Antibacterial Properties of Main-Chain Cationic Polymers Prepared through Amine-Epoxy ‘Click’ Polymerization

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Experimental Details

Materials

Poly(ethylene glycol) diglycidyl ether \((M_n = 500)\), 1,4 butanediol glycidyl ether, poly(propylene oxide) diglycidyl ether \((M_n = 380)\), propyglyamine, butylamine, pentylamine, hexylamine, heptylamine, iodomethane, iodoethane, iodopropane, iodobutane, hydrochloric acid (37%), silver tetrafluoroborate, acetonitrile were purchased from Sigma Aldrich. Ethylene glycol diglycidyl ether was purchased from TCI (Tokyo Chemical Industry).

Escherichia coli (ATCC25922), Staphylococcus aureus (ATCC6538), Mycobacterium smegmatis (ATCC19420), PBS buffer (0.1 M PBS with 0.05 M NaCl, pH 7.4), Luria–Bertani (LB) medium (tryptone 10 g/L, yeast extract 5 g/L, and NaCl 10 g/L), tryptic soy broth (TSB) medium (tryptone 17 g/L, soytone 3 g/L, dextrose 2.5 g/L, NaCl 5 g/L, dipotassium phosphate 2.5 g/L) with tween 80 supplement and red blood cells (sterile defibrinated sheep’s blood, RBC) were used as purchased.

Characterizations

The bacterial surfaces were characterized by a field emission-scanning electron microscope (FE-SEM, Hitach S-4800) operated at 10 kV.

GPC measurements were conducted with three styragel HR 0.5, HR 2, and HR 4 Columns. PS standards in THF were used for the relative molecular weight determinations.

NMR spectra were measured on a Varian NMR System 500 MHz spectrometer, using CDCl₃ DMSO-d₆ and D₂O as the deuterated solvents at 500 MHz for ¹H-NMR. ¹H-NMR spectra were measured on UNITY-INOVA 500 from VARIAN INC., using D₂O at 500 MHz.

The bacteria were viewed under an Olympus BX53 microscope equipped with a 470 nm excitation filter and a 540 nm emission filter for green fluorescence and 530 nm excitation filter and a 590 nm emission filter for red fluorescence.

General procedure for polymerization

Alkane amine monomer (2 eq.) is added very slowly and drop-wise to a diglycidyl ether monomer (1 eq.) solution (50 wt%) in water. Both monomers are used as received from commercial suppliers without any further purification. After complete addition of the amine monomer, the reaction mixture is stirred at room temperature for 48 hours. After this time, the reaction mixture is freeze-dried and used for further studies without any purification.

PEG N(Bu) (1): ¹H NMR (500 MHz, Deuterium Oxide) δ 4.01 – 3.39 (broad multiplet, OCH), 2.75 – 2.45 (broad multiplet, NCH₂), 1.47 (broad signal, CH₂CH₂CH₃), 1.30 (broad signal, CH₃CH₂), 0.90 (t, J = 7.4 Hz, CH₃). GPC(THF): \(M_n = 5700, M_w = 7300\), PDI \((M_w/M_n) = 1.28\).

PPO N(Bu) (4): ¹H NMR (500 MHz, Chloroform-d) δ 3.90 – 3.24 (broad multiplet, OCH), 2.50 (broad multiplet, NCH₂), 1.40 (broad signal, CH₂CH₂CH₃), 1.27 (broad signal, CH₃CH₂), 1.13 (broad signal, CHCH₃), 0.94 – 0.83 (broad signal, CH₂CH₃). GPC(THF): \(M_n = 2200, M_w = 3800\), PDI \((M_w/M_n) = 1.72\).
EO N (Bu) (7): 1H NMR (500 MHz, Deuterium Oxide) δ 4.01 – 3.43 (broad multiplet, OCH), 2.85 – 2.42 (broad multiplet, NCH2), 1.48 (broad signal, CH2CH2CH3), 1.31 (broad signal, CH2CH3), 0.91 – 0.84 (broad signal, CH3). GPC(THF): Mn = 900, Mw = 1300, PDI (Mw/Mn) = 1.44.

BD N(Bu) (10): 1H NMR (500 MHz, Chloroform-d) δ 3.80 (broad signal, HOCH), 3.52 – 3.29 (broad multiplet, OCH), 2.64 – 2.39 (broad multiplet, NCH2), 1.68 – 1.57 (broad multiplet, OCH2CH2), 1.41 (broad signal, CH2CH2CH3), 1.33 – 1.20 (broad signal, CH2CH3), 0.89 (t, J = 7.3 Hz, CH3). GPC(THF): Mn = 3800, Mw = 6200, PDI (Mw/Mn) = 1.63.

BD N(Pr) (13): 1H NMR (500 MHz, Chloroform-d) δ 3.81 (broad signal, HOCH), 3.53 – 3.28 (broad multiplet, OCH2CH2), 2.61 – 2.41 (broad multiplet, NCH2), 1.62 (broad signal, OCH2CH3), 1.52 – 1.36 (broad signal, CH2CH3), 0.86 (t, CH3). GPC(THF): Mn = 4200, Mw = 4200, PDI (Mw/Mn) = 1.40.

BD N(Pen) (14): 1H NMR (500 MHz, Chloroform-d) δ 3.85 (broad signal, HOCH), 3.55 – 3.21 (broad multiplet, OCH), 2.59 (broad signal, NCH2), 1.63 (broad signal, OCH2CH), 1.46 (broad signal, NCH2CH2), 1.36 – 1.17 (broad multiplet, CH2), 0.87 (t, J = 9.5 Hz, CH3). GPC(THF): Mn = 4500, Mw = 6100, PDI (Mw/Mn) = 1.35.

BD N(Hex) (15): 1H NMR (500 MHz, Chloroform-d) δ 3.86 (broad signal, HOCH), 3.53 – 3.31 (broad multiplet, OCH2), 2.61 (broad multiplet, NCH2), 1.63 (broad signal, OCH2CH), 1.47 (broad signal, NCH2CH2), 1.27 (broad signal, CH2), 0.87 (t, CH3). GPC(THF): Mn = 5200, Mw = 5200, PDI (Mw/Mn) = 1.86.

BD N(Hep) (16): 1H NMR (500 MHz, Chloroform-d) δ 3.86 (broad signal, HOCH), 3.43 (broad multiplet, OCH), 2.61 (broad multiplet, NCH2), 1.63 (broad signal, OCH2CH), 1.47 (broad signal, NCH2CH2), 1.26 (broad signal, CH2), 0.87 (t, CH3). GPC(THF): Mn = 3700, Mw = 5900, PDI (Mw/Mn) = 1.61.

**General procedure for protonation of the polymers**

The polymer to be protonated was dissolved in DI water. Hydrochloric acid was then added and the reaction mixture was stirred for a few minutes. After this time, the polymer was purified using a dialysis membrane for overnight. Finally, the aqueous solution is freeze-dried and used for further studies without any purification.

PEG N(H/Bu) (2): 1H NMR (500 MHz, Deuterium Oxide) δ 4.33 – 4.17 (broad multiplet, HOCH), 3.90 – 3.22 (broad multiplet, OCH, NCH), 1.69 (broad signal, CH2CH2CH3), 1.39 (broad signal, CH2CH3), 0.93 (broad signal, CH3).

PPO N(H/Bu) (5): 1H NMR (500 MHz, Chloroform-d) δ 4.26 (broad signal, HOCH), 3.94 – 3.27 (broad multiplet, OCH, OCH2, NCH2), 1.74 (broad signal, CH2CH2CH3), 1.41 (broad signal, CH2CH3), 1.17 (broad signal, CH3), 0.95 (t, J = 7.4 Hz, CH2CH3).

EO N (H/Bu) (8): 1H NMR (500 MHz, Deuterium Oxide) δ 4.32 – 2.89 (broad multiplet, OCH, OCH2, NCH2), 1.64 (broad multiplet, CH2CH2CH3), 1.41 – 1.25 (broad multiplet, CH2CH3), 0.89 (broad signal, CH3).

BD N(H/Bu) (11): 1H NMR (500 MHz, Deuterium Oxide) δ 4.27 – 4.14 (broad multiplet, HOCH), 3.62 – 3.48 (broad multiplet, OCH2), 3.42 – 3.20 (broad multiplet, NCH2), 1.69 (broad signal, CH2CH2CH3), 1.60 (broad signal, OCH2CH2), 1.42 – 1.28 (broad signal, CH2CH3), 0.97 – 0.85 (broad signal, CH3).

**General procedure for alkylation of polymers**
The polymer (0.2 g) and silver tetrafluoroborate (1.2 eq., per repeating unit) was dissolved in acetonitrile (2 mL). After that, excess alkyl iodide (4 eq., per repeating unit) is added very slowly and drop-wise to the reaction mixture. After complete addition, the reaction mixture is stirred at 65 °C for 48 hours. After this time, the acetonitrile solution is precipitated into cold diethyl ether. The precipitate was separated by centrifugation and the collected solid was purified using a dialysis membrane for overnight. Finally, the aqueous solution is freeze-dried and used for further studies without purification.

**PEG N(Bu/Bu) (3):** $^1$H NMR (500 MHz, Deuterium Oxide) δ 4.39 – 4.22 (broad multiplet, HOCH), 3.95 – 3.15 (broad multiplet, OCH$_2$, NCH$_2$), 1.48 – 1.29 (broad multiplet, CH$_2$CH$_3$), 1.01 – 0.83 (broad multiplet, CH$_3$).

**PPO N(Bu/Bu) (6):** $^1$H NMR (500 MHz, Deuterium Oxide) δ 4.26 (broad multiplet, HOCH), 3.99 – 3.25 (broad multiplet, OCH, OCH$_2$, NCH$_2$), 1.74 (broad multiplet, CH$_2$CH$_2$CH$_3$), 1.40 (broad multiplet, CH$_3$CH$_3$), 1.18 (broad multiplet, CHCH$_3$), 0.97 (t, $J = 7.2$ Hz, CH$_3$).

**EO N(Bu/Bu) (9):** $^1$H NMR (500 MHz, Deuterium Oxide) δ 4.27 (broad multiplet, HOCH), 3.94 – 3.16 (broad multiplet, OCH, OCH$_2$, NCH$_2$), 1.71 (broad multiplet, CH$_2$CH$_2$CH$_3$), 1.39 (broad multiplet, CH$_3$CH$_3$), 0.96 (broad signal, CH$_3$).

**BD N(Bu/Bu) (12):** $^1$H NMR (500 MHz, Deuterium Oxide) δ 4.25 (br m, HOCH), 3.65 – 3.24 (broad multiplet, OCH$_2$, NCH$_2$), 1.84 – 1.56 (broad multiplet, CH$_2$CH$_2$CH$_3$), 1.47 – 1.30 (broad multiplet, CH$_3$CH$_3$), 1.02-0.88 (broad multiplet, CH$_3$).

**BD N(Pr/Pen) (17):** $^1$H NMR (500 MHz, DMSO-$d_6$) δ 5.45 (broad multiplet, OH), 4.15 (broad multiplet, HOCH), 3.57 – 3.17 (broad multiplet, OCH$_2$, NCH$_2$), 1.58 (broad multiplet, CH$_2$CH$_2$CH$_3$), 1.28 (broad signal, CH$_3$CH$_3$), 0.91 (broad signal, CH$_3$).

**BD N(Et/Hex) (18):** $^1$H NMR (500 MHz, DMSO-$d_6$) δ 5.63 – 5.32 (broad multiplet, OH), 4.14 (broad multiplet, HOCH), 3.62- 3.17 (broad multiplet, OCH, NCH$_2$), 1.74 - 1.48 (broad multiplet, OCH$_2$CH$_2$, NCH$_2$CH$_2$), 1.37 – 1.05 (broad multiplet, NCH$_2$CH$_3$, CH$_3$), 0.87 (broad signal, CH$_3$).

**BD N(Me/Hep) (19):** $^1$H NMR (500 MHz, DMSO-$d_6$) δ 5.45 (broad multiplet, OH), 4.18 (broad multiplet, HOCH), 3.53 – 3.23 (broad multiplet, OCH, NCH$_2$), 3.08 (broad multiplet, NCH$_3$), 1.70-1.47 (broad multiplet, OCH$_2$CH$_2$, NCH$_2$CH$_2$), 1.35-1.19 (broad multiplet, CH$_3$), 0.87 (broad signal, CH$_3$).

**Minimum Inhibitory Concentration (MIC$_{90}$) determination**

Bacterial suspensions of *Escherichia coli* and *Staphylococcus aureus* were grown in Luria-Bertani (LB) medium and *Mycobacterium smegmatis* was grown in tryptic soy broth (TSB) medium at 37 °C overnight. *E. coli* cell suspension was diluted with fresh LB medium to an optical density of 0.01 (inoculum of 10$^7$ CFU mL$^{-1}$) at 600 nm (OD$_{600}$) and suspension of *S. aureus* was diluted with OD$_{600}$ ~ 0.05 (inoculum of 10$^7$ CFU mL$^{-1}$). *M. smegmatis* was diluted with fresh TSB medium to an OD$_{600}$ ~ 0.03 (inoculum of 10$^6$ CFU mL$^{-1}$).

Polymers were first dissolved in DMSO at 50 mg/mL and diluted with PBS buffer (pH 7.4) to different concentrations and sterilized by irradiation in a UV light for 15 min before mixing 1:1 with bacterial cell suspensions in a 96 well plate.
LB medium without any polymer solution was inoculated and used as a positive control and LB medium without any bacteria was used as a negative control. Samples in all experiments were tested in quadruplicate. All samples and controls were incubated at 37 °C for overnight. Inhibitory percentages were calculated by the following equation.

\[
\text{inhibition} \% = \frac{OD_{600(\text{sample})} - OD_{600(\text{negative control})}}{OD_{600(\text{positive control})} - OD_{600(\text{negative control})}} \times 100 \%
\]

\(OD_{600(\text{sample})}\) indicates the \(OD_{600}\) value for bacteria after overnight incubation with polymer sample.

\(OD_{600(\text{negative control})}\) indicates the \(OD_{600}\) value for overnight incubation without any bacteria.

\(OD_{600(\text{positive control})}\) indicates the \(OD_{600}\) value for bacteria after overnight incubation without any polymer.

MIC is defined as the lowest polymer concentration to inhibit >90% bacterial growth (MIC_{>90}).

**Hemolysis assay**

Hemolytic activity of the polymers was determined using sheep’s blood. Red blood cells (RBCs) were pelletized by centrifuging 1 mL of the blood and washing the pellet at least four times with PBS buffer solution. A polymer solution of known concentration was diluted with PBS buffer (pH 7.4) to different concentrations and vigorously stirred. Appropriate amounts of polymers and 25 µL of the RBC suspension were added to PBS solution, achieving desired concentrations in a total volume of 1000 µL. The samples were incubated at room temperature for 2 h and afterward, the samples were centrifuged to re-pelletize the blood cells. 200 µL of the supernatant of each sample were placed into wells in a 96-well plate and the absorbance at 540 nm was measured. The percent hemolysis was calculated by following equation.

\[
\text{Percent Hemolysis} = \frac{OD_{540(\text{Sample})} - OD_{540(\text{negative control})}}{OD_{540(\text{positive control})} - OD_{540(\text{negative control})}} \times 100 \%
\]

\(OD_{540(\text{sample})}\) indicates the \(OD_{540}\) value after 2 h incubation with polymer sample.

\(OD_{540(\text{negative control})}\) indicates the \(OD_{540}\) value after 2 h incubation with PBS buffer.

\(OD_{540(\text{positive control})}\) indicates the \(OD_{540}\) value after 2 h incubation with DI water.

**Microorganism morphology**

*E. Coli* and *S. aureus* with or without polymers were examined using Field Emission Scanning Electron Microscopy (FE-SEM, Hitachi S-4800) operated at 10 kV. *E. Coli* and *S. aureus* were grown in LB medium at 37 °C overnight. Cell suspensions were diluted to OD_{600} = 1.0 (inoculum of ~10^9 CFU mL^{-1}). After making the suspensions, 40 µg of **BD N(Bu/Bu)** was added to a 1 mL cell stock solution of *E. coli* or *S. Aureus*. For the control, cell suspensions without any polymer were used. After incubation at 37 °C for 24 h, all samples were then fixed in glutaraldehyde solution (2.5%) for 2 h at room temperature. The bacteria cells were dehydrated using graded ethanol solutions (30, 50, 70, 90 and 100% v/v in water) and dried under vacuum chamber and observed by FE-SEM.
LIVE/DEAD bacterial viability assays

_E. Coli_ were grown in LB medium at 37 °C overnight. Cell suspensions were diluted to OD<sub>600</sub> = 1.0 (inoculum of ~10<sup>9</sup> CFU mL<sup>-1</sup>). After making the suspensions, 80 µg of BD N(Bu) or 1000 µg of EO N(Bu) were added to 1 mL cell stock solution of _E. coli_. For control, cell suspensions without any polymer were used. After incubation at 37 °C for 4 h or 24 h, 1.0 mL of LIVE/DEAD BacLight (Kit L7012) commercial solution, which was prepared by adding 3 µL of SYTO (3.34 mM) and 3 µL of propidium iodide (20 mM) to 2 mL of PBS buffer, was added to the solutions. After incubation in the dark for 15 min, cells were viewed under an Olympus BX53 microscope equipped with a 470 nm excitation filter and a 540 nm emission filter for green fluorescence, and 530 nm excitation filter and a 590 nm emission filter for red fluorescence.

Kinetic assay

Cultures of _E. coli_ and _S. aureus_ (inoculum of 10<sup>5</sup> CFU mL<sup>-1</sup>) were incubated with 2 x MIC 12 in LB broth at 37 °C. At defined intervals (10, 30, 60, 120 min), 100 µL of each culture was plated onto LB agar containing no antibiotics and incubated at 37 °C 24 h. Cell viability was assessed by determining CFU·mL<sup>-1</sup> values. Each assay was performed in triplicate.

_Streptomyces venezuelae_ (ATCC 15439) assay

Polymers were first dissolved in DMSO at 50 mg/mL and diluted with PBS buffer (pH 7.4) to different concentrations and sterilized by irradiation in a UV light for 15 min before mixing 1:1 with bacterial cell suspensions.

Polymers and Cultures of _Streptomyces venezuelae_ (inoculum of 10<sup>6</sup> CFU mL<sup>-1</sup>) was grown in tryptic soy broth (TSB) medium at 30 °C overnight.

Antibiotic assay

Bacterial suspensions of _Escherichia coli_ and _Staphylococcus aureus_ were grown in Luria-Bertani (LB) medium and _Mycobacterium smegmatis_ was grown in tryptic soy broth (TSB) medium at 37 °C overnight. _E. coli_ cell suspension was diluted with fresh LB medium to an optical density of 0.01 at 600 nm (inoculum of 10<sup>7</sup> CFU mL<sup>-1</sup>) (OD<sub>600</sub>) and suspension of _S. aureus_ was diluted with OD<sub>600</sub> ~ 0.05 (inoculum of 10<sup>7</sup> CFU mL<sup>-1</sup>). _M. smegmatis_ was diluted with fresh TSB medium to an OD<sub>600</sub> ~ 0.03 (inoculum of 10<sup>6</sup> CFU mL<sup>-1</sup>). Antibiotics (kanamycin and carbenicillin) dissolved in 50% H<sub>2</sub>O solution at 50 mg/mL were diluted with PBS buffer (pH 7.4) to different concentrations and sterilized by irradiation in a UV light for 15 min before mixing 1:1 with bacterial cell suspensions in a 96 well plate.

LB medium without any polymer solution was inoculated and used as a positive control and LB medium without any bacteria was used as a negative control. Samples in all experiments were tested in quadruplicate. All samples and controls were incubated at 37 °C for overnight. Inhibitory percentages were calculated as mentioned before.

Outer membrane permeabilization assay

The outer membrane permeabilization activity of polymer 12 was determined by an NPN (N-phenylnapthylamine) assay. _E. coli_ cells (OD<sub>600</sub> = 0.05, ~10<sup>8</sup> CFU mL<sup>-1</sup>) were harvested (4000 rpm, 4 °C, 10 min), washed, and re-suspended in 5 mM glucose/5 mM HEPES buffer at pH 7.2. Then, 30 µg of polymer 12 in HEPES buffer (3 X MIC<sub>90</sub>) was added to a cuvette containing 1 mL of cells and 10 µM NPN. The excitation and emission wavelengths used
were 350 nm and 420 nm, respectively (slit width was 10 nm in both cases). The uptake of NPN as a measure of outer membrane permeabilization was monitored by the increase in fluorescence of NPN for 10 min. The control experiment was carried out any polymer.

**Inner membrane permeabilization assay**

*E. coli* cells (OD$_{600} = 0.05$, $\sim 10^8$ CFU mL$^{-1}$) were harvested (4000 rpm, 4 °C, 10 min), washed, and re-suspended in PBS buffer at pH 7.2. Then, 30 μg of polymer 12 in PBS buffer (3 X MIC$_{90}$) was added to a cuvette containing 1 mL of cells and 15 μM propidium iodide (PI). The excitation wavelength was 535 nm (slit width: 10 nm), and the emission wavelength was 617 nm (slit width: 10 nm). The uptake of PI was measured by the increase in fluorescence of PI for 10 min. The control experiment was carried out any polymer.
Figure S1. $^1$H-NMR of polymer PEG N(Bu) (1) in D$_2$O.

Figure S2. $^1$H-NMR of polymer PEG N(H/Bu) (2) in D$_2$O.
Figure S3. $^1$H-NMR of polymer PEG N(Bu/Bu) (3) in D$_2$O.

Figure S4. $^1$H-NMR of polymer PPO N(Bu) (4) in CDCl$_3$. 
Figure S5. $^1$H-NMR of polymer PPO N(H/Bu) (5) in D$_2$O

Figure S6. $^1$H-NMR of polymer PPO N(Bu/Bu) (6) in D$_2$O
Figure S7. $^1$H-NMR of polymer EO N(Bu) (7) in D$_2$O.

Figure S8. $^1$H-NMR of polymer EO N(H/Bu) (8) in D$_2$O.
Figure S9. $^1$H-NMR of polymer $\text{EO N(Bu/Bu)}$ (9) in $\text{D}_2\text{O}$.

Figure S10. $^1$H-NMR of polymer $\text{BD N(Bu)}$ (10) in $\text{CDCl}_3$. 
Figure S11. $^1$H-NMR of polymer BD N(H/Bu) (11) in D$_2$O

Figure S12a. $^1$H-NMR of polymer BD N(Bu/Bu) (12) in D$_2$O
Figure S12. $^1$H-NMR of polymer \textbf{BD N(Bu/Bu)} (12) in DMSO-$d_6$.

Figure S13. $^1$H-NMR of polymer \textbf{BD N(Pr)} (13) in CDCl$_3$. 
Figure S14. $^1$H-NMR of polymer **BD N(Pen)** (14) in CDCl$_3$.

Figure S15. $^1$H-NMR of polymer **BD N(Hex)** (15) in CDCl$_3$. 
Figure S16. $^1$H-NMR of polymer BD N(Hep) (16) in CDCl$_3$.

Figure S17. $^1$H-NMR of polymer BD N(Pr/Pen) (17) in deuterated DMSO-$d_6$. 
Figure S18. $^1$H-NMR of polymer BD N(Et/Hex) (18) in deuterated DMSO-$d_6$.

Figure S19. $^1$H-NMR of polymer BD N(Me/Hep) (19) in deuterated DMSO-$d_6$. 
Figure S20. GPC trace (THF) of polymer **PEG N(Bu)** (1).

Figure S21. GPC trace (THF) of polymer **PPO N(Bu)** (4).
Figure S22. GPC trace (THF) of polymer **EO N(Bu)** (7).

Figure S23. GPC trace (THF) of polymer **BD N(Bu)** (10).
Figure S24. GPC trace (THF) of polymer **BD N(Pr)** (13).

Figure S25. GPC trace (THF) of polymer **BD N(Pen)** (14).
Figure S26. GPC trace (THF) of polymer **BD N(Hex)** (15).

Figure S27. GPC trace (THF) of polymer **BD N(Hep)** (16).
Figure S28. Clearing of the antibiotic producing *streptomyces venezuelae* bacterial suspension (left) upon treatment with 20 (second to left), 10 (second to right), and 5 (right) \( \mu \text{g/mL} \) concentration of polymer 12.

S29. Bacterial death as a function of time while using polymer 12 as an antimicrobial agent.
S30. Outer-membrane permeabilization of *E. coli* by cationic polymer 12 as measured by the increase in 1-N-phenyl-naphthylamine fluorescence (red line, left image). Inner-membrane permeabilization of *E. coli* by cationic polymer 12 as measured by the increase in propidium iodide fluorescence (red line, right). The black trace is from the control experiments carried out in absence of 12.