

Supporting Information

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Poly(ethylene imine)s as antimicrobial agents with selective activity

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1. Experimental procedure

1.1. Potentiometric titration. Branched PEIs were titrated using a procedure from the literature with slight modifications.^[1] B-PEIs (5 mg) were dissolved in aqueous saline (10 mL, [NaCl] =150 mM) in a glass scintillation vial to give a concentration of 0.5 mg/mL. However, L-PEIs have limited solubility in the saline solution, which resulted in turbid mixtures. Thus the L-PEIs were not titrated. Each solution of the B-PEIs was purged with nitrogen gas for 15 min with constant stirring to remove CO₂. The pH of the solution was measured at room temperature by Fischer Scientific Accumet AB15 pH meter, equipped with Accumet 3-in-1 pH/ATC combination electrode. The pH electrode was introduced into the PEI solution and the pH was adjusted to ~11 using standardized 0.1 N sodium hydroxide. Titration was accomplished by the sequential addition of 40 μL of standardized 0.1 N hydrochloric acid with the solution being constantly stirred. Prior to each addition, the solution was equilibrated until the pH reading was stable within ± 0.01 pH units. Ethanolamine was used as a standard to verify the validity of the method used and its pKa was found to be 9.8, which is in close agreement to a reported literature pKa value of 9.5.^[2] Two trials were performed for each control and sample solutions. Buffer capacity β between two data points (pH (A) and pH (B)) was approximated by the following equation:

$$\beta \approx \Delta c (\text{HCl}) / \Delta \text{pH}$$

$$\Delta c (\text{HCl}) = 40\mu\text{L} * 0.1 \text{ N HCl} / (\text{total volume of solution at pH(B) in } \mu\text{L})$$

$$\Delta \text{pH} = \text{pH(A)} - \text{pH(B)}$$

The calculated values for each data point were reported as β at the midpoint pH = ½* (pH(A) + pH (B)). The result from one of two experiments is presented in Figure 3 in the manuscript.

PEIs	pKa1		Average ^{a)}	Range (%) ^{b)}	pKa2			Range (%) ^{b)}
	1	2			1	2		
Ethanolamine	9.685	9.915	9.8	2.3	-	-	-	-
B-PEI0.5	9.555	9.19	9.4	3.9	6.215	6.125	6.2	1.5
B-PEI1.1	9.475	9.71	9.6	2.4	6.19	6.145	6.2	0.7
B-PEI12	8.99	9.04	9.0	0.6	5.83	5.745	5.8	1.5

a) The average value from two experiments.

b) Range (%) = 100* range of values / average value

1.2. Gel permeation chromatography. GPC experiments were performed on an Alliance Waters 2695 separation module equipped with a 2487 dual wavelength UV absorbance detector (Waters Corporation), a Wyatt HELEOS Multi Angle Laser Light Scattering (MALLS) detector, and an Optilab rEX differential refractometer (Wyatt Technology Corporation). The DAWN® HELEOS II utilized a 120 mW solid-state laser operating at 658 nm and the Refractive Index detector was a Differential RI detector operating at 658 nm. The dn/dc was measured at a fixed wavelength, as mentioned above, by considering a 100% mass recovery for the sample. Columns employed were TosoHaas TSK-Gel Guard PHW 06762 (75 mm \times 7.5 mm, 12 μ m), G 2000 PW 05761 (300 mm \times 7.5 mm, 10 μ m, 125 Å), G 3000 PW 05762 (300 mm \times 7.5 mm, 10 μ m, 200 Å), and G 4000 PW (300 mm \times 7.5 mm, 17 μ m, 500 Å). Column temperature was maintained at 25 ± 0.1 °C with a Waters temperature control module. The isocratic mobile phase was 0.1 M citric acid (aqueous) and 0.025 wt % sodium azide, pH 2.74, at a flow rate of 1 mL/min. The sample concentration was 10 mg/5 mL with an injection volume of 100 μ L. The weight average molecular weight, M_w , has been determined by GPC, and the number average molecular weight, M_n , was calculated with Astra 5.3.2 software (Wyatt Technology Corporation) based on the molecular weight distribution.

1.3. Reverse-phase HPLC. HPLC analysis was carried out on a Waters Acquity Peptide Mapping System equipped with a Waters photodiode array detector, a column manager that facilitates 4 column housing, and a sample manager. The instrument is controlled by Empower 3 software. For characterization, calibration and quantitation studies, PEIs were ran on an Acquity BEH C4 column (100 x 2.1 mm, 1.7 μ m). The analysis was carried out using a gradient elution beginning with 99:1 (v/v) water/acetonitrile (ACN) reaching 20:80 water/acetonitrile (ACN) in 13.40 minutes. Trifluoroacetic acid (TFA) at 0.14 wt.% concentration was added in water as well as in ACN as a counter ion to make the PEI surfaces hydrophobic. The gradient was then re-equilibrated back to starting conditions in the next 1.0 minute. Flow rate was maintained at 0.208 mL/min. and The software is equipped with three different injection options. A 3 mL of sample was injected using one such option (“partial loop with needle overfill”). The column temperature was maintained at 35 °C. The concentration of PEIs was maintained at ~ 1 mg/mL.

1.4. Dynamic light scattering.

Samples were prepared for dynamic light scattering experiments by preparing concentrated stock solutions of L-PEIs in ethanol (50 mg/mL) and B-PEIs in water (100 mg/mL). These solutions were then diluted in either phosphate buffered saline (PBS; 50 mM phosphate, 100 mM NaCl, pH 7) or Mueller-Hinton broth to a final concentration of 1 mg/mL. These samples were then serially diluted in four fold increments over the range of 1 mg/mL through 0.0039 mg/mL. Particle size measurements were performed on a 90 Plus Particle Size Analyzer (Brookhaven Instruments Corporation) at 25 °C using a refractive index of 1.453 for the sample and a dust cutoff of 120.0. Scattering data was analyzed using the 9kdlsw software package. Individual samples were analyzed in quintuplicate at a rate of one minute per run and data are reported as averages and standard deviations. A commercially provided standard solution of polystyrene nanoparticles of fixed size (92 nm ± 3.7) was used as a reference (Duke Scientific Corporation, Catalog # 3090A).

1.5. Antimicrobial assay.^[3] Antibacterial activity of polymers was determined in a standard microbroth dilution assay according to the Clinical and Laboratory Standards Institute guidelines^[4] with suggested modifications by R.E.W. Hancock Laboratory (University of British Columbia, Vancouver, British Columbia, Canada)^[5] and Giacometti *et al.*^[6] for testing cationic agents. B-PEIs were dissolved in TBS buffer, and stock solutions were prepared by 2-fold serial dilutions with 0.01 TBS buffer to give 8 different concentrations. TBS was used as a control. Similarly, L-PEIs were dissolved in ethanol, and stock solutions were prepared by 2-fold serial dilutions with 0.01% acetic acid. Acetic acid or ethanol/acetic acid mixtures were used as a control. There was no difference in bacterial growth or OD₆₀₀ in the presence of solvents (TBS, ethanol/acetic acid, acetic acid) for the control experiments. The bacterial strains *Escherichia coli* ATCC[®] 25922[™] and *Staphylococcus aureus* ATCC[®] 25923[™], were aerobically cultured in MHB. An overnight culture of bacterial strains was regrown to exponential phase (OD₆₀₀ of 0.5-0.6) and diluted to give the final concentration of bacteria on the microplate approximately 5 × 10⁵ CFU/mL. After addition of the test compounds at a 1/10 volume into a 96-well sterile assay plate (Corning #3359), the assay plate was incubated at 37 °C for 18 h. Bacterial growth was detected at OD₆₀₀ using Varioskan Flash microplate reader (Thermo Fisher). Each MIC experiment was independently repeated at least three times in triplicate on different days. The

minimum inhibitory concentration (MIC) was defined as the lowest polymer concentration to completely inhibit bacterial growth.

1.6. Hemolysis assay. A solution of fresh erythrocytes in TBS (10 mM Tris buffer, 150 mM NaCl, pH = 7.3) 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 (B-PEIs) or ethanol with water (L-PEIs) and the RBC stock solution were added to a 96-well microplate to achieve a final red blood cell concentration of 15×10^6 cells per well. After an incubation period of 1 hr at 37° 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. The assay was performed in triplicate and repeated independently at least three times.

1.7. Lactate dehydrogenase (LDH) assay. Human epithelial HEP-2 cells isolated from a larynx carcinoma were grown in minimum essential medium (MEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 0.1 mM nonessential amino acids and 1 mM sodium pyruvate at 37°C, 5% CO₂, and 95% relative humidity. The HEP-2 cell line indicates a likelihood of contamination with HeLa cells derived from a cervical cancer.^[7] Cells at a concentration of 10^4 per well were seeded in MEM/FBS into the wells of a sterile 96-well microplate. After a 24 hr incubation period at 37 ° C and 5% CO₂, 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 ethanol/water. TBS or ethanol/water, 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 37° C and 5% CO₂. 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 activity of pure LDH from the test kit and LDH extracted from HEp-2 cells in the presence of the polymers showed that the polymers themselves had no effect on LDH activity.

1.8. 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 EC₅₀ 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.

1.9. *E. coli* outer membrane-permeabilization assay. A colony of *E. coli* D31 was inoculated in a solution of LB Broth supplemented with ampicillin (100 µg/mL). The culture was incubated at 37 °C with shaking for 18 hours and then diluted 1:100 in fresh media and incubated at 37 °C until the OD₆₀₀ was approximately 0.200. The culture was then centrifuged to pellet the cells and resuspended in an equal volume of PBS buffer (10 mM phosphate, 200 mM NaCl, pH 7.0). Immediately before measurement, nitrocefin was added to the solution to a final concentration of 50 µg/mL. Subsequently this bacterial suspension (90 µL) was dispensed into 96-well plate, each well containing the PEI (10 µL) or control compounds at appropriate concentrations for a final volume reaction of 100 µL. PolymyxinB was used as a positive control. The absorbance at 486 nm of each well was measured on a ThermoSkan absorbance plate reader for 90 minutes (at 5 minute intervals) with brief shaking between readings. Data shown is the average of 3-6 samples.

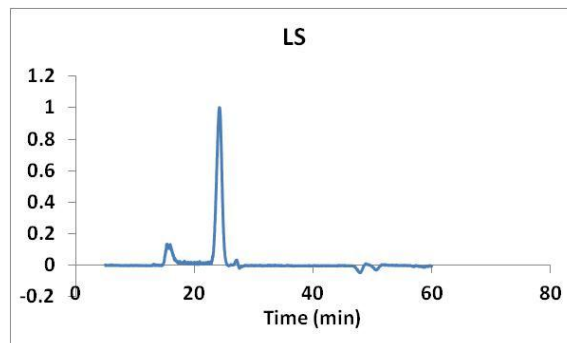
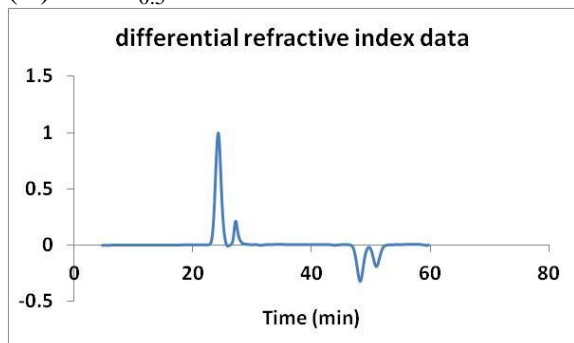
1.10. *E. coli* inner membrane-permeabilization assay. A single colony of *E. coli* D31 was inoculated in a solution of LB Broth (Difco) supplemented with Isopropyl β-D-1-

thiogalactopyranoside (IPTG) (2 mM) and incubated with shaking at 37 °C for 18 hours. The overnight culture was then diluted 1:100 in fresh LB media supplemented with IPTG and incubated with shaking at 37 °C until the OD₆₀₀ was 0.2-0.5. PEIs and CTAB (cetyl trimethyl ammonium bromide) were serially diluted from stocks in individual wells of a 96-well polystyrene plate over appropriate concentration ranges. Z-buffer (56.25 µL) was added to each well followed the *E. coli* culture (18.75 µL). Just prior to reading, ortho-Nitrophenyl-β-galactoside (ONPG) dissolved in Z-buffer (15 µL of a 4 mg/ml stock solution) was added to the wells. The absorbance at 420 nm was then monitored using a ThermoSkan plate reader for 90 minutes with shaking between individual readings to prevent cell settling. All assays were performed at least in triplicate.

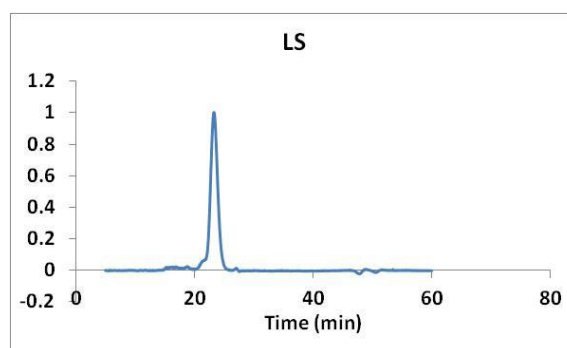
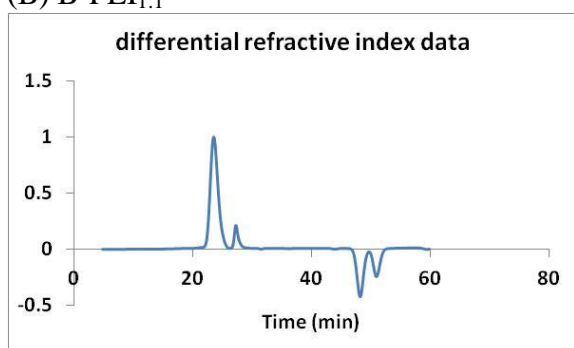
1.11. *S. aureus* membrane depolarization assay.^[8-10] Cytoplasmic membrane disruption was evaluated for both linear and branched PEIs against *S. aureus* using the membrane potential sensitive dye DiSC₃-(5). A single colony of *S. aureus* was inoculated in MH broth for 18 h at 37 °C, and mid-logarithmic phase cells (OD₆₀₀ = 0.5–0.6) were collected. The cells were re-suspended in buffer (5 mM HEPES, 5 mM sucrose, 100 mM KCl, pH 7.2) to OD = 0.05 at 600 nm. A stock solution of DiSC₃-(5) in ethanol (0.3 mM; 5 µL) was added to *S. aureus* suspension (3 mL). The final dye concentration is 0.5 µM. The cell suspension with DiSC₃-(5) dye was stirred at room temperature until a stable reduction in fluorescence intensity was achieved due to quenching upon accumulation of dye on the *S. aureus* membrane. At 100 s, a solution of melittin (3.6 mg/mL; 5 µL) in 0.01% acetic acid or PEIs (9.7 mg/mL for B-PEI_{0.5} and B-PEI₁₂, 19.3 mg/mL for B-PEI_{1.1}, 4.9 mg/mL of L-PEI_{4.4} and L-PEI_{6.5}; 5 µL) in HEPES buffer was added to the bacterial suspension to give the final concentration of their MICs. It should be noted that L-PEIs were initially dissolved in ethanol prior to dilution in HEPES buffer to give the desired concentration due to its limited solubility in the buffer. The fluorescence intensity was monitored using (FP-6200 JASCO Spectrofluorometer) with an excitation and emission wavelengths of 622 and 670 nm, respectively. Finally, melittin in 0.01% acetic acid buffer was added after 200 s to a final concentration of 2x MIC of melittin. As a control, mixtures of dye and PEIs in buffer show no significant change in fluorescence intensity compared to only dye in buffer (Supporting Information). The experiment was repeated at least 2 times from bacterial cell culture, and similar results were obtained. One of the data set was presented as a representative data.

2. GPC

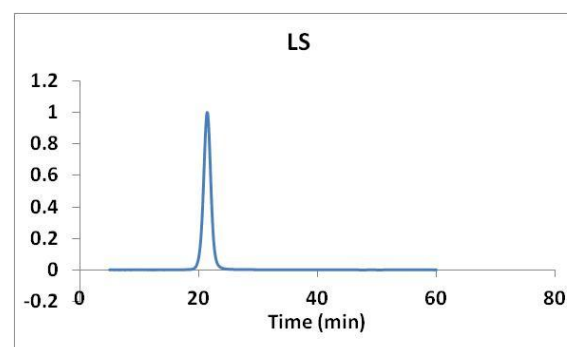
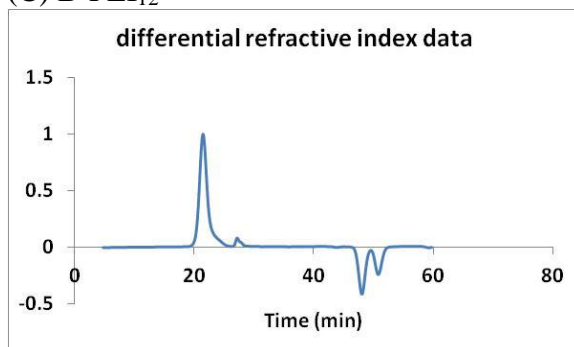
(A) B-PEI_{0.5}



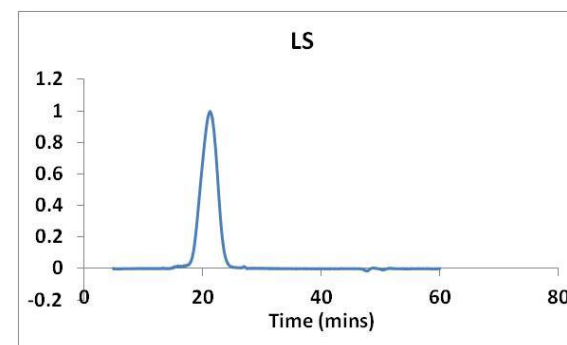
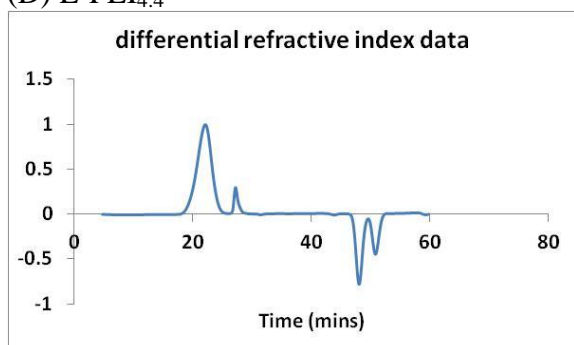
(B) B-PEI_{1.1}



(C) B-PEI_{1.2}



(D) L-PEI_{4.4}



(E) L-PEI_{6.5}

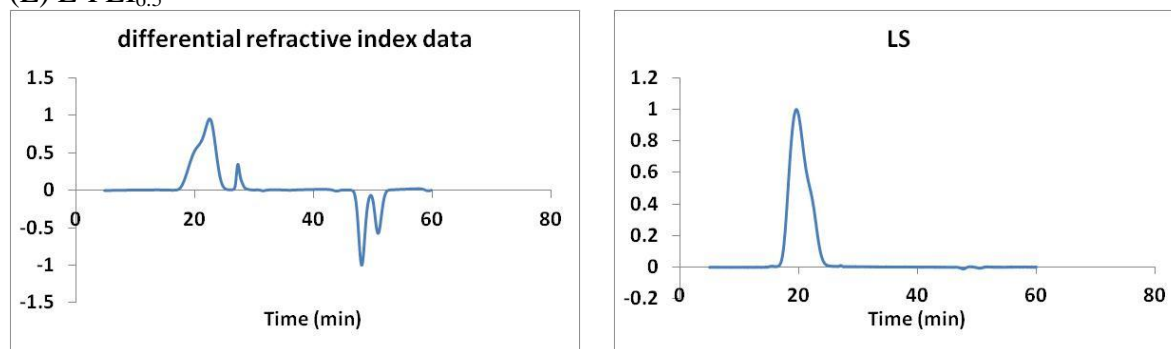


Figure S1. GPC elution curves for B-PEIs (A-C) and L-PEIs (D, E). Detector: Refractive index (Left) and light scattering (right).

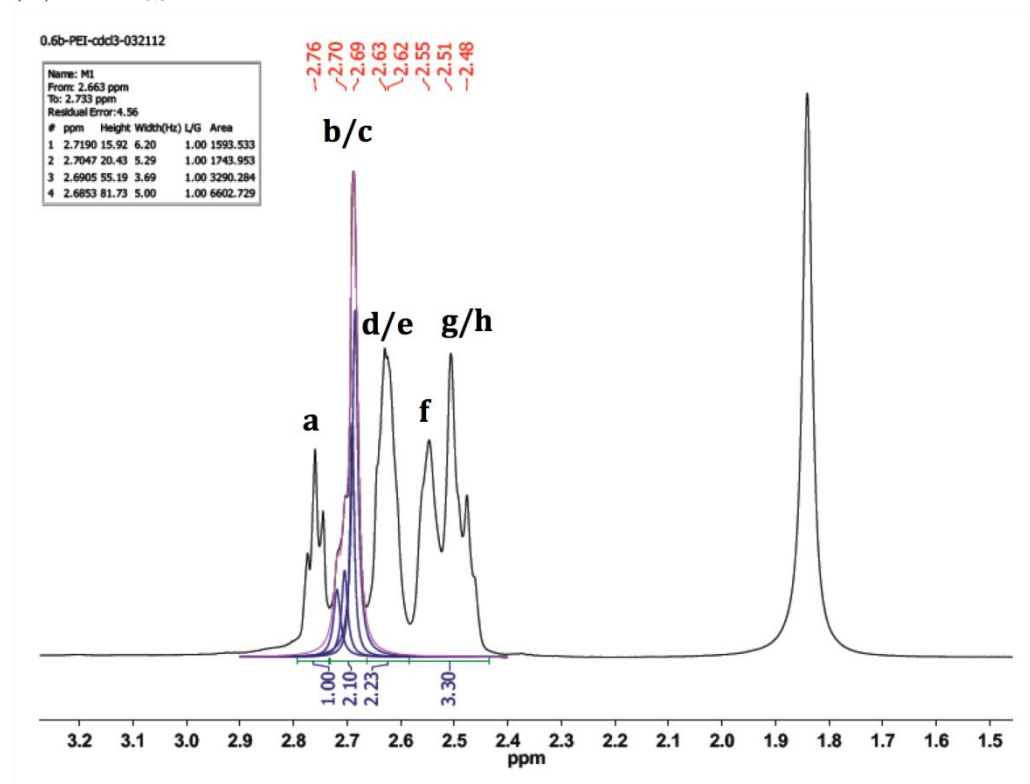
3. NMR Analysis.

3.1. ^1H NMR spectra

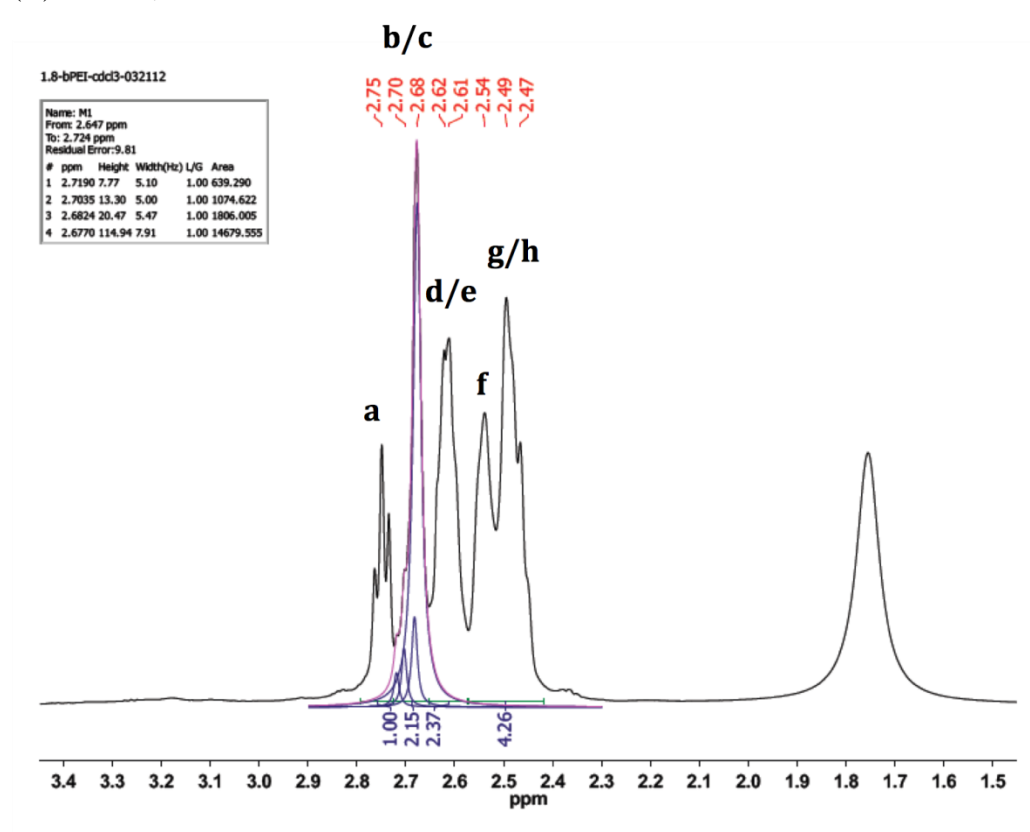
Table S1. Assignments and Integration of ^1H NMR spectra for Branched PEIs

Label	Structural Unit	ppm	Integration		
			B-PEI _{0.5}	B-PEI _{1.1}	B-PEI ₁₂
a	$\text{NH}_2\text{-CH}_2\text{-CH}_2\text{-NH-}$	2.76/2.75	1.00	1.00	1.00
b	$\text{NH}_2\text{-CH}_2\text{-CH}_2\text{-N-}$ 	2.70	2.10	2.15	1.85
c	$\text{-NH-CH}_2\text{-CH}_2\text{-NH-}$	2.69/2.68			
d	$\text{-NH-CH}_2\text{-CH}_2\text{-N-}$ 	2.63	2.23	2.37	2.28
e	$\text{-NH-CH}_2\text{-CH}_2\text{-NH}_2$	2.62/2.61			
f	$\text{-N-CH}_2\text{-CH}_2\text{-NH-}$ 	2.55	3.30	4.26	4.52
g	$\text{-N-CH}_2\text{-CH}_2\text{-N-}$ 	2.51			
h	$\text{-N-CH}_2\text{-CH}_2\text{-NH}_2$ 	2.48			

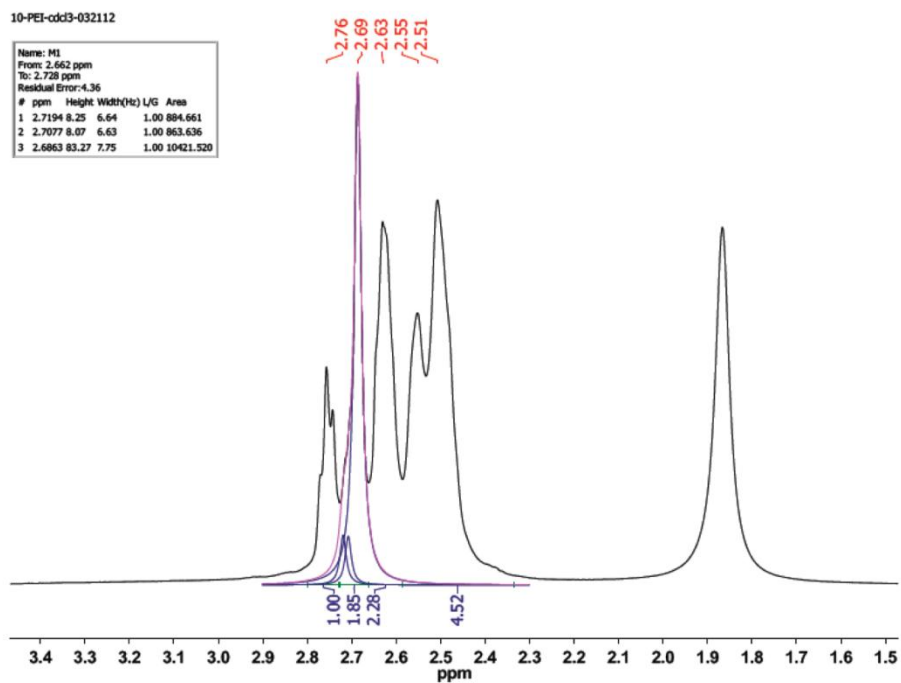
(A) B-PEI_{0.5}



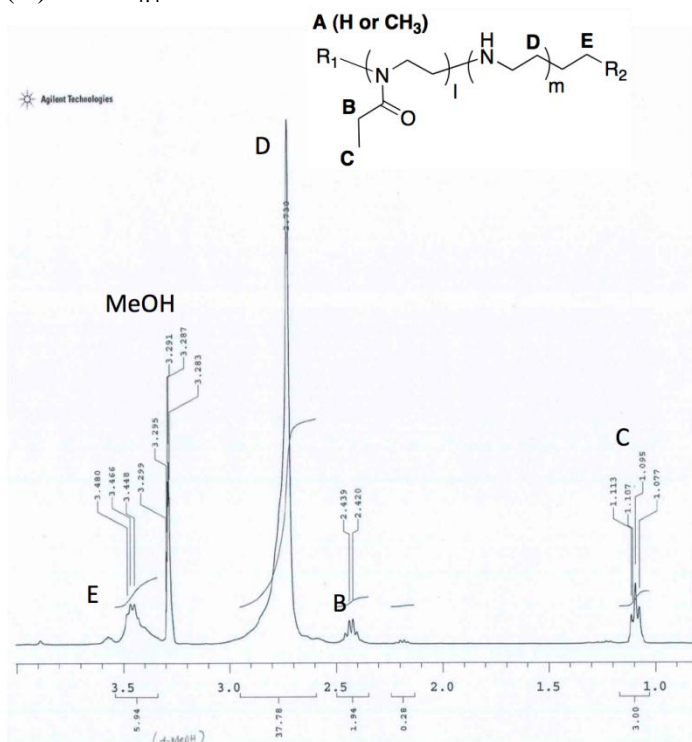
(B) B-PEI_{1.1}



(C) B-PEI₁₂



(D) L-PEI_{4,4}



(E) LPEI_{6,5}

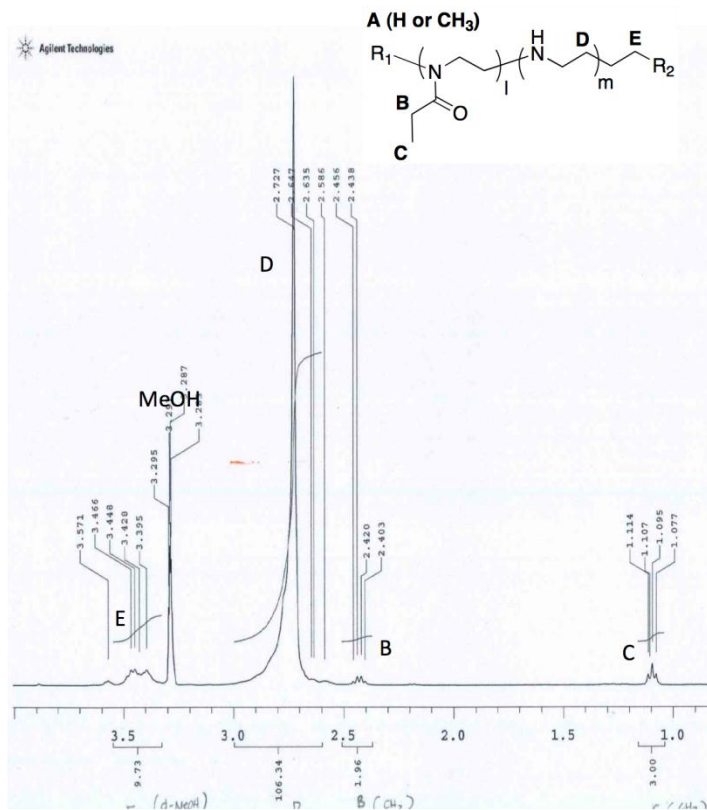


Figure S2. ¹H NMR spectra of B-PEIs (A-C) (CDCl₃) and L-PEIs (CD₃OD). See Table S1 for peak assignment of B-PEIs.

3.2. Determination of ratio of different amine groups. The sum of the integration of the protons adjacent to each amine groups (primary, secondary or tertiary) was used to estimate the degree of branching of the B-PEIs. Among other peaks, the overlap between b and c needed deconvolution to differentiate the protons adjacent to the primary and secondary amines. MestReNova chemistry software was used for the deconvolution of peaks b and c after phase and baseline correction. Residual errors were maintained at < 10 units during fitting. Deconvolution of overlapping peak b and c was performed as follows:

$$\begin{aligned} \text{Total peak area for peak b} &= \sum \text{peak area with frequency} < 2.70 \text{ ppm} \\ \text{Total peak area for peak c} &= \sum \text{peak area with frequency} > 2.70 \text{ ppm} \end{aligned}$$

As an example, for the B-PEI_{0,6},

$$\begin{aligned} \text{Total peak area for peak b} &= 1593.533 + 1743.953 = 3337.486 \\ \text{Total peak area for peak c} &= 3290.284 + 6602.729 = 9893.013 \end{aligned}$$

Therefore, the ratio peak b: peak c is 1:3. This ratio is applied to the values obtained from the integration (see Table S1) to approximate separate integration values for peak b and c. Thus,

$$\begin{aligned} \text{peak b integration} &= 2.10 * \frac{1}{4} = 0.525 \\ \text{peak c integration} &= 2.10 * \frac{3}{4} = 1.575 \end{aligned}$$

The ratio of 1°, 2° and 3° amines was then estimated as follows:

$$\begin{aligned} 1^\circ: a + b &= 1 + 0.525 = 1.525 \\ 2^\circ: [c + d + e] * \frac{1}{2} &= [1.575 + 2.23] * \frac{1}{2} = 1.90 \\ 3^\circ: [f + g + h] * \frac{1}{3} &= 3.30 * \frac{1}{3} = 1.10 \end{aligned}$$

The ratio of amine groups of other B-PEIs were calculated using the same method (Table S2).

Table S2. Estimated relative ratio of 1°: 2°: 3° amine groups of B-PEIs

Amine Type	Relevant Proton Label	B-PEI _{0.5}	B-PEI _{1.1}	B-PEI ₁₁
1°	a, b	1.5 (33%)	1.2 (25%)	1.3 (28%)
2°	c, d, e	1.9 (42%)	2.2 (46%)	1.9 (40%)
3°	f, g, h	1.1 (24%)	1.4 (29%)	1.5 (32%)

3.3. Determination of percentage of *N*-propionyl groups of L-PEIs. The mole percentage of *N*- propionyl groups of L-PEIs relative to the total number of repeating units was determined by using the following equation:

$$\text{N- propionyl groups [mol.\%]} = 100 * [A_C(\text{COCH}_2\text{-CH}_3) * (1/3)] / [A_D(\text{NCH}_2\text{-CH}_2\text{-}) * (1/4)]$$

A_C and A_D are integrated areas of C and D, respectively.

Table S3. Mole percentage of *N*- propionyl groups of L-PEIs

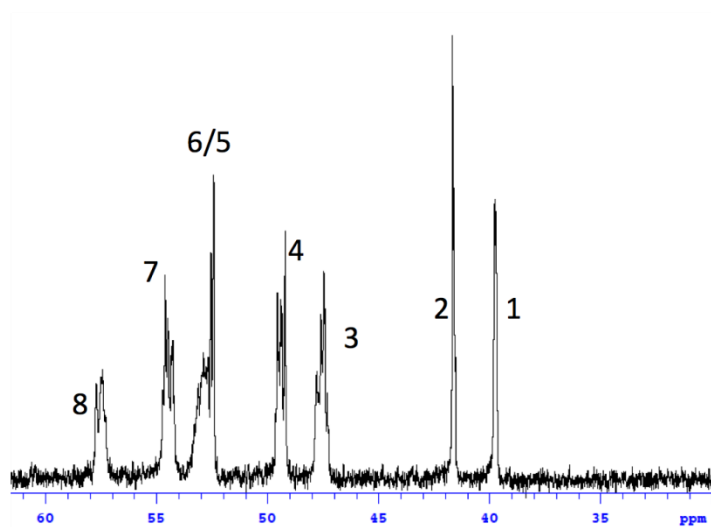
L-PEI	<i>N</i> - propionyl groups (mol.\%)
L-PEI _{4.4}	10.6
L-PEI _{6.5}	3.8

3.4. ^{13}C NMR spectra

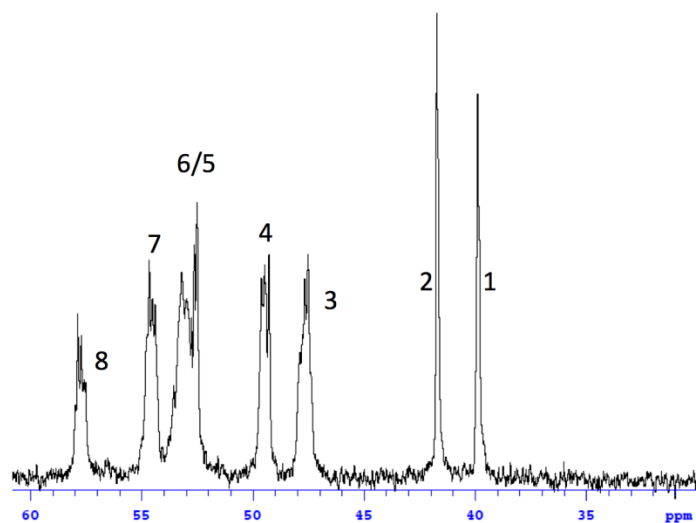
Table S4. Assignments of ^1H NMR spectra for B-PEIs^[11, 12]

Amine	Label	Structural Unit
1°	1	$\text{NH}_2\text{-CH}_2\text{-CH}_2\text{-N-}$
	2	$\text{NH}_2\text{-CH}_2\text{-CH}_2\text{-NH-}$
2°	3	$\text{-NH-CH}_2\text{-CH}_2\text{-N-}$
	4	$\text{-NH-CH}_2\text{-CH}_2\text{-NH-}$
	5	$\text{-NH-CH}_2\text{-CH}_2\text{-NH}_2$
3°	6	$\text{-N-CH}_2\text{-CH}_2\text{-N-}$
	7	$\text{-N-CH}_2\text{-CH}_2\text{-NH-}$
	8	$\text{-N-CH}_2\text{-CH}_2\text{-NH}_2$

(A) B-PEI_{0.5}



(B) B-PEI_{1,1}



(C) B-PEI_{1,2}

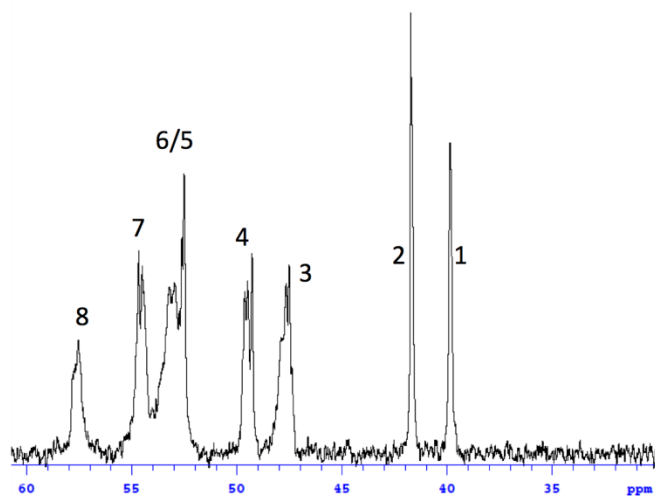


Figure S3. ¹³C NMR spectra of B-PEIs (CDCl₃). See Table S4 for the peak assignments.

4. Reverse-phase HPLC

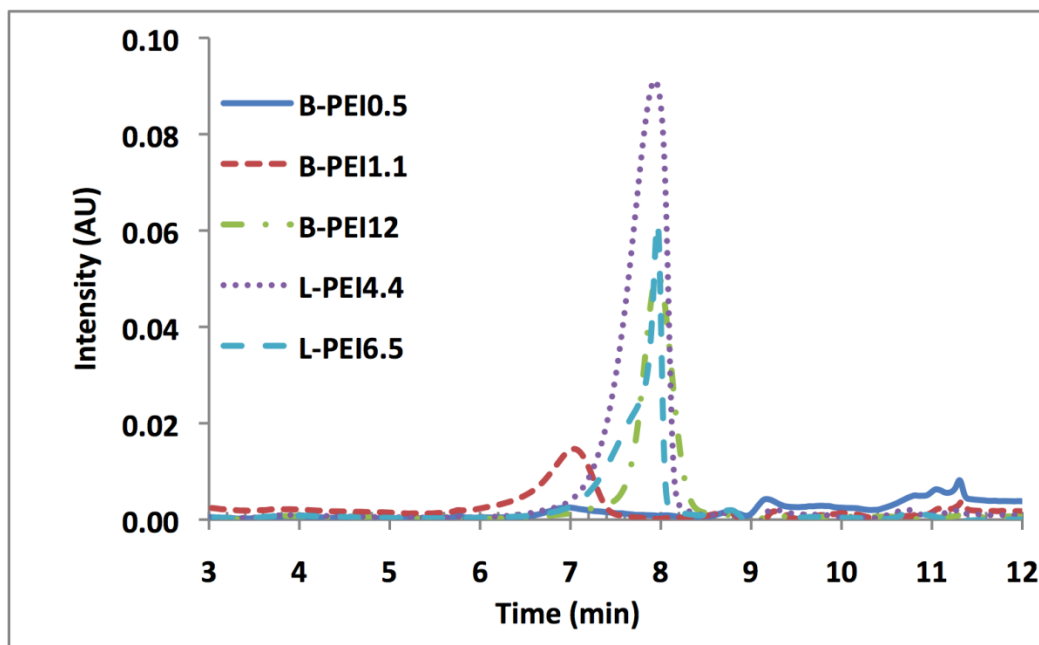
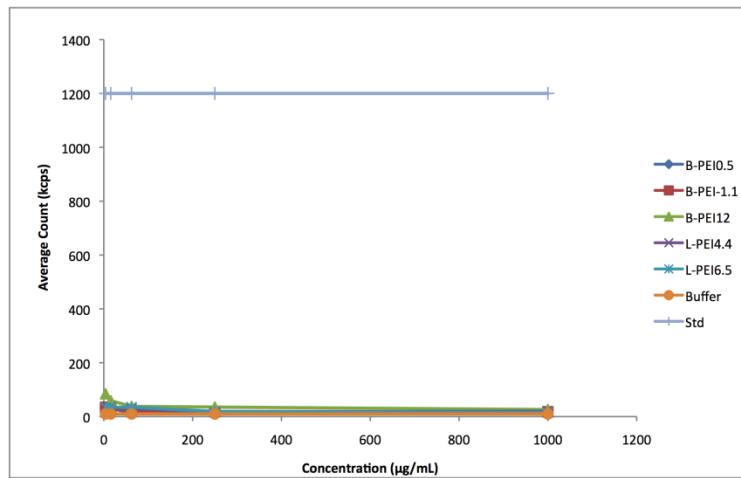


Figure S4. Reverse-phase HPLC elution curves.

5. DLS data

(A) Buffer



(B) MH broth

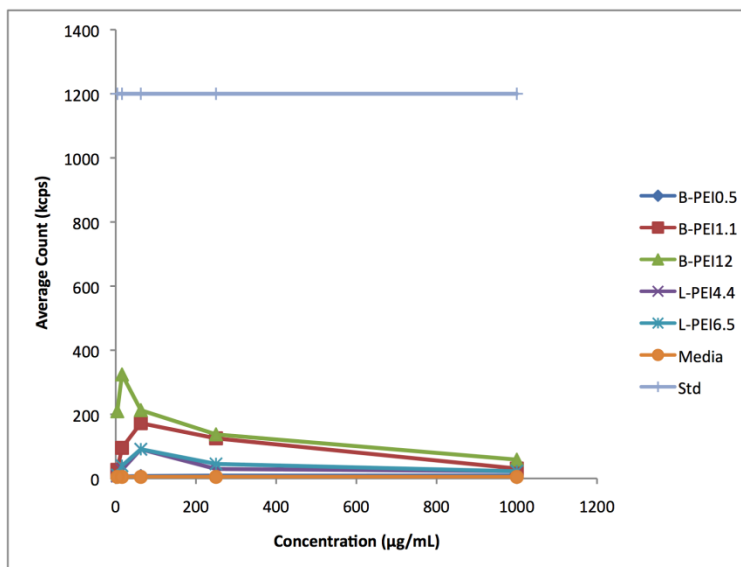


Figure S5. Dynamic light scattering intensity vs. PEI concentration in phosphate buffer solution (A) and MH broth (B). The standard used was a suspension of ~90nm polystyrene nanoparticles dispersed in phosphate buffer.

6. *E. coli* inner membrane permeabilization

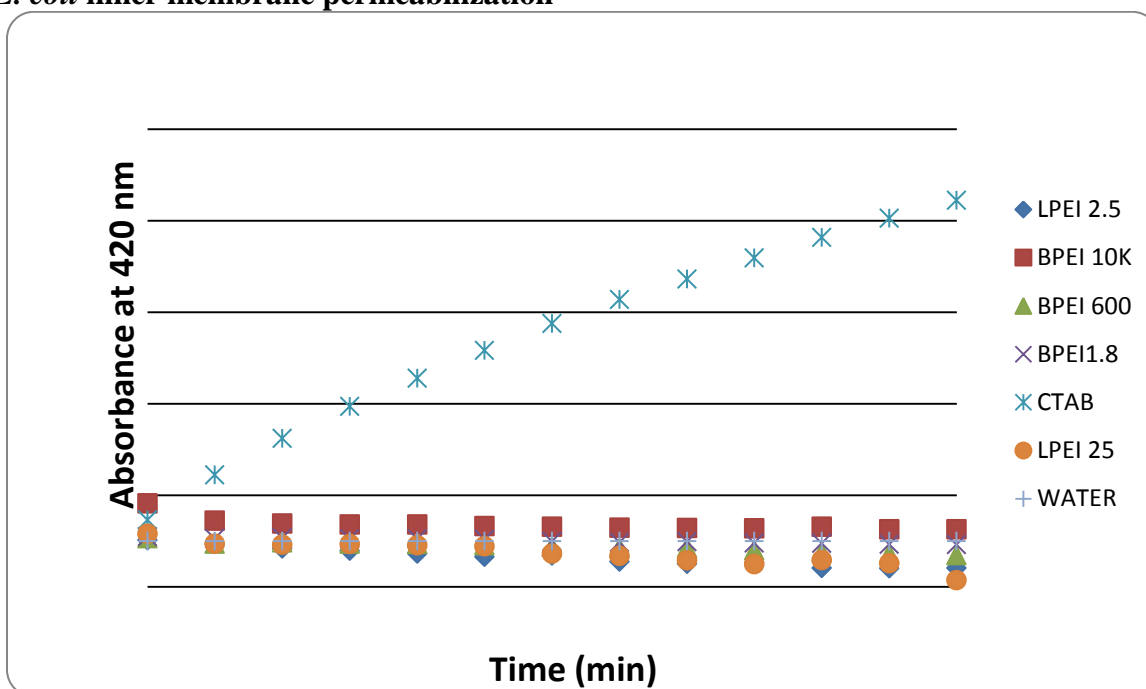


Figure S6. Permeabilization of *E. coli* cytoplasmic membrane by PEIs and CTAB. CTAB and water are a positive and negative control, respectively. PEI concentrations: L-PEI2.5, 31 $\mu\text{g}/\text{mL}$; L-PEI25, 31 $\mu\text{g}/\text{mL}$; B-PEI600, 350 $\mu\text{g}/\text{mL}$; B-PEI1.8, 175 $\mu\text{g}/\text{mL}$; CTAB, 13 $\mu\text{g}/\text{mL}$. Error bars are not presented for clarification.

7. *S. aureus* membrane depolarization

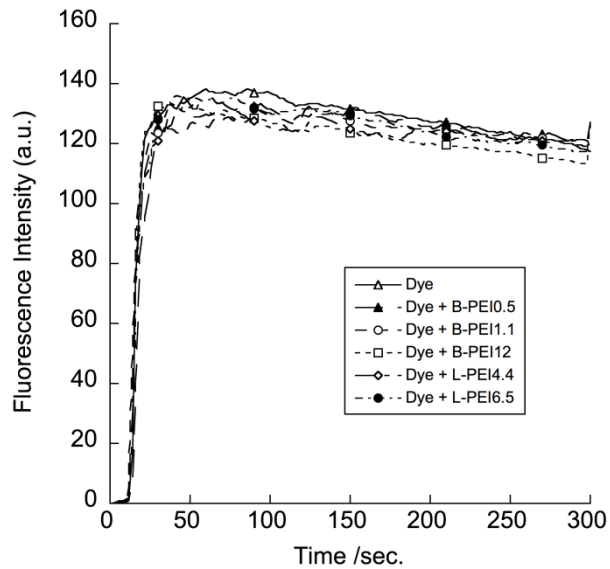


Figure S7. Fluorescence of dye in the presence of PEIs. The dye concentration is 0.5 μM . PEI concentrations are 1 x MIC determined in broth. Both linear and branched PEIs did not show any significant fluorescence intensity changes when mixed with the dye.

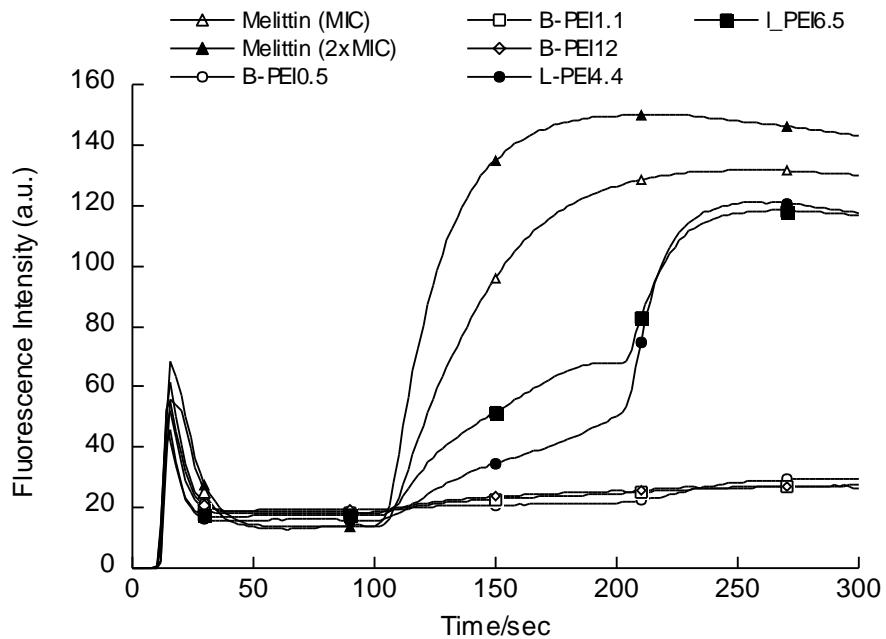


Figure S8. *S. aureus* membrane depolarization by PEIs at 0.5 x MIC. A membrane potential-sensitive DiSC3(5) dye was added to *S. aureus* suspension at 20 sec. After the fluorescence

intensity was leveled due to dye uptake by *S. aureus*, PEIs or melittin were added to the suspension. At 200 sec, melittin was added to the assay solution containing PEIs. The MIC concentrations of PEIs and melittin determined in MH broth were used for the assay.

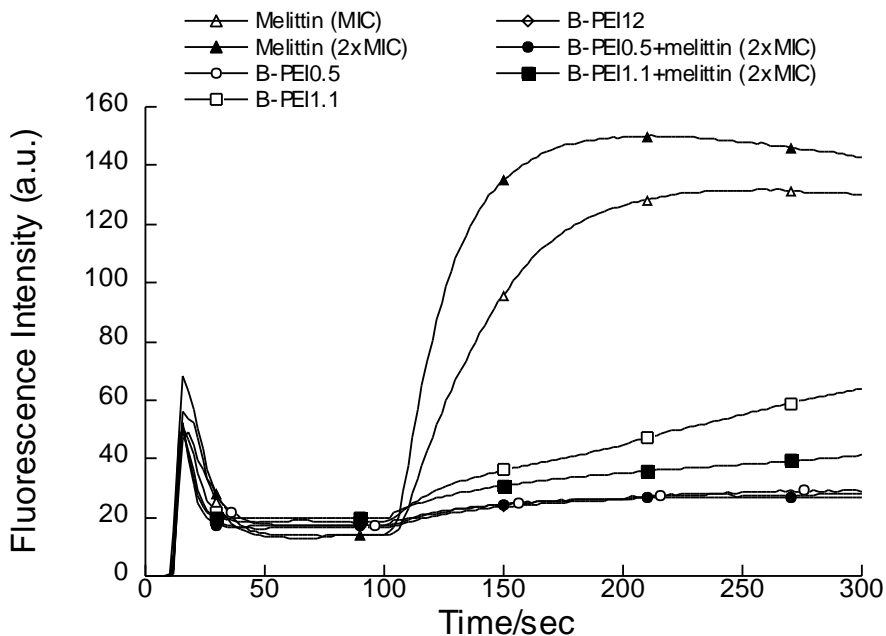


Figure S9. *S. aureus* membrane depolarization by B-PEIs at 5 x MIC. A membrane potential-sensitive DiSC3(5) dye was added to *S. aureus* suspension at 20 sec. After the fluorescence intensity was leveled due to dye uptake by *S. aureus*, PEIs or melittin were added to the suspension. At 200 sec, melittin was added to the assay solution containing PEIs. The MIC concentrations of PEIs and melittin determined in MH broth were used for the assay. B-PEI0.5 or B-PEI1.1 was mixed with melittin, and the mixture was added to the *S. aureus* suspension.

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