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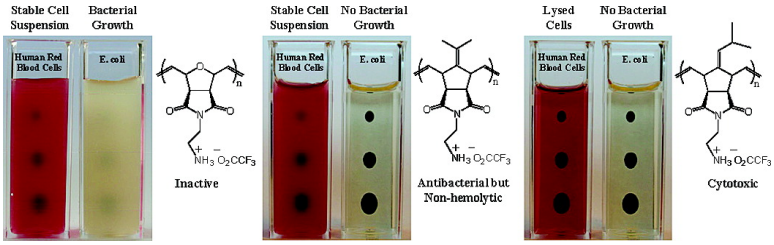


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# Tuning the Hemolytic and Antibacterial Activities of Amphiphilic Polynorbornene Derivatives

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***Abstract:*** Amphiphilic cationic polynorbornene derivatives, soluble in water, were prepared from modular norbornene monomers, with a wide range of molecular weights ($M_n$ = 1600−137 500 g/mol) and narrow polydispersities (PDI = 1.1−1.3). The antibacterial activity determined by growth inhibition assays and the hemolytic activity against human red blood cells were measured and compared to determine the selectivity of the polymers for bacterial over mammalian cells. The effects of monomer repeat unit hydrophobicity and polymer molecular weight on antibacterial and hemolytic activities were determined. The hydrophobicity of the repeat unit was observed to have dramatic effects on antibacterial and hemolytic activities. Lipid membrane disruption activities of the polymers was confirmed by measuring polymer-induced dye leakage from large unilamellar vesicles. By tuning the overall hydrophobicity of the polymer through random copolymerizations of modular norbornene derivatives, highly selective, nonhemolytic antibacterial activities were obtained. For appropriate monomer composition, selectivity against bacteria versus human red blood cells was determined to be over 100.

## Introduction

Antibacterial activities of macromolecules, including oligomeric compounds, have been studied under two major thrusts, for the most part independent from each other. One group of studies has focused on the structure−property relationships of natural host-defense peptides derived from multicellular organisms.[1−4] These peptides have great diversity with regard to their length, amino acid composition, and antimicrobial activities ranging from very potent to weak. Despite this diversity, most are cationic peptides with a certain degree of hydrophobicity. Extensive studies on the mechanism of action suggest that antimicrobial peptides act by permeabilizing the cell membranes of microorganisms through favorable interactions with negatively charged and hydrophobic components of the membranes followed by aggregation and subsequent disruption.[1,2,5,6] This mechanism is suggested to be responsible for the wide spectrum of potency and speed of action for these antibacterial peptides.[3] Host-defense peptides and their synthetic analogues are reported to exhibit varying degrees of activity against different bacteria and mammalian cells.[1] While host-defense peptides may show selectivity against the membranes of microbes versus the host organism, a number of them are antibacterial and not toxic to human cells, within certain concentration limits, and are thus considered as potential therapeutic agents.[1−3] Hemolytic activity is conventionally used as a measure of cytotoxicity and model for mammalian cells because red blood cells are, in general, extremely fragile.[4,5] The selective action has been suggested to be due to the balance and spatial arrangement of hydrophobic and hydrophilic components of the peptide that distinguishes between the more negatively charged outer surface of microbial membranes and the neutral and cholesterol rich membranes of multicellular animals. Studies aimed at understanding the structure−property relationships of natural peptides have recently evolved into a number of research efforts targeting the preparation of synthetic mimics of antimicrobial peptides. These include stereoisomers of natural peptides,[7,8] α-peptides,[9] β-peptides,[10−13] cyclic α-peptides,[14] peptoids,[15] and polyarylamides,[16] all of which are oligomeric with molecular weight below 3000 g/mol. Many of these examples target an amphiphilic secondary structure, typically helical, in addition to their cationic nature. Depending

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on the type of peptide, a facially amphiphilic structure results in the gain, or loss, of selective activity, which reveals that a stable amphiphilic secondary structure is not a precondition for selective antibacterial activity.[7,9,13] Resistance to enzymatic degradation was also targeted in some cases for potential use in therapeutic applications.[4,8,10,14,15]

Independent from the antimicrobial peptide research, a second thrust involves studies of synthetic cationic polymers that exhibit varying degrees of antibacterial activities.[17−23] This class of polymeric compounds is relatively inexpensive and less cumbersome to prepare, when compared to peptide mimics. In many instances, cationic polymers were reported to exhibit enhanced antibacterial activities compared to their small molecule counterparts. The most common polymers are quaternary ammonium or phosphonium functionalized. This class of cationic polymers was predominantly targeted for use in the solid state as potent disinfectants, biocidal coatings, or filters, due to their toxicity to human cells at relatively low concentrations which is an important distinction from the work on peptide mimics.[17,18] Consistent with the target applications of these cationic polymers, in most cases only antibacterial activity was reported without any report of hemolytic activity. In one instance, a soluble pyridinium polymer was reported to have low acute toxicity against the skin of test animals.[24] An example of antibacterial cationic polymers that have found large industrial use as disinfectants and biocides is poly(hexamethylene biguanide) (PHMB). Different levels of toxicity against various mammalian cells were reported for PHMB and similar biguanide functionalized polymers.[25−29] To the best of our knowledge, a direct comparison of antibacterial and hemolytic action has not been reported for this class of antimicrobial polymers. Gelman et al. have recently reported the antibacterial activity of low molecular weight, hydrophobically modified, cationic polystyrene derivatives in comparison with a potent derivative of magainin II.[30] In their initial study, a crossover between the research on antimicrobial peptide mimics and polymeric disinfectants, cationic polystyrene derivatives have shown similar antibacterial activities as the magainin derivative but were highly hemolytic. Recently, two of us reported selective activities of facially amphiphilic low molecular weight polyphenyleneethynylenes with activity and selectivity similar to those of a magainin derivative.[31] The successful design for nonhemolytic, antibacterial, and high molecular weight polymers has not been achieved thus far.

Ring-opening metathesis polymerization (ROMP) has been successfully used in the preparation of biologically active well-defined polymeric materials,[32] due to its living nature and functional group tolerance.[33,34] Remarkable examples included polymers carrying oligopeptides,[35] oligonucleotides,[36] carbohydrates,[37−39] anti-cancer drugs,[40] and antibiotic agents.[41] ROMP-based techniques are evolving into a powerful synthetic toolbox for the introduction of multiple functionalities into polymeric materials in pursuit of obtaining potent biological activities. We have recently reported the synthesis and ROMP of modular norbornene derivatives for the preparation of well-defined amphiphilic polymers exhibiting lipid membrane disruption activities.[42] Cationic amphiphilic polymers above certain molecular weights were reported to show the highest membrane disruption activities on lipid vesicles as rough models for bacterial membranes.

The current report presents the antibacterial and hemolytic activities of narrow polydispersity homopolymers and random copolymers of modular norbornene derivatives, spanning a large range of molecular weights. Our results show that, by controlling the hydrophobic/hydrophilic balance of water-soluble amphiphilic polymers, it is possible to obtain high selectivity between antibacterial and hemolytic activities without a predisposed amphiphilic secondary structure as part of the synthetic design. The overall efficacy toward both Gram-negative and Gram-positive bacteria is strongly dependent on the length of alkyl substituents on the repeat units. This report shows that it is possible to design simple polymers that are potent against bacteria yet nonhemolytic.

## Experimental Section

**Materials**. (Tricyclohexylphosphine)(1,3-dimesitylimidazolidine-2-ylidine)benzylideneruthenium dichloride, the second generation Grubbs' catalyst, was purchased from Strem Chemical. Stearoyl-oleoyl-phosphatidylcholine (SOPC) and phosphatidylserine (SOPS) were purchased from Avanti Polar-Lipids, Inc.. Cyclopentadiene for the synthesis of fulvene derivatives was obtained by the thermally induced cracking of dicyclopentadiene followed by distillation. Compounds **1**−**3**,[42] homopolymers of **1**−**4**,[42] and $[(H_2Imes)(3\text{-Br-py})_2\text{-}(Cl)_2Ru{=}CHPh]$[43] were prepared according to literature procedures. All other reagents were obtained from Aldrich. Deuterated chloroform and dichloromethane were passed through columns of basic activated alumina prior to use.


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**Instrumentation.** $^1$H (300 MHz), and $^{13}$C NMR (75 MHz) spectra were obtained on a Bruker DPX-300 NMR spectrometer. Gel permeation chromatography (GPC) was performed with a Polymer Lab LC1120 high-performance liquid chromatography (HPLC) pump equipped with a Waters differential refractometer detector. The mobile phase was tetrahydrofuran (THF) with a flow rate of 1.0 mL/min. Separations were performed with $10^5$, $10^4$, and $10^3$ Å Polymer Lab columns. Molecular weights were calibrated versus narrow molecular weight polystyrene standards. Fluorescence spectroscopy was recorded with a Perkin-Elmer LS50B luminescence spectrometer. Optical density and absorbance spectroscopy were recorded with a Molecular Devices SpectraMAX 190 plate reader.

**Preparation of 4.** Compound **4** was prepared by a slight modification of the literature procedure that was used for the preparation of compounds **2** and **3**.[42] To a solution of 4-heptanone (20 mmol, 2.28 g) and cyclopentadiene (20 mmol, 1.32 g) in methanol (20 mL) was added pyrrolidine (20 mmol, 1.42 g). The mixture was stirred at room temperature for 1 h, and acetic acid was added (20.1 mmol, 1.21 g). The reaction mixture was diluted with ether (50 mL) and water (50 mL). The ether portion was separated, washed with water (50 mL) and brine (50 mL), and dried over $MgSO_4$. Ether was removed under reduced pressure, and the product, di-*n*-propylfulvene, was used without further purification for the cycloaddition with maleic anhydride. The Diels−Alder reaction between di-*n*-propylfulvene (20 mmol, 3.24 g) and maleic anhydride (20 mmol, 1.96 g) was performed in ethyl acetate (50 mL) at 80 °C for 2 h in a sealed pressure tube. Upon removal of ethyl acetate under reduced pressure, the adduct was obtained in high yield as an oil (85:15 *exo*−*endo* ratio) and used without further purification. Previously reported mono protected diamine[44] (6.8 g, 42.3 mmol) was added to the Diels−Alder adduct (6.1 g, 23.5 mmol) in DMAc (*N*,*N*-Dimethylacetamide, 6 mL) at 60 °C and stirred for 20 min. A catalytic amount of cobalt acetate (0.5 mmol, 88.5 mg) dissolved in DMAc was added to this mixture followed by the addition of acetic anhydride (25 mmol, 255 mg) and the reaction mixture was stirred for 4 h at 80°C. After cooling to room temperature, the solution was diluted with ethyl acetate, washed with water and dilute HCl, dried, and evaporated under reduced pressure to afford 95% yield of an *exo*−*endo* (87:13) mixture of **4**. Recrystallization from cold diethyl ether afforded pure *exo* isomer **4** (50%). $^1$H NMR (300 MHz, $CDCl_3$, ppm): $\delta$ 6.42 (2H, t, $J = 2.1$ Hz), 5.05 (1H, s), 3.70 (2H, t, $J = 1.9$ Hz), 3.53 (2H, t, $J = 5.4$ Hz), 3.25 (2H, broad d, $J = 5.0$ Hz), 2.75 (2H, s), 1.82 (4H, t, $J = 7.8$ Hz), 1.42 (9H, s), 1.22 (4H, m), 0.81 (6H, t, $J = 7.3$ Hz). $^{13}$C NMR (75 MHz, $CDCl_3$, ppm): $\delta$ 177.6, 155.8, 141.9, 137.8, 123.2, 78.9, 47.8, 45.1, 38.8, 38.4, 33.1, 28.2, 21.7, 13.9. HRMS (FAB) calcd for $C_{23}H_{35}N_2O_4$: 403.260. Found: 403.260.

**Preparation of Poly4.** Homopolymerizations of **4** and subsequent deprotection of primary amine groups to obtain poly**4** were performed according to the previously reported literature procedure,[42] using bromopyridine-substituted derivative of second generation Grubbs' catalyst, [$(H_2Imes)(3\text{-Br-py})_2$-$(Cl)_2Ru{=}CHPh$].[45] $^1$H NMR (300 MHz, $D_2O$, ppm): $\delta$ 5.70−5.20 (2H, br), 4.10−3.50 (4H, br), 3.40−3.05 (4H, br), 2.20−1.70 (4H, br), 1.55−1.10 (4H, br), 1.00−0.60 (6H, s). $^{13}$C NMR (75 MHz, *d*-DMSO, ppm): $\delta$ 178.6 (br), 138.1 (br), 135.8, 132.4 (br), 51.3 (br), 47.9 (br), 44.2, 36.2, 33.5, 21.0, 13.8.

**Preparation of Random Copolymers.** The preparation of poly($\mathbf{2}_2$-*co*-$\mathbf{3}_1$) ($M_n = 15\,300$ g/mol) will be described as a representative procedure for the preparation of random copolymers of **2** and **3**. The comonomer feed ratio and catalyst-to-monomer ratio were changed in order to obtain random copolymers with desired comonomer content and molecular weights. A mixture of **2** (0.58 mmol) and **3** (0.29 mmol) was dissolved in dichloromethane or deuterated chloroform (1.5 mL), and a solution of catalyst (0.015 mmol in 0.05 mL of dichloromethane or deuterated chloroform) [$(H_2Imes)(3\text{-Br-py})_2$-$(Cl)_2Ru{=}CHPh$] was added at room temperature, under an inert atmosphere. The mixture was allowed to react for 90 min at 40 °C. The random progression of the copolymerization was monitored using in situ $^1$H NMR analysis, by probing the disappearance rates of the peaks at 1.53 ppm from **2**, and 2.24 ppm from **3** in deuterated chloroform solutions. Polymerization was terminated by addition of ethyl vinyl ether (0.2 mL) followed by precipitation in pentane resulting in a white polymer precipitate and brown supernatant. The product was filtered and dried overnight under reduced pressure at room temperature. A small sample was used for molecular weight determination. Deprotection of primary amine pendant groups was performed by dissolution of the polymer in trifluoroacetic acid and stirring at 45 °C for 8 h. Polymer was recovered by evaporation of trifluoroacetic acid under reduced pressure and dissolution in water followed by freeze-drying overnight. The isolated yield was 85% (275 mg). $^1$H NMR (300 MHz, $D_2O$, ppm): $\delta$ 5.90−5.10 (2H, br), 4.35−3.55 (4H, br), 3.55−2.90 (4H, br), 2.65−2.30 (33% of 1H, br), 2.00−1.20 (66% of 6H, br), 1.10−0.60 (33% of 6H, br).$^{13}$C NMR (75 MHz, $D_2O$, ppm): $\delta$ 180.4 (br), 163.7, 163.4, 163.2, 162.8, 162.3, 139.4 (br), 136.0 (br), 134.9 (br), 132. 2 (br), 131.4 (br), 130.6 (br), 122.6, 118.7, 114.9, 111.0, 52.8, 51.6 (br), 50.0 (br), 48.5 (br), 46.4 (br), 37.8, 36.7, 28.8 (br), 22.5, 21.0.

**Measurement of Hemolytic Activity.** Hemolytic activity measurements were performed with slight modifications of literature procedures.[7,12,45] Freshly drawn human red blood cells (HRBC, 30 $\mu$L), were suspended in 10 mL of TRIS saline (10 mM TRIS, 150 mM NaCl, pH 7.2, filtered through polyethersulfone membrane with 0.22 $\mu$m pore size) and rinsed 3 times by centrifugation (5 min at 1500 rpm) and resuspension in TRIS saline. Polymer solutions were prepared by dissolution in TRIS saline (10 mM TRIS, 150 mM NaCl, pH 7.2) at concentration of 8 mg/mL and further diluted as necessary. After the complete dissolution, the pH of the solution was adjusted to pH values between 6.5 and 7.0 depending on the solubility of polymer. TRIS saline solutions of poly**1**, poly**2**, and poly(**2**-*co*-**3**)s were adjusted to pH 7.0. TRIS saline solutions of poly**3** and poly**4** were adjusted to pH 6.5 because of slow precipitation of these polymers at higher pH values. After the pH adjustments, polymer solutions were filtered through polyethersulfone membranes (0.45 $\mu$m pore size). Freshly prepared polymer solutions with different concentrations were added to 100 $\mu$L of the above-prepared HRBC suspension to reach a final volume of 200 $\mu$L on a 96-well plate. The resulting mixture was kept at 37 °C for 30 min on a stirring plate. Then the plate was centrifuged (IEC Centra-4B, 10 min at 1500 rpm), and the supernatant in each well was transferred to a new plate. Hemolysis was monitored by measuring the absorbance of the released hemoglobin at 414 nm. 100% Hemolysis was obtained by adding 10 $\mu$L of TRITON-X solution (20 vol % in DMSO), a strong surfactant, to the above-prepared HRBC suspension. The upper limit of polymer concentration that was required to cause 50% hemolysis is reported as $HC_{50}$, where the absorbance from TRIS saline containing no polymer was used as 0% hemolysis. The value of percent hemolysis was reported in cases where it was below 50% hemolysis at the highest polymer concentration tested or above 50% hemolysis at the lowest polymer concentration tested. Relatively small absorbances of polymer solution due to residual catalyst at 414 nm, at the corresponding concentrations, were measured and subtracted from polymer−HRBC mixtures. All experiments were run in quadruplicate. Control experiments were run to monitor the hemolytic activity of TFA-treated ruthenium catalyst that may be present in trace amounts in polymer solutions. Catalyst was dissolved and stirred for 8 h at 45 °C in TFA followed by evaporation of TFA and dissolution in DMSO due to the insolubility of TFA-treated catalyst in TRIS saline. It must be noted that as a result of successive precipitations of the protected polymer in pentane, the majority of the initial ruthenium catalyst was removed from the polymer. Elemental analysis, which was performed to determine the residual ruthenium in the deprotected polymer, did

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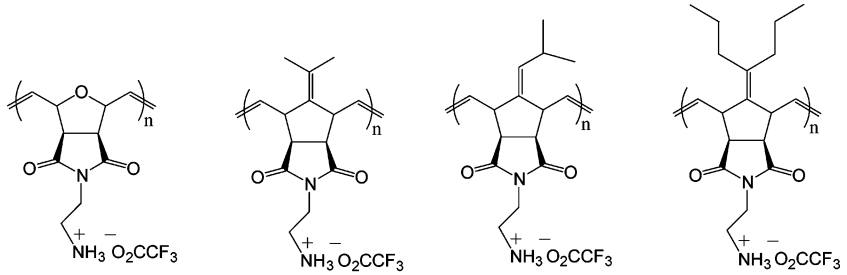


***Figure 1.*** Amphiphilic polynorbornene derivatives.

not show the presence of ruthenium down to 0.3 wt % for poly**2** ($M_n$ = 9950 g/mol) and poly**3** ($M_n$ = 10050 g/mol). No hemolytic activity was observed from the TFA-treated catalyst up to a concentration of 100 $\mu$g/mL (10 $\mu$L of 2 mg/mL solution in DMSO), a higher concentration than the possible residual catalyst at the highest polymer concentrations, within the time limits that were used for the hemolysis assays.

**Measurement of Antibacterial Activity.** Antibacterial activity measurements were performed with slight modifications of literature procedures.[7,12,31] Bacteria suspensions (*E. coli* D31 and *B. subtilis* ATCC 8037) were grown in Mueller−Hinton Broth (MHB) overnight at 37 °C, diluted with fresh MHB to an optical density of 0.1 at 600 nm ($OD_{600}$) and further diluted by a factor of 10. This suspension was mixed with different concentrations of freshly prepared polymer solutions in TRIS saline (pH 6.5−7.0), by serial dilutions in a 96-well plate, and incubated for 6 h at 37 °C. The $OD_{600}$ was measured for bacteria suspensions that were incubated in the presence of polymer solution or only TRIS saline. Antibacterial activity was expressed as minimal inhibitory concentration (MIC), the concentration at which more than 90% inhibition of growth was observed after 6 h. All experiments were run in quadruplicate. In a control experiment, the TFA-treated ruthenium catalyst did not show any antibacterial activity up to a concentration of 100 $\mu$g/mL, a higher concentration than the possible residual catalyst at the highest polymer concentrations, within the time limits that were used for antibacterial activity assays.

**Determination of Polymer-Induced Leakage of Vesicle Content.** The lipid vesicles were prepared with slight modifications of literature procedures.[7,42] Cholesterol (1.7 $\mu$mol) was dissolved in a chloroform solution of SOPC (17.2 $\mu$mol), and the chloroform was subsequently removed under a nitrogen stream followed by drying under reduced pressure for 3 h at room temperature to obtain the mixture as a dry film. The dried film was hydrated by addition of 2 mL of buffer containing calcein (40 mM) and sodium phosphate (10 mM, pH 7.0). The suspension was vortexed for 10 min. The suspension was sonicated 3 times in a bath type sonicator (Aquasonic 150 HT) at room temperature and freeze−thawed after each sonication. The nonencapsulated calcein was removed by eluting through a size exclusion Sephadex G-25-150 column with 90 mM sodium chloride, 10 mM sodium phosphate buffer (pH 7) as eluent. The average size of vesicles was measured to be 1 to 3 $\mu$m in diameter, as determined using optical microscopy (ZEISS Axiovert S100TV). The preparation of negatively charged SOPS/SOPC vesicles and the measurement of polymer-induced calcein leakage from lipid vesicles were performed according to a literature procedure.[42]

## Results and Discussion

**Amphiphilic Polynorbornene Derivatives.** We probed the biological activities of a class of amphiphilic polymers that were previously shown to exhibit lipid membrane disruption activities.[42] The amphiphilic polynorbornene derivatives bearing primary amine and variable length alkyl moieties as pendant groups were prepared by ROMP of modular norbornene derivatives using the [$(H_2Imes)(3\text{-Br-py})_2$-$(Cl)_2$Ru=CHPh] variant of Grubbs' catalyst.[43] These amphiphilic polymers provide a well-defined model for testing the effect of hydrophobicity and molecular weight of cationic polymers on antibacterial and hemolytic activities. The current study involves four types of repeating units (**1**−**4**) as shown in Figure 1. All homo- and copolymers of these monomers have narrow polydispersities, less than 1.3, and encompass a large range of molecular weight from oligomers to high polymers, up to 137 500 g/mol, as determined by THF GPC relative to polystyrene standards prior to the deprotection of polymer.[42] No preformed and stable polymeric secondary structure is expected from these macromolecules considering the imperfect tacticity of polynorbornene derivatives prepared by homogeneous ruthenium catalyst[34,46] and the presence of *cis*−*trans* isomers on the backbone unsaturations. Furthermore, the asymmetry in the isobutylidene group of poly**3** resulting from head-to-head and head-to-tail insertions leads to multiple dyad possibilities. In the case of random copolymers, there is the factor of additional compositional heterogeneity. All polymers are soluble in TRIS saline solutions at appropriate pH values (6.5−7.0).

**Antibacterial and Hemolytic Activities of Homopolymers.** The hydrophobicity of the repeating unit was observed to have dramatic effect on antibacterial and hemolytic activities of the amphiphilic polymers. The activity of each homopolymer with similar molecular weights (near 10 000 g/mol, $M_n$) was probed against Gram-negative bacteria (*E. coli*), Gram-positive bacteria (*B. subtilis)*, and human red blood cells (Table 1). Poly**1**, a cationic polymer with no substantial hydrophobic group, did not show any antibacterial or hemolytic activity within the measured concentrations. This result is consistent with the previously reported lack of activity against phospholipid membranes.[42] Introduction of a hydrophobic group at the repeat unit level produced an increase in antibacterial and hemolytic activities, which depended on the size of hydrophobic group. Poly**2**, with an isopropylidene pendant group, exhibited antibacterial activity with an MIC of 200 $\mu$g/mL against *E. coli*, which is less efficacious than most antimicrobial peptides and their mimics that have MICs typically ranging between 1 and 50 $\mu$g/mL.[1,3,8,9,11−16,47] However, poly**2** remained nonhemolytic up to the measured concentration of 4000 $\mu$g/mL, thus giving a selectivity, defined as the ratio of $HC_{50}$ to MIC,[16] greater than 20. Poly**3** with an additional carbon atom per repeat unit is more hydrophobic than poly**2**, and has additional mobility of the pendant alkyl group. Poly**3** exhibited a substantial increase in

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***Table 1.*** Antibacterial and Hemolytic Activities of Homopolymers[a]

| | MIC [$\mu$g/mL, ($\mu$M)] | | | selectivity ($HC_{50}$/MIC) | |
|---|---|---|---|---|---|
| polymer | *E. coli* | *B. subtilis* | $HC_{50}$ [$\mu$g/mL, ($\mu$M)][b] | *E. coli* | *B. subtilis* |
| poly**1** | >500, (>49) | >500, (>49) | >1000, (>98) | | |
| poly**2** | 200, (20) | 300, (30) | >4000, (>400) | >20 | >13 |
| poly**3** | 25, (2.5) | 25, (2.5) | <1, (<0.1) | <0.04 | <0.04 |
| poly**4** | 200, (19) | 200, (19) | <1, (<0.1) | <0.005 | <0.005 |

[a] $M_n$ and PDI values are 10 250 g/mol, 1.07 for poly**1**; 9950 g/mol, 1.10 for poly**2**; 10 050 g/mol, 1.13 for poly**3**; and 10 300 g/mol, 1.08 for poly**4**. $M_n$ and PDI values were determined by THF GPC relative to polystyrene standards, prior to deprotection of the polymer. [b] Poly**1** caused 5% hemolysis at 1000 $\mu$g/mL, the highest concentration measured. Poly**2** caused 25% hemolysis at 4000 $\mu$g/mL. Poly**3** caused 80% hemolysis at 1 $\mu$g/mL, and poly**4** caused 100% hemolysis at 1 $\mu$g/mL, the lowest concentrations measured.

***Table 2.*** Effect of Molecular Weight on Antibacterial and Hemolytic Activities[a]

| | | | MIC [$\mu$g/mL, ($\mu$M)] | | |
|---|---|---|---|---|---|
| polymer | $M_n$ (g/mol) | PDI | *E. coli* | *B. subtilis* | $HC_{50}$ [$\mu$g/mL, ($\mu$M)][b] |
| poly**2** | 1600 | 1.15 | 200, (125) | 300, (188) | >4000, (>2500) |
| | 24 100 | 1.10 | 200, (8.3) | 200, (8.3) | >4000, (>164) |
| | 49 600 | 1.14 | 200, (4.0) | 200, (4.0) | >4000, (>81) |
| | 137 500 | 1.27 | 200, (1.5) | 200, (1.5) | >4000, (>29) |
| poly**3** | 1650 | 1.26 | 25, (15) | 25, (15) | <1, (<0.6) |
| | 25 500 | 1.17 | 40, (1.6) | 40, (1.6) | <1, (<0.04) |
| | 57 200 | 1.70 | 80, (1.4) | 80, (1.4) | <1, (<0.02) |
| poly**4** | 5300 | 1.09 | 200, (38) | 200, (38) | <1, (<0.2) |
| | 32 200 | 1.13 | 200, (6.2) | 200, (6.2) | <1, (<0.04) |
| | 57 000 | 1.19 | 200, (3.5) | 200, (3.5) | <1, (<0.02) |

[a] $M_n$ and PDI values were determined by THF GPC relative to polystyrene standards, prior to the deprotection of polymer. [b]Poly**2**s caused 20−25% hemolysis at 4000 $\mu$g/mL. Poly**3**s caused 70−80% hemolysis at 1 $\mu$g/mL. Poly**4**s caused 100% hemolysis at 1 $\mu$g/mL.

antibacterial activity, with an MIC of 25 $\mu$g/mL for both *E. coli* and *B. subtilis*, as well as hemolytic activity, an $HC_{50}$ of less than 1 $\mu$g/mL (Table 1). This increase in antibacterial and hemolytic activity with increasing hydrophobicity is in accordance with literature reports that predict larger hydrophobic groups will have stronger interactions with the inner core of cell membranes leading to loss of selectivity.[1,4] However, in the case of poly**4**, when the hydrophobic size was further increased the hemolytic activity was retained, but the antibacterial activity decreased to an MIC of 200 $\mu$g/mL. In many instances, hydrophobic interactions have been reported to control hemolytic activities; whereas charge interactions are suggested to be more important for antibacterial activity.[1,9] These results show that the presence and balance of hydrophobic and hydrophilic groups dictate the antibacterial and hemolytic activities of the amphiphilic nonnatural polymer in agreement with natural peptide studies.

The effect of molecular weight on antibacterial and hemolytic activities was investigated for poly**2**, poly**3**, and poly**4** (Table 2). Changes in molecular weights over a large range did not result in significant changes in antibacterial and hemolytic activities of poly**2** and poly**4**. The antibacterial activity of poly**3** was observed to increase moderately as the molecular weight decreased from 57 200 g/mol to 10 300 g/mol or lower. Overall there was no substantial molecular weight dependence on antibacterial or hemolytic activities of these homopolymers if activity is reported in mass/volume rather than molarity. In the most commonly suggested mechanisms for membrane disruption based on amphiphilic peptides, there is some type of cooperative action, in either pore formation or coverage of the surface in a carpetlike manner.[2,5] If the membrane disruption activity is associated with the accumulation of the macromolecule on the membrane surface, it is a germane approach to report MIC values in units of mass/volume. Otherwise at the same molar concentrations higher molecular weight polymers would cover larger surfaces than lower molecular weight polymers. However, it should be noted that this approach underestimates the possible effect of the increase in the number of electrostatic and hydrophobic interactions at the membrane surface as a consequence of covalent connectivity resulting from higher molecular weights. One of many possible advantages of high molecular weight polymeric systems would be the ability of using them at relatively low molar concentrations if that is a requirement of the target application.

**Antibacterial and Hemolytic Activities of Random Copolymers.** The results from homopolymerization studies have shown the strong influence of subtle structural changes on the biological activities of these amphiphilic polymers. The low hemolytic activity of poly**2** and strong antibacterial activity of poly**3** suggest that copolymerization of monomers **2** and **3** would be a facile synthetic approach to optimize activity and selectivity. Random copolymers consisting of different comonomer ratios of **2** and **3** were prepared without compromising narrow polydispersities. In situ $^1$H NMR analysis revealed the equal disappearance rates of both monomers, suggesting random copolymer formation. This synthetic approach easily allows various compositions to be explored in contrast to polycondensation approaches earlier reported.[31,48] Poly($\mathbf{2}_9$-*co*-$\mathbf{3}_1$), the random copolymer of **2** and **3** with a final comonomer molar ratio of 9/1 respectively and an $M_n$ of 12 000 g/mol showed antibacterial activity near that of poly**3** while retaining the nonhemolytic character of poly**2** (Table 3). Remarkably, 10% of comonomer **3** content was enough to bring the antibacterial activity near homopolymers of **3** and exhibit excellent selectivity, a ratio greater than 100. In comparison, a Magainin derivative, MSI-78, showed a selectivity of 9.6 as determined

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***Table 3.*** Activities of Random Copolymers of **2** and **3**[a]

| | | | | MIC [$\mu$g/mL, ($\mu$M)] | | | selectivity ($HC_{50}$/MIC) | |
|---|---|---|---|---|---|---|---|---|
| polymer | $M_n$(g/mol) | PDI | **2**/**3**[b] | *E. coli* | *B. subtilis* | $HC_{50}$ [$\mu$g/mL, ($\mu$M)][c] | *E. coli* | *B. subtilis* |
| poly($\mathbf{2}_9$-*co*-$\mathbf{3}_1$) | 12000 | 1.09 | 9/1 | 40, (3.3) | 40, (3.3) | >4000, (>333) | >100 | >100 |
| poly($\mathbf{2}_2$-*co*-$\mathbf{3}_1$) | 15300 | 1.15 | 2/1 | 40, (2.6) | 40, (2.6) | >4000, (>261) | >100 | >100 |
| | 93700 | 1.21 | 2/1 | 80, (0.9) | 80, (0.9) | >4000, (>43) | >50 | >50 |
| poly($\mathbf{2}_1$-*co*-$\mathbf{3}_2$) | 8500 | 1.09 | 1/2 | 40, (4.7) | 40, (4.7) | <1, (<0.12) | <0.025 | <0.025 |
| | 32600 | 1.19 | 1/2 | 80, (2.5) | 80, (2.5) | <1, (<0.03) | <0.013 | <0.013 |
| poly($\mathbf{2}_1$-*co*-$\mathbf{3}_4$) | 11800 | 1.15 | 1/4 | 40, (3.4) | 40, (3.4) | <1, (0.08) | <0.025 | <0.025 |

[a] $M_n$ and PDI values were determined by THF GPC relative to polystyrene standards, prior to the deprotection of polymer. [b] The comonomer ratios in the copolymers were determined by $^1$H NMR analysis. [c] Poly($\mathbf{2}_9$-*co*-$\mathbf{3}_1$) caused 15% hemolysis and poly($\mathbf{2}_2$-*co*-$\mathbf{3}_1$)s caused 20−25% hemolysis at 4000 $\mu$g/mL. Poly($\mathbf{2}_1$-*co*-$\mathbf{3}_2$)s caused 60−70% hemolysis and poly($\mathbf{2}_1$-*co*-$\mathbf{3}_4$) caused 75% hemolysis at 1 $\mu$g/mL.

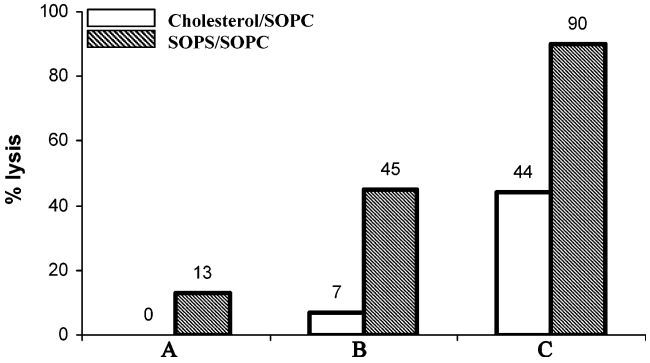


***Figure 2.*** Lysis of neutral vesicles (Cholesterol/SOPC) and negatively charged vesicles (SOPS/SOPC), at 3 min, caused by 25 $\mu$g/mL poly**2** (A), $M_n$ = 9950 g/mol; poly($\mathbf{2}_2$-*co*-$\mathbf{3}_1$) (B), $M_n$ = 15 300 g/mol; and poly(**3**) (C), $M_n$ = 10 500 g/mol. Percent lysis values are given on top of the bars.

in control experiment.[49] Poly($\mathbf{2}_2$-*co*-$\mathbf{3}_1$)s have also shown high selectivity where antibacterial activity was slightly decreased with increasing molecular weight as in the case of poly**3**. These copolymers, with selectivity values reaching over 100, are powerful examples of the ability to obtain good antibacterial activity from nonhemolytic polymers by fine-tuning the hydrophobic/hydrophilic balance and molecular weight. Poly($\mathbf{2}_1$-*co*-$\mathbf{3}_2$)s and poly($\mathbf{2}_1$-*co*-$\mathbf{3}_4$)s exhibited high hemolytic activities in accordance with the increased content of hemolytic comonomer **3**.

**Disruption of Lipid Vesicle Membranes.** Polymer-induced fluorescent dye leakage from negatively charged and neutral large unilamellar vesicles (LUV) were measured. Lipid vesicles provide simplified models for bacterial and mammalian cell membranes, although they underestimate several factors such as cell walls and lipopolysaccharides in bacterial cell membranes. At the same time, these assays are well documented in the literature and provide useful insight.[7,12,48] Therefore, these tests were used to study the overall membrane disruption activities of polymers but not to make direct comparisons of the activities against vesicles or biological cells. Poly**2** was inactive against neutral vesicles and showed little disruption of negatively charged vesicles at the measured concentrations (Figure 2). Poly($\mathbf{2}_2$-*co*-$\mathbf{3}_1$)s were found to exhibit increased activity against negatively charged vesicles while retaining low activities against neutral vesicles, with a selectivity near 6. Poly**3** was highly active against both types of membranes with a lower selectivity of 2. The above results confirm the membrane activity of these biologically active high molecular weight polymers but underestimate the degree of selectivity measured for poly($\mathbf{2}_2$-*co*-$\mathbf{3}_1$)s during in vitro experiments.

(49) Table S1 in the Supporting Information.

## Conclusion

Amphiphilic polymers based on modular norbornene derivatives were shown to exhibit good antibacterial activities and high selectivity for bacteria versus red blood cells. This class of polymers was prepared through a ROMP-based facile synthetic strategy that allows excellent control over monomer composition, molecular weight, polydispersity, and amphiphilicity. Small modifications to the hydrophobic character of the cationic amphiphilic polymer were shown to dramatically change the antibacterial and hemolytic activities. Tuning the hydrophobic/hydrophilic balance and molecular weights of these copolymers allowed preparation of highly selective, antibacterial nonhemolytic macromolecules. Desired biological activities were maintained across a large range of molecular weights. Furthermore, this study showed the preparation of fully synthetic high molecular weight polymers that mimic the activities of host-defense peptides in the absence of a specific secondary structure.

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**Supporting Information Available:** Antibacterial and hemolytic activities of a Magainin derivative (MSI-78) as reference. Polymer concentration versus percent hemolysis curves. This material is available free of charge via the Internet at http://pubs.acs.org.

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