ± 23	6	12
Magainin-2	125 [d)]	>500 [d)]	<0.3	>250 (9%) [c),d)]	>2	–
Melittin	13 [d)]	6 [d)]	2	2 [d)] ± 0.1	0.2	0.3

[a)]The ratio of MIC for *E. coli* (*E. c.*) to MIC for *S. aureus* (*S. a.*); [b)]polymer concentration for 50% hemolysis. The presented data and error are average and standard deviation from at least three independent experiments in triplicate; [c)]the hemolysis percentage at the highest polymer concentration is given if the HC_{50} was not determined; [d)]the data were previously reported.[56]

is 125 $\mu g \cdot mL^{-1}$ under the same assay conditions. The titration results indicated that B-PEI_{12} has 68 mol% of cationic ammonium groups, slightly lower than other B-PEIs, and the RP-HPLC results showed the intrinsic hydrophobicity of B-PEI_{12} is higher than B-$PEI_{1.1}$. Although it has been previously reported that increasing the hydrophobicity of cationic amphiphilic PEIs[18] and poly(propylene imine) dendrimers,[32,33] increases their antimicrobial activity, PEI_{12} showed lower activity against *E. coli* as compared to other PEIs. On the other hand, both L-PEIs exhibited MIC values of 31 $\mu g \cdot mL^{-1}$ against *E. coli* possibly due to the similar molecular weight ($\overline{M}_n$ = 4 400 and 6 500). Additionally, the MICs for the L-PEIs are eight times lower than those for the most active B-$PEI_{1.1}$ (MIC = 250 $\mu g \cdot mL^{-1}$), suggesting that the L-PEIs are more effective at inhibiting *E. coli* growth.

It has been reported that the excess hydrophobicity of B-PEIs quaternized with long alkyl groups decreases their antibacterial activity because the formation of aggregates is likely to reduce the number of polymer chains available to interact with bacterial cell membranes.[18] Dynamic light scattering showed that the unmodified B- and L-PEIs studied here showed little or no light scattering over the concentration range of MICs in phosphate buffer solution or the assay medium (MH broth) compared to a sample containing a suspension of polystyrene nanoparticles of known, standard size (Figure S5 in Supporting Information). This indicates that the B- and L-PEIs do not form any significant or measurable aggregates under the assay conditions. This result implies that the polymers are not "trapped" in an aggregated form that could reduce the ability to interact with the bacterial cell membranes, decreasing their efficacy. Compared to hydrophobically modified PEIs, the hydrophilic nature of unmodified B-PEIs may also be beneficial to maximize the number of active polymer chains against bacterial cells.

When tested for activity against the Gram-positive bacteria *S. aureus*, the MICs of B-PEIs were 16–31 $\mu g \cdot mL^{-1}$ while magainin-2 did not display potent activity against the same strain (MIC > 250 $\mu g \cdot mL^{-1}$). The MIC values are orders of magnitude smaller than those for *E. coli* (Table 3). Although B-PEI_{12} did not display potent activity against *E. coli* (MIC > 1 000 $\mu g \cdot mL^{-1}$), the MIC value for *S. aureus* is 16 $\mu g \cdot mL^{-1}$, yielding a MIC selectivity index MIC (*E. coli*)/MIC (*S. aureus*) of >64 (Table 3). Other B-PEIs also showed the MIC selectivity index larger than 8. These results indicate that the B-PEIs are selectively active against *S. aureus* over *E. coli*. Similarly, both L-PEIs also had an MIC value of 8 $\mu g \cdot mL^{-1}$ against *S. aureus*, which is fourfold lower than that for *E. coli* (Table 3). This suggests that the L-BEIs are also selective to *S. aureus* over *E. coli* (the MIC selective index = 4).

It has been reported that the identity of counter anions for ammonium groups of synthetic polymers and dendrimers can affect antibacterial activity, although the mechanism is not clear yet.[33–35] Although there is no information on the presence or identity of counter anions in the commercially available PEIs studied here, counter anions, if present, may also affect the antibacterial activity of PEIs. Determination of the effect of counter ions on their antibacterial activity would be of interest for further investigation to modulate PEI antibacterial activity.

3.6. *E. coli* Membrane Permeabilization

As many natural cationic-amphiphilic peptides exhibit the ability to disrupt lipid bilayers, we assessed the ability of the PEIs to permeabilize bacterial membranes to gain

further insight into the antibacterial mechanism of these molecules. The periplasmic protein β-lactamase and the colorimetric substrate nitrocefin are used as reporters for changes in the permeability of the outer membrane (OM) of *E. coli*.[36–38] Under normal conditions, the cephalosporin analog nitrocefin cannot easily diffuse across the *E. coli* OM and therefore shows a low degree of conversion into the chromophore product. Once the PEIs (or other compounds) permeabilize the OM, the nitrocefin can diffuse into the periplasm and the β-lactamase can cleave the substrate, yielding a compound with absorbance at 486 nm. Since this assay monitors the enzymatic reaction, the rate of chromophoric product formation (rate of increase in absorbance) is related to the ability of the nitrocefin to cross the OM which can be altered by the interaction of the PEIs with the membrane.

Figure 4A shows typical kinetic curves of the production of the nitrocefin cleavage product in cells exposed to L-PEI$_{6.5}$. This membrane permeabilizing activity of the L-PEIs was shown to be dose-dependent (Figure 4B). The samples treated with L-PEIs exhibited increasing amounts of chromophore production at low polymer concentrations, followed by a plateau above 6 $\mu g \cdot mL^{-1}$ (Figure 4B). Both L-PEI$_{4.4}$ and L-PEI$_{6.5}$ showed similar concentration dependence even though they have different MWs. On the other hand, the B-PEIs displayed much lower levels of chromophore production compared to L-PEIs (Figure 4C), which mirrors the lower activity of B-PEIs against *E. coli* compared to L-PEIs.

The experimental design to test the integrity of the inner membrane is similar to that for the OM except that the cytoplasmic enzyme β-galactosidase and the chromogenic substrate ONPG are used as the reporter for the assay. The results of this assay showed very little, if any, permeabilization of the inner membrane of the *E. coli* upon exposure to the B-PEIs or L-PEIs tested even though the L-PEIs displayed significant permeabilization of the *E. coli* OM (Figure S6 in Supporting Information). Although, we hypothesized that the cationic amphiphilic structures of PEIs may exert antibacterial effects by disrupting bacterial cell membranes similar to natural antibacterial peptides, this result indicates that molecular mechanisms other than the disruption of the *E. coli* inner membrane may factor in the activity. It has been reported that some cationic antimicrobial peptides are translocated though the IM without significant disruption and subsequently bind to cytoplasmic enzymes and DNA/RNA, inhibiting macromolecular synthesis.[39–41] It has also been reported that B-PEI with a MW of 50 kDa strongly permeabilized the OMs of Gram-negative bacteria, but the PEI did not significantly inhibit the bacterial growth.[42] However, Tashiro reported bactericidal activity of B-PEI with MW of 600 on *E. coli* at the concentrations of 25–100 $\mu g \cdot mL^{-1}$ in saline.[43,44] We found that the B-PEI$_{12}$ are not active against *E. coli*

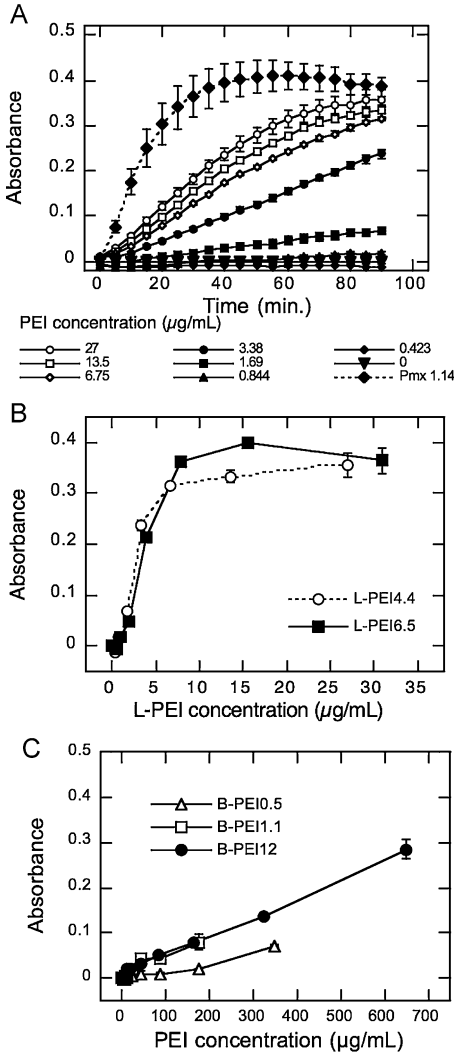


Figure 4. E. coli OM leakage. Absorbance was measured at 486 nm. (A) L-PEI$_{2.5}$ kinetics. (B) Endpoint absorbance after 90 min for L-PEIs. (C) Endpoint absorbance after 90 min for B-PEIs.

(MIC > 1 000 $\mu g \cdot mL^{-1}$) in MH broth and do not significantly permeabilize the OM of *E. coli*. However, the lower MW B-PEIs displayed relatively weak inhibitory effects against *E. coli* (MIC = 250–500 $\mu g \cdot mL^{-1}$) although they are also not strong membrane permeabilizers. The low MW B-PEIs could have better ability to penetrate the cell wall structure without becoming trapped in the anionic peptidoglycan layers and liposaccharides (LPA) because of the lower density of cationic charges on the PEI surfaces due to smaller molecular size.

3.7. *S. aureus* Membrane Permeabilization

Considering that the PEIs show selective activity against *S. aureus* over *E. coli*, the permeabilizing ability of PEIs was also tested against *S. aureus*. The single membrane

architecture of this Gram-positive bacteria allows for the direct interrogation of bilayer integrity by assaying the membrane potential using the potential-sensitive fluorophore DiSC3(5).[45–50] This compound is a membrane potential-sensitive dye that accumulates in the *S. aureus* membrane and undergoes self-quenching when the membrane is intact. Upon depolarization of the membrane (in this case caused by PEIs), the self-quenching is alleviated and thus the fluorescence emission from DiSC3(5) increases. Therefore, an increase in the fluorescence emission intensity indicates the ability of PEIs to disrupt the *S. aureus* cell membrane.

As a control, both L-PEIs and B-PEIs did not show any significant fluorescence intensity changes when mixed with the fluorophore in the absence of bacteria (Supporting Information). The B-PEIs displayed little or no effect on the membrane depolarization up to concentrations of five times the MIC (Figure 5 and Supporting Information). The lytic peptide melittin at its MIC induced rapid increase in fluorescence intensity, suggesting that the peptide permeabilizes the cytoplasmic membrane of *S. aureus*. Interestingly, addition of melittin to the assay solutions at 200 s did not cause any significant fluorescence change even though complete membrane depolarization was expected. This result suggests that the melittin action against *S. aureus* cell membranes was inhibited. To investigate this inhibitory effect, melittin and B-PEIs were mixed prior to addition to *S. aureus* suspension. Under these conditions, melittin did not appear to induce membrane depolarization (Supporting Information). These results indicate that the melittin activity was inhibited likely because of complex formation between melittin and PEIs.

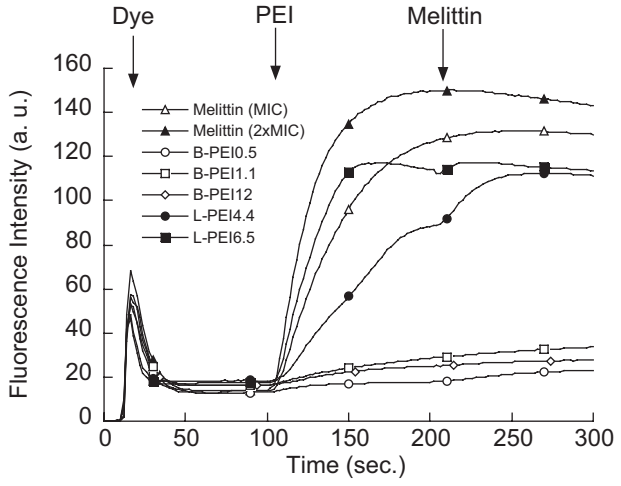


Figure 5. S. aureus membrane depolarization by PEIs and melittin in HEPES buffer. A membrane potential-sensitive DiSC3(5) dye in ethanol was added to *S. aureus* suspension at 20 s. After the fluorescence intensity was leveled due to dye uptake by *S. aureus*, PEI or melittin was added to the suspension to give final concentrations equal to their MICs as determined in MH broth (Table 1). At 200 s, melittin was added to the assay solution containing PEIs.

Epand et al. reported that an acyl-Lys oligomer is selective toward *S. aureus* over *E. coli*.[46] The oligomer is bacteriostatic and did not exhibit a strong permeabilization ability against the *S. aureus* membrane and that the oligomer did not interact with DNA, which supports the hypothesis that the oligomer does not interact with cytoplasmic targets. The authors propose that the acyl-Lys oligomers bound to cell wall block extracellular nutrients, resulting in starvation. In addition, Raafat et al. reported that the cationic property of the polysaccharide chitosan is a key factor in the antibacterial activity against *S. aureus*.[51] The authors speculate that chitosan binds to anionic biopolymer teichoic acids in the cell wall, which causes a sequence of "untargeted" molecular events including membrane depolarization, resulting in bacterial cell death. These reports suggest that the B-PEIs may also exhibit multiple targets and steps in the antibacterial mechanism against *S. aureus*. It has also been reported that cationic polynorbornene derivatives display selective activity to *S. aureus* over *E. coli*.[52,53] The authors proposed that the double membrane structure of the *E. coil* cell wall, which controls the polymer's transit to the cytoplasmic membrane, is responsible for the selective activity toward *E. coli* over *S. aureus*.[52] Although these mechanisms proposed in literature may be also at work with PEIs, the antimicrobial mechanism of PEIs against *S. aureus* is not clear at this point.

In contrast, the L-PEIs exhibited the ability to disrupt the *S. aureus* membrane potential, where the membrane disruption induced by L-PEI$_{6.5}$ is comparable to the lytic peptide melittin (Figure 5). The L-PEIs may exert an antibacterial effect against *S. aureus*, at least in part, by membrane disruption, which could reflect the higher activity (lower MIC values) against *S. aureus* compared to the B-PEIs tested in this study.

3.8. Hemolysis

In order for a compound to be a viable antibiotic, it must be relativity non-toxic to human cells by selectively targeting bacterial cells. To assess the cytotoxicity of the polymers to human cells, we first determined the hemolytic activity of PEIs. The release of hemoglobin molecules from lysis of human RBCs in the presence of the PEIs was monitored by absorbance spectroscopy. In general, the RBC membrane surfaces are less negatively charged than the bacterial cell surface. When cationic amphiphilic polymers are extensively hydrophobic, they can non-selectively bind to the RBCs and cause cell lysis.[54,55] In this assay, hemolysis reflects damage to human cell membranes induced by PEIs. The HC$_{50}$ value for each polymer (the concentration necessary for 50% hemolysis) was determined from the dose-hemolysis curves (Figure 6). Lysis of all RBCs (100%

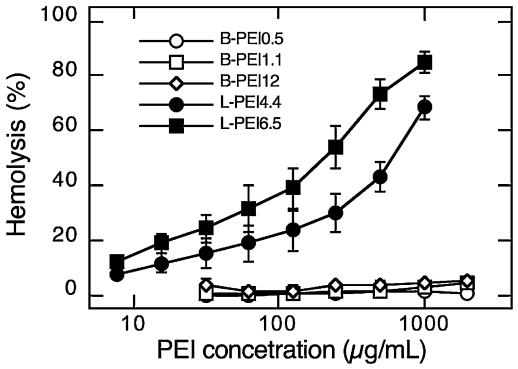


Figure 6. Hemolysis induced by PEIs. Each data point represents the average of three independent experiments in triplicate ± standard deviation.

hemolysis) was determined by exposing the cells to the surfactant Triton-X.

The B-PEIs showed little (less than 5%) or no hemolytic activity up to 2 000 $\mu g \cdot mL^{-1}$ (Figure 6) although they displayed antibacterial activity within this concentration range (Table 1). For instance, B-$PEI_{1.8}$ displayed antibacterial activity against *E. coli* (MIC = 250 $\mu g \cdot mL^{-1}$) and *S. aureus* (MIC = 31 $\mu g \cdot mL^{-1}$), giving HC_{50}/MIC selectivity indices of >8 for *E. coli* and >256 for *S. aureus*. These results indicate that the B-PEIs are selective agents for bacteria over RBCs. For comparison, the lytic peptide melittin displayed an HC_{50} of 2 $\mu g \cdot mL^{-1}$ under the same assay conditions and a corresponding selectivity index of 0.2–0.3.

On the other hand, both L-PEIs caused greater than 60% hemolysis at a polymer concentration of 1 000 $\mu g \cdot mL^{-1}$. L-$PEI_{6.5}$ displayed higher hemolytic activity (HC_{50} = 195 $\mu g \cdot mL^{-1}$) compared to L-$PEI_{4.4}$ (HC_{50} = 577 $\mu g \cdot mL^{-1}$), indicating the higher MW PEIs are more hemolytic although the antimicrobial activity of these L-PEIs was same. Although the L-PEIs caused appreciable hemolysis, their HC_{50} values are orders of magnitudes higher than the MIC values; the selectivity index of L-$PEI_{4.4}$ is 18 for *E. coli* and 32 for *S. aureus*. This suggests that the L-PEIs are also selective to bacteria over RBCs.

The result of antibacterial assays showed that the PEIs exhibit selectively enhanced activity against *S. aureus* over *E. coil* (Table 3). Based on the hemolysis results, the activity of PEIs is also selective to the tested bacteria over human RBCs, indicating the PEIs have desirable cell-selectivity. It has been also reported that cationic polynorbornene derivatives displayed similar selectivity against *S. aureus* over *E. coli* and RBCs, which was referred as "double selectivity".[52] An acyl-Lys oligomer also showed selective toward *S. aureus* over *E. coli*.[46] These indicate that the cationic and amphiphilic properties of polymers or oligomers may be the determining factor for their cell-selective antibacterial activity rather than the polymer molecular structures.

3.9. Lactate Dehydrogenase (LDH) Assay

We further evaluated the effect of PEIs on human HEp-2 cells, isolated from larynx epidermoid carcinoma with HeLa markers, as a gauge of PEI-induced cytotoxicity to human cells as well as to tumor cells. We first examined cell membrane integrity of HEp-2 cells in the presence of these polymers. We measured the amount of LDH that leaks from cells human HEp-2 cells after exposure to the PEIs. This LDH assay reports the damage of membrane damage caused by the polymers in general (Figure 7 and Table 2). In contrast to the hemolysis assay using RBCs, HEp-2 cells are metabolically active cells, therefore the LDH assay reports the effect of PEIs on the membrane of actively proliferating cells.

B-$PEI_{0.5}$ and B-$PEI_{1.1}$ caused little or no LDH leakage (Figure 7). Interestingly, B-PEI_{12} caused LDH release in as low as ≈4 $\mu g \cdot mL^{-1}$, and the LDH release leveled off above ≈250 $\mu g \cdot mL^{-1}$, giving 30% release (Figure 8) although B-PEI_{12} was not hemolytic (Figure 4). In the same assay, melittin caused significant LDH release, giving EC_{50} (peptide concentration for 50% LDH release) of 1.5 $\mu g \cdot mL^{-1}$ although magainin displayed only little effect (9%) up to 250 $\mu g \cdot mL^{-1}$.

Fischer et al. reported that B-PEI with MW 600–1000 kDa caused 30–80% LDH release after 1 h from L929 mouse fibroblasts in the PEI concentration range from 0.01 to 1 $mg \cdot mL^{-1}$ for 60 min.[24] Hong et al. also demonstrated that B-PEI with an MW of 78 220 induced significant LDH release from human KB cells (oral carcinoma origin) after 3 h, and rat Rt2 cells (derived from glioma). These results suggest that while the cells are tolerant to low MW PEIs, high MW PEIs are potentially toxic to many different types of the mammalian cells by compromising the cell membrane structure. On the other hand, L-$PEI_{6.5}$ and L-$PEI_{4.4}$ caused 27 and 10% LDH leakage at 250 $\mu g \cdot mL^{-1}$, respectively, as both L-PEIs caused membrane damage to RBCs (hemolysis). Similar to the B-PEIs, the low MW L-PEIs are also less toxic.

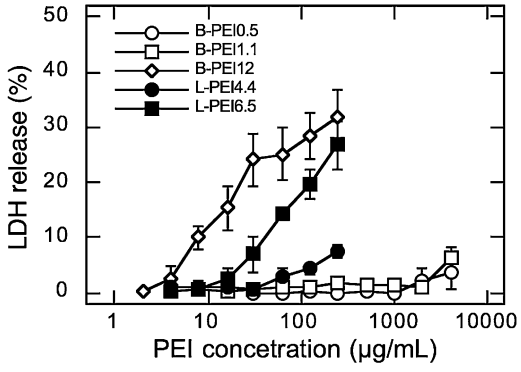


Figure 7. PEI-induced LDH release from HEp-2 cells. Each data point represents the average of three independent experiments in triplicate ± standard deviation. Lines are present to guide the eye and do not represent a mathematical fit of the data.

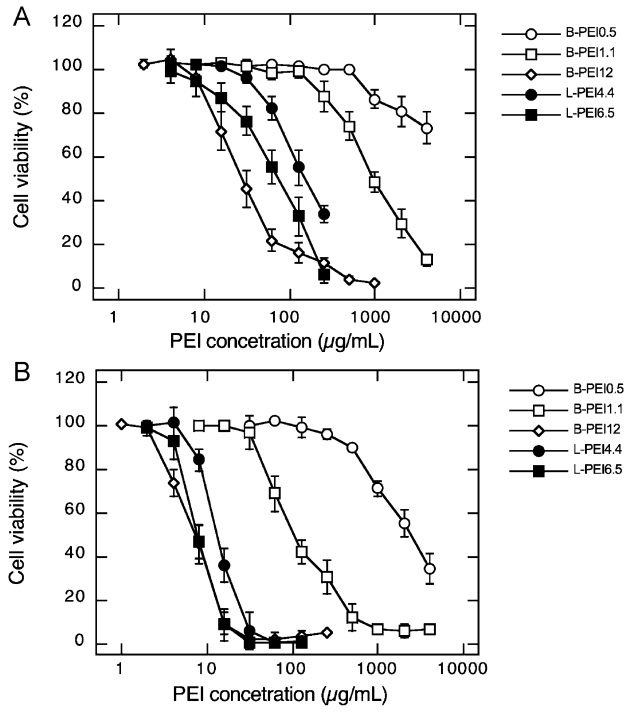


Figure 8. Cell viability after exposure to PEIs for (A) 1 h and (B) 24 h. Each data point represents the average of three independent experiments in triplicate ± standard deviation.

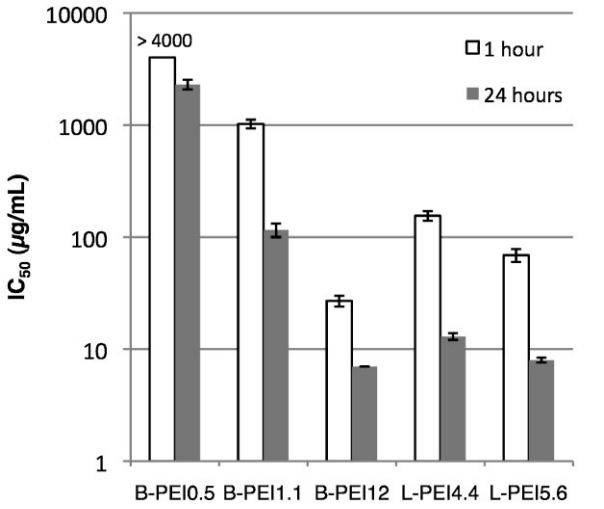


Figure 9. Cell viability after 1 h (open box) and 24 h (shaded box) exposure time to PEIs. Each data point represents the average of three independent experiments in triplicate ± standard deviation.

3.10. Cell Viability Assay

The XTT assay reports the inhibition of metabolic activity of cells (Table 4), providing information on cell viability in the presence of polymers that is not directly tied to membrane permeability. All PEIs displayed concentration- and molecular weight- dependent effects on the viability of HEp-2 cells after 1 h exposure (Figure 8). B-PEI$_{12}$ displayed highest toxic effect on cell viability; the viability reduced to 80% in the presence of 10 $\mu g \cdot mL^{-1}$ of B-PEI$_{12}$, and no viable cells were observed at 1 000 $\mu g \cdot mL^{-1}$, giving IC_{50} value of 26 $\mu g \cdot mL^{-1}$, which is the polymer concentration necessary for 50% inhibition of metabolic activity of cells (Table 4), B-PEIs with lower MWs (B-PEI$_{0.5}$ and B-PEI$_{1.1}$) only reduced the cell viability at high polymer concentrations, although they did not display LDH release.

Similarly, the L-PEIs also reduced cell viability as the polymer concentration increased. As the molecular weights of L-PEIs were increased from 4 400 to 6 500, the corresponding IC_{50} values decreased from 153 to 70 $\mu g \cdot mL^{-1}$ (Figure 9 and Table 4). Considering that the

Table 4. Cytotoxicity of PEIs to human epithelial HEp2 cells.

PEI or peptide	EC_{50} [$\mu g \cdot mL^{-1}$][a)]	IC_{50} [$\mu g \cdot mL^{-1}$][b)]	
		1 h[c)]	24 h[c)]
B-PEI$_{0.5}$	>4 000 (4%)[d)]	>4 000 (73%)[d)]	2 305 ± 225
B-PEI$_{1.1}$	>4 000 (6%)[d)]	1 026 ± 90	116 ± 16
B-PEI$_{12}$	>4 000 (30%)[d)]	27 ± 3	7 ± 0.4
L-PEI$_{4.4}$	>250 (8%)[d)]	155 ± 15	13 ± 0.9
L-PEI$_{6.5}$	>250 (27%)[d)]	69 ± 9	8 ± 0.4
Magainin-2	>250 (3%)[d)]	>250 (94%)[d)]	>250 (100%)[d)]
Melittin	1.5 ± 0.1	1.3 ± 0.1	1.4 ± 0.1

[a)]PEI concentration for 50% LDH release in an LDH assay. The data represent the average of three independent experiments in triplicate ± standard deviation; [b)]PEI concentration for 50% viability in an XTT assay. The data represent the average of three independent experiments in triplicate ± standard deviation; [c)]incubation time of cells with PEIs in the XTT assay; [d)]the LDH leakage or cell viability percentage at the highest polymer concentration was given if the EC_{50} or IC_{50} was not determined.

L-PEIs induced LDH release, the membrane disruption may be partially responsible for the cell viability reduction.

We further examined the effect of longer time exposure to PEIs (24 h) on the viability of HEp-2 cells (Figure 8B). In this experiment, the transitions in the viability curves are shifted toward the lower PEI concentrations, indicating the PEIs are more toxic to the cells after longer exposure time. The IC_{50} values of PEIs decreased by an order of magnitude after 24-h exposure (Figure 9 and Table 4). L-PEIs and B-PEI_{10} showed an IC_{50} of $\approx 10\,\mu g \cdot mL^{-1}$, which is the lowest IC_{50} value of this PEI series. These results suggest that the use of these PEIs needs to be limited to short term topical treatment rather than systemic administration for long-term infection treatment.

4. Conclusion

In conclusion, we investigated a series of B-PEIs and L-PEIs with relatively low MWs to study the effect of molecular architecture and size on antibacterial activity. The activity against *E. coli* and *S. aureus* depended on both the PEI architecture and MW. Interestingly, the PEIs displayed selective activity against *S. aureus* over *E. coli*. The membrane permeabilization assays suggested that PEIs may exert their antibacterial activity by mechanisms other than membrane disruption, contrary to our original hypothesis. The PEIs are also selective to bacteria over RBCs, indicating the antimicrobial action of unmodified PEIs is cell-selective. The combined results of two independent cytotoxic assays (LDH release assay and XTT assay) show that the low MW B-PEIs are less cytotoxic to human HEp-2 cells than their linear counterparts. However, even these polymers significantly reduced cell viability after a 24 h exposure time.

The results presented here will be useful in optimizing the antibacterial activity and cytotoxicity of PEIs as well as in studying the antimicrobial mechanisms of these and similar cationic amphiphilic macromolecules. Although PEIs quaternized with alkyl groups have been extensively studied as membrane-active antibacterials, understanding the intrinsic antimicrobial mechanisms of unmodified PEIs is advantageous to designing antibacterial agents for optimal activity and cell selectivity. Synthetic polymers such PEIs are not cost or labor intensive to produce, and well established methods in polymer chemistry enables accessible modifications of their chemical and physical properties, which will facilitate their further development as new antibacterial agents.

Acknowledgements: This research was supported by the NSF CAREER Award (DMR-0845592 to KK), NIH (5R21DE020908-02 to KK and 1R15GM094330 to GC), and the Department of Biologic and Materials Sciences, University of Michigan School of Dentistry. We thank Dr. Robertson Davenport at the University of Michigan Hospital for supplying the red blood cells and Dr. Eric Krukonis at the University of Michigan School of Dentistry for providing HEp-2 cells. We also thank Dr. Mark Banaszak Holl, Dr. Ankur Desai, and Olga Lykhytska at Department of Chemistry and the Michigan Nanotechnology Institute for Medicine and Biological Sciences (MNIMBS), the University of Michigan for assistance with GPC and HPLC measurements. GC would like to thank the department of Physics and Astronomy at Rowan University for use of the DLS instrument.

Received: February 9, 2012; Revised: May 2, 2012; Published online: August 3, 2012; DOI: 10.1002/mabi.201200052

Keywords: antimicrobial; biomimetic; peptides; poly(ethylene imine); structure–property relations

[1] K. Bush, P. Courvalin, G. Dantas, J. Davies, B. Eisenstein, P. Huovinen, G. A. Jacoby, R. Kishony, B. N. Kreiswirth, E. Kutter, S. A. Lerner, S. Levy, K. Lewis, O. Lomovskaya, J. H. Miller, S. Mobashery, L. J. V. Piddock, S. Projan, C. M. Thomas, A. Tomasz, P. M. Tulkens, T. R. Walsh, J. D. Watson, J. Witkowski, W. Witte, G. Wright, P. Yeh, H. I. Zgurskaya, *Nat. Rev. Microbiol.* **2011**, *9*, 894.
[2] L. S. Miller, J. S. Cho, *Nat. Rev. Immunol.* **2011**, *11*, 505.
[3] R. R. Roberts, B. Hota, I. Ahmad, R. D. Scott, S. D. Foster, F. Abbasi, S. Schabowski, L. M. Kampe, G. G. Ciavarella, M. Supino, J. Naples, R. Cordell, S. B. Levy, R. A. Weinstein, *Clin. Infect. Dis.* **2009**, *49*, 1175.
[4] K. A. Brogden, *Nat. Rev. Microbiol.* **2005**, *3*, 238.
[5] M. Zasloff, *Nature* **2002**, *415*, 389.
[6] M. R. Yeaman, N. Y. Yount, *Pharmacol. Rev.* **2003**, *55*, 27.
[7] E. F. Palermo, K. Kuroda, *Appl. Microbiol. Biotechnol.* **2010**, *87*, 1605.
[8] G. N. Tew, R. W. Scott, M. L. Klein, W. F. Degrado, *Acc. Chem. Res.* **2010**, *43*, 30.
[9] S. Rotem, A. Mor, *Biochim. Biophys. Acta, Biomembr.* **2009**, *1788*, 1582.
[10] Y. W. Cho, J. D. Kim, K. Park, *J. Pharm. Pharmacol.* **2003**, *55*, 721.
[11] M. E. Davis, *Curr. Opin. Biotechnol.* **2002**, *13*, 128.
[12] J. H. Jang, T. L. Houchin, L. D. Shea, *Expert Rev. Med. Devices* **2004**, *1*, 127.
[13] T. Segura, L. D. Shea, *Ann. Rev. Mater. Res.* **2001**, *31*, 25.
[14] D. N. Nguyen, J. J. Green, J. M. Chan, R. Longer, D. G. Anderson, *Adv. Mater.* **2009**, *21*, 847.
[15] M. Breunig, U. Lungwitz, R. Liebl, C. Fontanari, J. Klar, A. Kurtz, T. Blunk, A. Goepferich, *J. Gene Med.* **2005**, *7*, 1287.
[16] M. Neu, D. Fischer, T. Kissel, *J. Gene Med.* **2005**, *7*, 992.
[17] B. J. Gao, X. Zhang, Y. Zhu, *J. Biomater. Sci., Polym. Ed.* **2007**, *18*, 531.
[18] N. Pasquier, H. Keul, E. Heine, M. Moeller, B. Angelov, S. Linser, R. Willumeit, *Macromol. Biosci.* **2008**, *8*, 903.
[19] V. P. Dhende, S. Samanta, D. M. Jones, I. R. Hardin, J. Locklin, *ACS Appl. Mater. Interfaces* **2011**, *3*, 2830.
[20] J. Haldar, A. K. Weight, A. M. Klibanov, *Nat. Protoc.* **2007**, *2*, 2412.
[21] J. Lin, S. Y. Qiu, K. Lewis, A. M. Klibanov, *Biotechnol. Bioeng.* **2003**, *83*, 168.

[22] K. Miyata, N. Nishiyama, K. Kataoka, *Chem. Soc. Rev.* **2012**, *41*, 2562.
[23] T. Xia, M. Kovochich, M. Liong, H. Meng, S. Kabehie, S. George, J. I. Zink, A. E. Nel, *ACS Nano* **2009**, *3*, 3273.
[24] D. Fischer, Y. X. Li, B. Ahlemeyer, J. Krieglstein, T. Kissel, *Biomaterials* **2003**, *24*, 1121.
[25] S. P. Hong, A. U. Bielinska, A. Mecke, B. Keszler, J. L. Beals, X. Y. Shi, L. Balogh, B. G. Orr, J. R. Baker, M. M. B. Holl, *Bioconjugate Chem.* **2004**, *15*, 774.
[26] A. von Harpe, H. Petersen, Y. X. Li, T. Kissel, *J. Controlled Release* **2000**, *69*, 309.
[27] S. A. Idris, O. A. Mkhatresh, F. Heatley, *Polym. Int.* **2006**, *55*, 1040.
[28] L. Tauhardt, K. Kempe, K. Knop, E. Altuntas, M. Jager, S. Schubert, D. Fischer, U. S. Schubert, *Macromol. Chem. Phys.* **2011**, *212*, 1918.
[29] M. Thomas, J. J. Lu, Q. Ge, C. C. Zhang, J. Z. Chen, A. M. Klibanov, *Proc. Natl. Acad. Sci. U. S. A.* **2005**, *102*, 5679.
[30] D. Fischer, A. von Harpe, K. Kunath, H. Petersen, Y. X. Li, T. Kissel, *Bioconjugate Chem.* **2002**, *13*, 1124.
[31] H. K. Hall, *J. Am. Chem. Soc.* **1957**, *79*, 5441.
[32] C. Z. S. Chen, S. L. Cooper, *Biomaterials* **2002**, *23*, 3359.
[33] C. Z. S. Chen, N. C. Beck-Tan, P. Dhurjati, T. K. van Dyk, R. A. LaRossa, S. L. Cooper, *Biomacromolecules* **2000**, *1*, 473.
[34] A. Kanazawa, T. Ikeda, T. Endo, *J. Polym. Sci. Polym. Chem.* **1993**, *31*, 1441.
[35] K. Lienkamp, A. E. Madkour, K. N. Kumar, K. Nusslein, G. N. Tew, *Chem. -Eur. J.* **2009**, *15*, 11715.
[36] R. F. Epand, M. A. Schmitt, S. H. Gellman, R. M. Epand, *Biochim. Biophys. Acta, Biomembr.* **2006**, *1758*, 1343.
[37] B. Mensa, Y. H. Kim, S. Choi, R. Scott, G. A. Caputo, W. F. DeGrado, *Antimicrob. Agents Chemother.* **2011**, *55*, 5043.
[38] R. E. W. Hancock, P. G. W. Wong, *Antimicrob. Agents Chemother.* **1984**, *26*, 48.
[39] C. B. Park, H. S. Kim, S. C. Kim, *Biochem. Biophys. Res. Commun.* **1998**, *244*, 253.
[40] H. Brotz, G. Bierbaum, K. Leopold, P. E. Reynolds, H. G. Sahl, *Antimicrob. Agents Chemother.* **1998**, *42*, 154.
[41] A. Patrzykat, C. L. Friedrich, L. J. Zhang, V. Mendoza, R. E. W. Hancock, *Antimicrob. Agents Chemother.* **2002**, *46*, 605.
[42] I. M. Helander, H. L. Alakomi, K. LatvaKala, P. Koski, *Microbiology-UK* **1997**, *143*, 3193.
[43] T. Tashiro, *J. Appl. Polym. Sci.* **1991**, *43*, 1369.
[44] T. Tashiro, *J. Appl. Polym. Sci.* **1992**, *46*, 899.
[45] S. Choi, A. Isaacs, D. Clements, D. H. Liu, H. Kim, R. W. Scott, J. D. Winkler, W. F. DeGrado, *Proc. Nat. Acad. Sci. U. S. A.* **2009**, *106*, 6968.
[46] R. F. Epand, H. Sarig, A. Mor, R. M. Epand, *Biophys. J.* **2009**, *97*, 2250.
[47] C. L. Friedrich, D. Moyles, T. J. Beveridge, R. E. W. Hancock, *Antimicrob. Agents Chemother.* **2000**, *44*, 2086.
[48] H. Sugiarto, P. L. Yu, *FEMS Microbiol. Lett.* **2007**, *270*, 195.
[49] M. H. Wu, R. E. W. Hancock, *J. Biol. Chem.* **1999**, *274*, 29.
[50] M. H. Wu, E. Maier, R. Benz, R. E. W. Hancock, *Biochemistry* **1999**, *38*, 7235.
[51] D. Raafat, K. von Bargen, A. Haas, H.-G. Sahl, *Appl. Environ. Microbiol.* **2008**, *74*, 3764.
[52] K. Lienkamp, K. N. Kumar, A. Som, K. Nusslein, G. N. Tew, *Chem. -A Eur. J.* **2009**, *15*, 11710.
[53] K. Lienkamp, A. E. Madkour, A. Musante, C. F. Nelson, K. Nusslein, G. N. Tew, *J. Am. Chem. Soc.* **2008**, *130*, 9836.
[54] K. Kuroda, G. A. Caputo, W. F. DeGrado, *Chem. Eur. J.* **2009**, *15*, 1123.
[55] I. Sovadinova, E. F. Palermo, R. Huang, L. M. Thoma, K. Kuroda, *Biomacromolecules* **2011**, *12*, 260.
[56] I. Sovadinova, E. F. Palermo, M. Urban, P. Mpiga, G. A. Caputo, K. Kuroda, *Polymers* **2011**, *3*, 1512.