Hydrophilic Modifications of an Amphiphilic Polynorbornene and the Effects on its Hemolytic and Antibacterial Activity

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Here we report the modification of an amphiphilic antibacterial polynorbornene, **Poly3**, via incorporation of hydrophilic, biocompatible groups. The sugar, zwitterionic, and polyethylene glycol based moieties were incorporated in varying ratios by copolymerization and postpolymerization techniques. Well-defined copolymers with molecular weights of 3 kDa and narrow polydispersity indices ranging from 1.08 to 1.15 were obtained. The effects of these modifications on the biological activity of these polymers were analyzed by determining their minimum inhibitory concentrations (MIC) and their hemolytic activities (HC_{50}).

Introduction

Antimicrobial peptides (AMPs) are part of the natural defense mechanism of many living organisms against pathogens.[1,2] They have broad spectrum activity and are highly selective for bacteria over mammalian cells. Though they can be found in very diverse structures, their common mechanism of action is the adoption of a specific conformation, where hydrophilic and hydrophobic components are segregated into two different faces of the molecule, which is known as facial amphiphilicity.[2] AMPs possess an overall positive charge in the hydrophilic region, which has been suggested to help them bind to the negatively charged outermost leaflet of the bacterial membrane through electrostatic attractions. These nonspecific interactions and the versatility of the AMPs have been proposed to limit bacterial resistance to them.[2] All these distinctive properties of AMPs led to increased interest in new synthetic mimics of antimicrobial peptides (SMAMPs), which include peptoids,[3−5] α- and β-amino acids,[6−13] aromatic oligomers,[14−16] and synthetic polymers.[17−24]

The first example of SMAMPs, based on ring opening metathesis polymerization (ROMP), was reported by Ilker et al.,[21] where a series of polymers was synthesized by varying the hydrophobicity of the backbone, at the repeat unit level, to tune the hemolytic and antibacterial properties of the facially amphiphilic polymers. It was shown that these SMAMPs could be transformed from nonselectively active (biocidal) to antimicrobial but nonhemolytic with changes in the backbone hydrophobicity. The most potent SMAMP was **Poly3** (Figure 1), with minimum inhibitory concentration (MIC, the lowest concentration required to inhibit 90% of the bacterial growth) of 25 μg/mL but selectivity (the ratio of HC_{50} (the concentration required to lyse 50% of human red blood cells) and MIC) of less than 1 due to the large hydrophobic groups. In an attempt to improve the selectivity of this SMAMP, the effects of increasing the charge density were studied by Al-Badri et al.[25] The number of amine groups on the polymer were varied by introducing groups carrying one, two, and three amines per repeat unit while keeping the backbone structure constant. It was concluded that increasing the number of amine groups on the polymer resulted in a reduction of its hemolytic activity, thus, increased selectivity was gained.

Here we report an alternative approach to fine-tune the properties of this interesting polymer, **Poly3**, by incorporating hydrophilic, biocompatible functionalities including sugar, zwitterionic, and polyethylene glycol (PEG) moieties (Figure 1). Carbohydrate based sugars, in addition to being used as solubilizing agents, play an important role in application areas of biomolecular chemistry.[26,27] PEG and various zwitterions have been widely used for improving the water-solubility of compounds.[28,29] Having similar properties, all these groups have been routinely employed in conjunction with each other as solubilizing agents in many biological systems,[28,30,31] where they were found to be nontoxic, thus, making them good candidates for this study. The importance of repeat unit level facial amphiphilicity, rather than globally (or the entire molecule), on the selectivity of SMAMPs was recently emphasized.[23] Easily synthesizable structures that contain both hydrophobic and hydrophilic units on the same monomer allowed successful tuning of the SMAMPs' selectivities. The advantages of the facial amphiphilicity repeat unit approach was confirmed with comparison to random copolymers of hydrophilic and hydrophobic monomers.[32] Therefore, in this study, facially amphiphilic monomers having the same hydrophobic backbone, but various hydrophilic pendant groups, were incorporated into **Poly3** via ROMP (Figure 1). The resulting effects on the overall biological activities of these polymers were studied via determination of MICs coupled with hemolytic activity (HC_{50}) studies.

Experimental Section

Materials and Instrumentation. Poly(ethylene glycol) methylether acrylate (PEG-acrylate; 99%), triphenylphosphine (Ph_3P; 99%), diisopropylazodicarboxylate (DIAD; 94%), second generation Grubbs' catalyst, and 3-bromopyridine (99%) were purchased from Sigma-Aldrich and used as received. Maleimide (98%) was obtained from Alfa Aesar and used as received. 1,2:3,4-Di-*O*-isopropylidene-D-galactopyranose (97%), *N*,*N*-dimethylethanolamine (99%), and ethylvinyl ether (EVE) (99%) were purchased from Acros Organics and used without further purification. 4-Bromobutanoic acid *tert*-butyl ester

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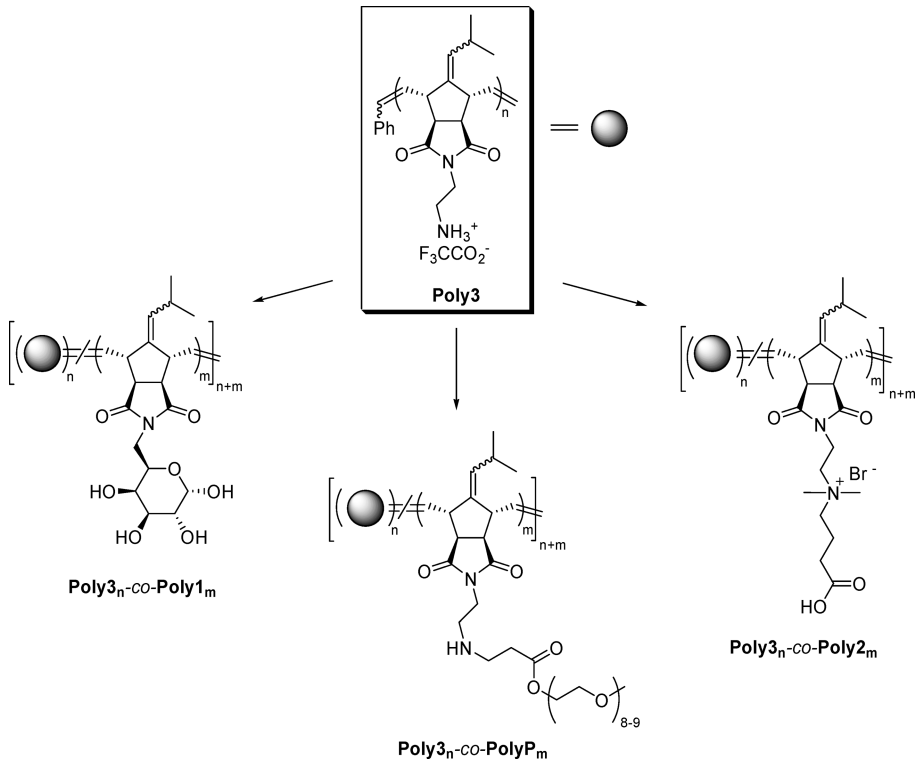


Figure 1. Schematic representation of modifications to **Poly3** via incorporation of hydrophilic groups. Explanation of nomenclature: **Poly3$_n$**-*co*-**Poly1$_m$**: **n** indicates the mol fraction of **M3** and **m** indicates the mol fraction of **M1** in the copolymer composition.

(95%) was purchased from Astatech, Inc. and used as is. Ethyl acetate, hexane, anhydrous diethyl ether, methanol, sodium bicarbonate ($NaHCO_3$), citric acid, and trifluoroacetic acid (TFA) were purchased from Fisher Scientific and used as received. Tetrahydrofuran (THF) was obtained from Fisher Scientific and was distilled form sodium/benzophenone under nitrogen before use. Dichloromethane (DCM; Fisher Scientific) was distilled from CaH_2, under nitrogen. Third generation Grubbs' catalyst (G3; dichloro-di(3-bromopyridino)-*N*,*N*′-dimethylenoimidazolino-Ru=CHPh) was synthesized according to a previously published procedure.[33]

^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker DPX300 spectrometer (Bruker, Madison, WI). Gel permeation chromatography (GPC) was performed using a Polymer Laboratories PL-GPC50 (Amherst, MA) instrument equipped with a PL Gel 5 μm precolumn and two 5 μm Mixed D columns. The mobile phase was THF with a flow rate of 1 mL/min and toluene was used as a flow marker. The instrument was calibrated with narrow molecular weight polystyrene standards using a Knauer RI detector. High resolution mass spectra were obtained on a JEOL JMS 700 instrument (JEOL, Peabody, MA).

Synthesis of M1. Isopropylfulvene (7.0 g, 58.3 mmol), synthesized according to a previously reported procedure,[34] and maleimide (4.7 g, 48.6 mmol) were charged in a Schlenk flask and dissolved in toluene (100 mL). The reaction mixture was placed in an oil bath at 135 °C, stirred for 12 h, then cooled to room temperature. Excess toluene was removed under reduced pressure to yield a mixture of exo/endo (9/1 by ^{1}H NMR) product. The pure exo product **1** was isolated by crystallization from diethyl ether as a colorless solid. Yield: 60%. ^{1}H NMR (300 MHz, $CDCl_3$, ppm) δ 0.87 (q, J = 6.6 Hz, 6H), 2.3 (m, 1H), 2.77 (m, 2H), 3.37 (s, 1H), 3.74 (s, 1H), 4.76 (d, J = 9.6 Hz, 1H), 6.4 (m, 2H), 8.24 (s, 1H). ^{13}C NMR (75 MHz, $CDCl_3$, ppm) δ 22.97, 23.47, 28.10, 44.53, 48.61, 49.04, 120.90, 136.99, 137.54, 143.62, 178.10, 178.21.

Compound **1** (2.0 g, 9.2 mmol), Ph_3P (2.4 g, 9.2 mmol), and 1,2:3,4-di-*O*-isopropylidene-D-galactopyranose (2.4 g, 9.2 mmol) were dissolved in dry THF (50 mL) in a 250 mL round-bottom flask under a N_2 atmosphere. The solution was then cooled to 0 °C in an ice bath. DIAD (1.8 g, 1.8 mL, 9.2 mmol) was added dropwise to the cooled reaction mixture. After addition was complete, the ice bath was removed and the solution was stirred at room temperature for 24 h. Excess THF was removed under reduced pressure to yield a yellow, oily product, which was recrystallized from toluene. After discarding the white precipitate, the excess toluene from the filtrate was removed under reduced pressure to yield an oily product. The pure **M1** was isolated by crystallization from cold diethyl ether to afford a colorless solid. Yield: 50%. ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.77 (q, J = 4.14 Hz, 3H), 0.85 (d, J = 6.59 Hz, 3H), 1.29 (d, J = 17.7 Hz, 6H), 1.45 (d, J = 9.2 Hz, 6H), 2.23 (m, 1H), 2.76 (m, 2H), 3.27 (d, J = 2.6 Hz, 1H), 3.34 (d, J = 2.6 Hz, 1H), 3.69 (d, J = 3.0 Hz, 1H), 3.95 (m, 1H), 4.17 (d, J = 8.3 Hz, 2H), 4.26 (q, J = 2.4 Hz, 1H), 4.58 (d, J = 2.5 Hz, 1H), 4.65 (d, J = 9.6 Hz, 1H), 5.43 (d, J = 5.1 Hz, 1H), 6.38 (m, 2H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 23.82, 24.45, 25.33, 25.79, 26.54, 26.73, 28.89, 39.76, 45.24, 48.32, 48.64, 49.71, 64.87, 71.17, 71.64, 72.23, 97.05, 109.54, 110.56, 121.43, 137.80, 138.65, 144.81, 177.67, 178.69. HR-MS (FAB): calcd, 459.53 g/mol; found, 460.20 g/mol.

Synthesis of M2. Compound **1** (4.0 g, 18.4 mmol), Ph_3P (4.8 g, 18.4 mmol), and *N*,*N*-dimethylethanolamine (1.6 g, 1.8 mL, 18.4 mmol) were dissolved in dry THF (150 mL) in a 250 mL round-bottom flask under a N_2 atmosphere. The solution was then cooled to 0 °C in an ice bath. DIAD (3.7 g, 3.6 mL, 18.4 mmol) was added dropwise to the cooled reaction mixture. After addition was complete, the ice bath was removed and the solution was stirred at room temperature for 24 h. Excess THF was removed under reduced pressure. The remaining product was extracted from ethyl acetate into citric acid solution (pH = 4.0), and the pH of the solution was adjusted to 13.0 by the addition of saturated $NaHCO_3$ solution. Compound **2** was then extracted with hexane/diethyl ether mixture. The excess solvent was removed under reduced pressure to yield a colorless powder. Yield = 70%. ^{1}H NMR (300 MHz, CD_3OD) δ 0.86 (q, J = 6.8 Hz, 6H), 2.27 (m, 7H), 2.5 (t, J = 6.7 Hz, 2H), 2.81 (dd, J = 7.2 Hz, 2H), 3.26 and 3.66 (s, 2H), 3.53 (t, J = 6.7 Hz, 2H), 4.63 (d, J = 9.4 Hz, 1H), 6.46 (m, 2H). ^{13}C NMR (75 MHz, CD_3OD) δ 23.49 and 24.01, 29.21, 36.93, 45.46 and 45.53, 49.07 and 49.13, 50.08, 57.06, 120.9, 138.22 and 138.95, 146.44, 179.39 and 179.52.

Compound **2** (2.0 g, 6.9 mmol) was dissolved in 50 mL of dry THF. To the solution 4-bromobutanoic acid *tert*-butyl ester (3.1 g, 13.8 mmol) was added and the reaction mixture was stirred at 50 °C for 36 h. The product was filtered, washed with excess dry THF, and dried under vacuum to yield **M2** as a colorless powder. Yield = 65%. ^{1}H NMR (300 MHz, CD_3OD) δ 0.88 (q, J = 6.7 Hz, 6H), 1.48 (s, 9H), 2.04 (m, 2H), 2.29 (m, 1H), 2.43 (t, J = 6.6 Hz, 2H), 2.9 (m, 2H), 3.19 (m, 6H), 3.45 (m, 4H), 3.72 (m, 2H), 3.91 (t, J = 7.2 Hz, 2H), 4.67 (d, J = 9.2 Hz, 1H), 6.48 (m, 2H). ^{13}C NMR (75 MHz, CD_3OD) δ 19.8, 24.3, 25.2, 29.2, 30.1, 32.8, 33.7, 46.4, 52.8, 61.5, 65.3, 83.0, 121.9, 139.1, 139.8, 147.1, 173.8 (ester, -C=O), 179.7 (imide, -C=O). HR-MS (FAB): calcd, 431.59 g/mol; found, 431.29 g/mol.

Homo and Copolymer Synthesis and Deprotection. *Homopolymerization of M1 and M2.* All the monomers were polymerized using third generation Grubbs' catalyst as an initiator. The same procedures were followed for the homopolymerization of M1, M2, and the deprotection of the corresponding polymers. In a typical experiment, M1 (0.4 g, 0.8 mmol) and G3 (0.013 g, 0.014 mmol) were weighed in separate reaction flasks and purged with N_2 gas. The monomer was dissolved in 1 mL and the catalyst was dissolved in 0.5 mL of THF. In the homopolymerization of M2, the monomer was dissolved in 1 mL of methanol due to its insolubility in THF. Both solutions were degassed by three freeze−pump−thaw cycles. After warming the solutions to room temperature, the monomer solution was cannulated into the catalyst solution. The reaction mixture was stirred at 60 °C for 2 h. The reaction was cooled in an ice bath and terminated by the addition of EVE (0.3 g, 0.6 mL, 6.3 mmol). The reaction mixture was further stirred for 1 h. The resultant homopolymers, **Poly1** and **Poly2**, were precipitated from excess diethyl ether to yield dark brown powders. Yield = 95%.

Poly1: ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.92 (br m, 6H), 1.27 (br m, 3H), 1.33 (br m, 3H), 1.48 (br m, 3H), 1.59 (br m, 3H), 2.41 (br m, 1H), 3.04 (br m, 2H), 3.37 (br m, 2H), 3.77 (br m, 1H), 3.99 (br m, 1H), 4.18 (br m, 2H), 4.27 (br m, 1H), 4.59 (br m, 1H), 5.16 (br m, 1H), 5.42 (br m, 2H), 5.61 (br m, 1H). **Poly2**: ^{1}H NMR (300 MHz, DMSO-d_6) δ 0.89 (br m, 6H), 1.42 (br m, 9H), 1.91 (br m, 2H), 2.32 (br m, 3H), 2.82 (br m, 2H), 3.15 (br m, 6H), 3.43 (br m, 4H), 3.64 (br m, 2H), 3.8 (br m, 2H), 4.6 (d, 1H), 5.18 (br m, 2H, cis), 5.33 (br m, 2H, trans).

Deprotection of Poly1 and Poly2. The deprotection reactions of all the polymers were done as follows: in a typical procedure, 0.25 g of **Poly1** was dissolved in 3 mL of neat TFA and stirred at room temperature for 24 h. The deprotected polymer was precipitated from diethyl ether. The deprotected **Poly1** was dried under vacuum for 24 h, then dissolved in 5 mL of methanol and 25 mL of RO water, and freeze-dried to remove all organic solvents. Yield = 85%.

Deprotected **Poly1**: ^{1}H NMR (300 MHz, DMSO-d_6) δ 0.87 (br m, 6H), 3.21 (br m, 2H), 3.49 (br m, 4H, -OH), 4.09 (br m, 1H), 4.84 (br m, 1H), 4.99 (br m, 1H), 5.14 (br m, 1H), 5.35 (br m, 1H), 5.58 (br m, 1H), 7.32 (br m, 2H, aromatic), 7.41 (br m, 3H, aromatic).

Deprotected **Poly2**: ^{1}H NMR (300 MHz, DMSO-d_6) δ 0.88 (br m, 6H), 1.92 (br m, 2H), 2.33 (br m, 3H), 2.81 (br m, 2H), 3.12 (br m, 6H), 3.4 (br m, 4H), 3.7 (br m, 4H), 5.2 (br m, 2H, cis), 5.3 (br m, 2H, trans).

Copolymerizations of M1 and M2 with M3. The same procedure described above for the homopolymerization of **Poly1** and **Poly2** was used for the synthesis of all **Poly3**-*co*-**Poly1** and **Poly3**-*co*-**Poly2** series. Corresponding monomers were mixed in the same reaction vessel prior to reacting with the catalyst. Copolymers with varying compositions were synthesized by adjusting the molar amounts of the monomers at the desired ratios.

The monomer composition of the copolymers was quantified by ^{1}H NMR analysis. For the **Poly3**-*co*-**Poly1** samples copolymer composition was determined by comparing the integration value of methyl protons (6H, at 0.92 ppm) at the backbone to the -NH_3 (3H, at 7.88 ppm) protons of **M3** after deprotection. In the case of **Poly3**-*co*-**Poly2** copolymer composition was determined from the comparison of the integration value of methyl protons (6H, at 0.92 ppm) at the backbone to the methyl protons (9H, at 1.39 ppm) of the *tert*-butyl group of the **M2**.

Poly3-*co*-**Poly1** (50:50): ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.92 (br m, 6H), 1.27 (br m, 3H), 1.33 (br m, 3H), 1.41 (br m, 9H), 1.47 (br m, 6H), 2.41 (br m, 1H), 3.04 (br m, 2H), 3.29 (br m, 2H), 3.61 (br m, 2H), 3.78 (br m, 1H), 3.98 (br m, 1H), 4.17 (br m, 2H), 4.27 (br m, 1H), 4.60 (br m, 1H), 5.17 (br m, 2H), 5.42 (br m, 1H), 5.62 (br m, 1H). Deprotected **Poly3**-*co*-**Poly1** (50:50): ^{1}H NMR (300 MHz, DMSO-d_6) δ 0.88 (br m, 6H), 2.95 (br m, 2H), 3.21 (br m, 2H), 3.43 (br m, 4H, -OH), 3.61 (br m, 2H), 3.87 (br m, 1H), 4.07 (br m, 1H), 4.85 (br m, 1H), 5.02 (br m, 1H), 5.16 (br m, 1H), 5.29 (br m, 2H), 5.46 (br m, 1H), 5.59 (br m, 1H), 7.32 (br m, 2H, aromatic), 7.40 (br m, 3H, aromatic), 7.88 (br m, 3H, -NH_3^+).

Poly3-*co*-**Poly2** (50:50): ^{1}H NMR (300 MHz, CD_3OD) δ 0.92 (br m, 6H), 1.39 (br m, 9H), 1.44 (br m, 9H), 2.03 (br m, 1H), 2.39 (br m, 2H), 3.19 (br m, 2H), 3.29 (br m, 6H), 3.52 (br m, 2H), 3.95 (br m, 2H), 5.54 (br m, 1H), 5.71 (br m, 1H), 7.28 (br m, 2H), 7.38 br m, 2H). Deprotected **Poly3**-*co*-**Poly2** (50:50): ^{1}H NMR (300 MHz, DMSO-d_6) δ 0.87 (br m, 6H), 1.91 (br m, 1H), 2.33 (br m, 2H), 3.09 (br m, 2H), 3.39 (br m, 6H), 3.61 (br m, 2H), 3.91 (br m, 2H), 5.54 (br m, 1H), 5.62 (br m, 1H), 7.5 (br m, 5H), 8.04 (br m, 3H).

Synthesis of Poly3-co-PolyP. A postfunctionalization approach was utilized for the synthesis of **Poly3**-*co*-**PolyP** copolymer. **Poly3** was synthesized and deprotected according to a previous literature procedure.[34] The repeat unit determination was done by GPC analysis. In a typical reaction, deprotected **Poly3** (0.15 g, 0.4 mmol) was dissolved in 10 mL of DMSO. Triethyl amine (0.02 g, 0.20 mmol) was added to the reaction mixture and stirred for 30 min, followed by the addition of PEG-acrylate (0.15 g, 0.3 mmol). The reaction mixture was stirred at room temperature for 24 h. The pure polymer was isolated by crystallization three times from cold diethyl ether, affording a light brown solid. Yield: 70%.

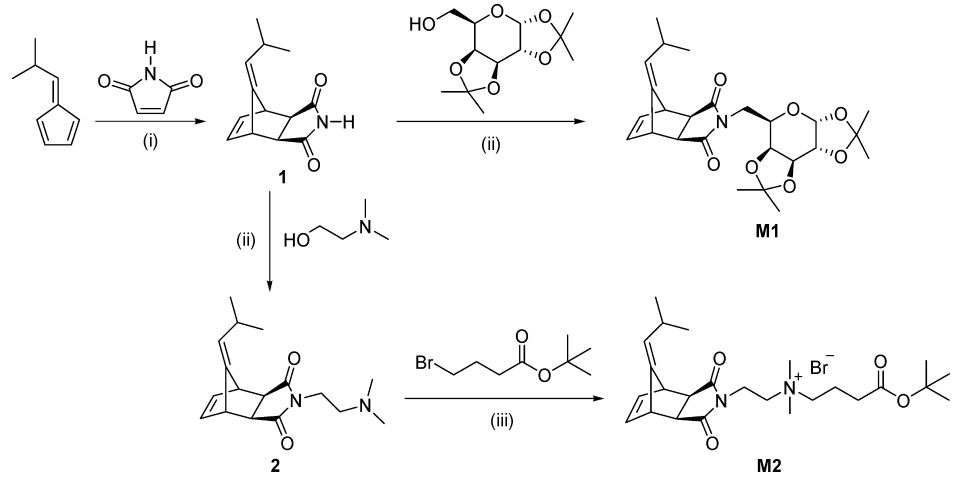
Copolymer composition was determined by the ratio of the integration values of methyl protons of the backbone (6H) appearing at 0.75 ppm to the methylene peaks next to the ester linkage of the PEG-acrylate (2H) appearing at 4.07 ppm. ^{1}H NMR (300 MHz, D_2O) δ 0.85 (br m, 6H), 2.42 (br m, 1H), 2.84 (br m, 2H), 3.05 (br m, 2H), 3.15 (br m, 3H, -CH_3 of PEG), 3.46 (br m, 32H), 3.55 (br m, 2H), 4.07 (br m, 2H, -$COOCH_2$-), 5.18 (br m, 1H), 5.48 (br m, 1H), 7.10 (br m, 5H, aromatic).

Results and Discussion

Polymer Synthesis and Characterization. It has been previously reported that the facial amphiphilicity at the repeat unit level has an important effect on the overall selectivity of the SMAMPs compared to random copolymers of hydrophobic and hydrophilic monomers.[21,23,32,35] Selectivity values were improved in the prior cases by fine-tuning these systems by copolymerization. Therefore, to preserve the facial amphiphilicity at the repeat unit level, in this study, monomers composed of the same hydrophobic backbone and varying biocompatible hydrophilic pendant moieties were designed and synthesized (Figure 1).

ROMP has been successfully used to polymerize norbornene based monomers bearing various functionalities to obtain well-defined systems.[24,34,36−38] However, it has also been reported that the presence of particular additives, such as pyridine, secondary amines, thiols, or benzoic acid, can significantly slow or shut down the kinetics of ROMP.[39,40] Therefore, to prevent these retardation effects and to be able to successfully perform ROMP, a protecting group approach was utilized to synthesize the highly functionalized monomers (**M1**−**M3**). In addition to facilitating the polymerization process, the protective group approach also provided ease in the later polymer characterization by gel permeation chromatography (GPC) and nuclear magnetic

Scheme 1. Synthesis of **M1** and **M2**[a]



[a] Reagents and conditions: (i) toluene, at 135 °C for 12 h; (ii) THF, Ph_3P, DIAD, at RT for 24 h; (iii) THF, at 50 °C for 36 h.

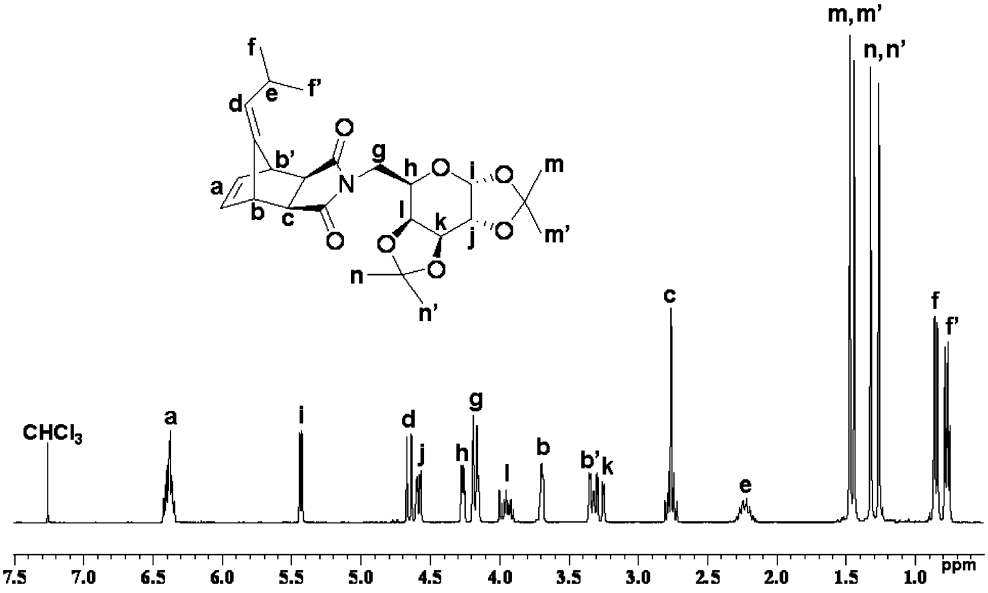


Figure 2. ^{1}H NMR of **M1** in $CDCl_3$.

resonance (NMR). Upon complete polymerization, the protective groups were removed to yield the corresponding antimicrobially active polymers.

The first step of the monomer synthesis is the Diels−Alder reaction of maleimide with isopropylfulvene to yield product **1**. **M1**, the sugar functional norbornene based monomer, was synthesized via Misunobu coupling[41] of **1** with the corresponding alcohol (Scheme 1). The same reaction conditions were employed to obtain **2**, which was further alkylated with 4-bromobutanoic acid *tert*-butyl ester to yield the positively charged **M2** as the quaternary ammonium salt, leading to the zwitterionic functionality upon deprotection (Scheme 1).[40] **M3** was synthesized according to a previously published procedure.[34] All monomers were characterized using NMR techniques. Figures 2 and 3 show the resulting ^{1}H NMR spectra of **M1** and **M2**, respectively.

All homopolymers (**Poly1**, **Poly2**, and **Poly3**) and copolymers (**Poly3**$_n$-*co*-**Poly1**$_m$, **Poly3**$_n$-*co*-**Poly2**$_m$, and **Poly3**$_n$-*co*-**PolyP**$_m$, where **n** indicates the mol fraction of **M3** and **m** indicates the mol fraction of **M1**, **M2**, or **MP** (PEG functionality) in the copolymer composition) were synthesized using third generation Grubbs' catalyst as an initiator. Upon reaction completion, the polymerizations were terminated via addition of excess ethyl vinyl ether. ROMP yielded polymers with approximate molecular weights of 3 kDa and narrow polydispersity indices ranging from 1.08 to 1.15, as determined by GPC (see Table 1). All polymers were deprotected using trifluoroacetic acid to obtain the antimicrobially active polymers.

Syntheses of **Poly3**$_n$-*co*-**Poly1**$_m$ (sugar) and **Poly3**$_n$-*co*-**Poly2**$_m$ (zwitterionic) copolymers were done via random copolymerization of the corresponding facially amphiphilic monomers. To vary the amphiphilicity of the resulting polymers, different molar ratios of the monomers were used in the copolymerization reactions (Table 1 and Table S1). Depending on the solubility of the monomers and the catalyst, different solvent systems were used to provide homogeneous reaction media for all polymerizations. **Poly3**$_n$-*co*-**Poly1**$_m$ was synthesized using only THF, whereas **Poly3**$_n$-*co*-**Poly2**$_m$ was synthesized in a THF−methanol solvent mixture (Scheme 2). The copolymerization kinetics were studied via ^{1}H NMR analysis by recording spectra every 30 min until the reaction was completed within 2 h. The constant decrease in the intensity of the broad peak at 6.5 ppm, corresponding to the alkene functionalities of both norbornene monomers, indicated successful copolymer formation. The complete disappearance of the same peak and the simultaneous appearance of two new peaks at approximately 5.4 and 5.6 ppm,

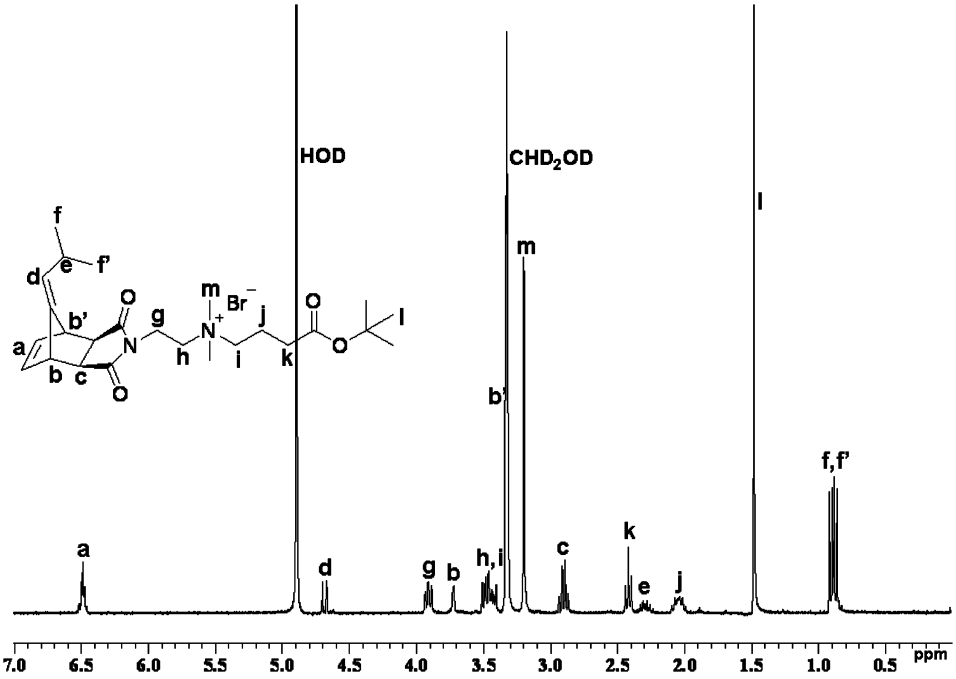


Figure 3. ^{1}H NMR of **M2** in CD_3OD.

Table 1. Biological Activity Data of **Poly3$_n$-*co*-PolyX$_m$** Copolymers

copolymer ratio (**n**/**m**)	M_n (kDa)	PDI	MIC [μg/mL] *E. coli*	MIC [μg/mL] *S. aureus*	HC_{50} [μg/mL]	selectivity (HC_{50}/MIC) *E. coli*	selectivity (HC_{50}/MIC) *S. aureus*
Poly3	2.8[a]	1.15	<50	<50	<1	<0.02	<0.02
Poly3$_{0.7}$-*co*-Poly1$_{0.3}$	2.9[a]	1.10	100	100	50	0.2	0.2
Poly3$_{0.5}$-*co*-Poly1$_{0.5}$	2.9[a]	1.10	200	200	100	0.5	0.5
Poly3$_{0.3}$-*co*-Poly1$_{0.7}$	3.0[a]	1.09	>200	>200	400	<2.0	2.0
Poly1	3.0[a]	1.08	>200	>200	2000	<10.0	<10.0
Poly3$_{0.7}$-*co*-Poly2$_{0.3}$	3.6[b]	n.d.	100	100	250	2.5	2.5
Poly3$_{0.5}$-*co*-Poly2$_{0.5}$	4.1[b]	n.d.	150	200	1500	10.0	7.5
Poly3$_{0.3}$-*co*-Poly2$_{0.7}$	4.3[b]	n.d.	>200	>200	750	<3.8	<3.8
Poly2	4.0[b]	n.d.	>400	>400	750	<1.9	<1.9
Poly3$_{0.8}$-*co*-PolyP$_{0.2}$	3.3[c,d]	1.15	15	25	<50	<3.3	<2
Poly3$_{0.7}$-*co*-PolyP$_{0.3}$	3.6[c,d]	1.15	20	100	<50	<2.5	<0.2
Poly3$_{0.5}$-*co*-PolyP$_{0.5}$	4.1[c,d]	1.15	>200	>200	1000	<5	<5

[a] M_n determined by GPC of the protected polymers. [b] M_n determined by ^{1}H NMR of the deprotected polymers. [c] M_n reported after subtracting the corresponding weights of the protecting groups. [d] M_n includes the molecular weight of the PEG chains.

corresponding to the *cis*- and *trans*-conformations of the double bonds of the polymer backbone, indicated complete conversion.

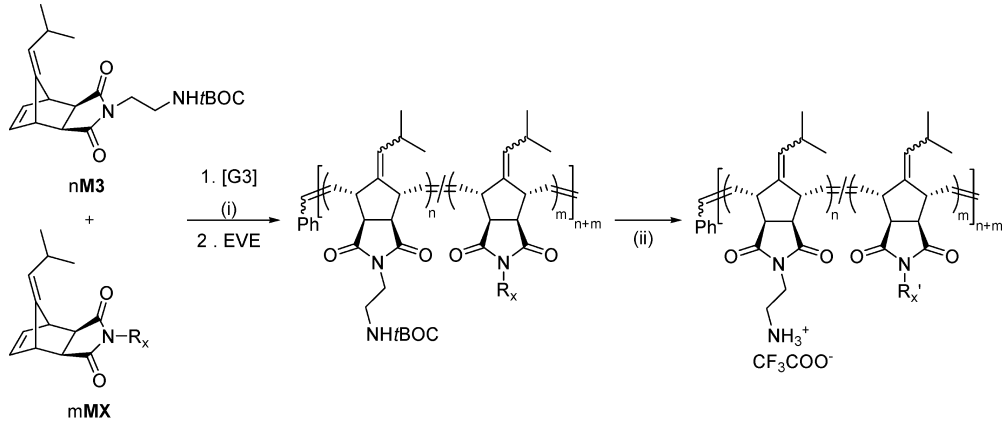
Molecular weight characterization of **Poly3$_n$-*co*-Poly1$_m$** was performed by gel permeation chromatography (GPC) with THF as the eluent, and calibration was done against polystyrene standards. The experimental molecular weights were in accordance with the theoretical (calculated) values (Table 1 and Table S1). Representative GPC traces of **Poly1** (homopolymer of M1) and the copolymer, **Poly3$_{0.5}$-*co*-Poly1$_{0.5}$**, are shown in the Supporting Information.

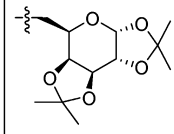
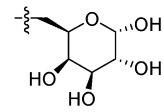
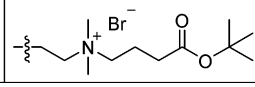
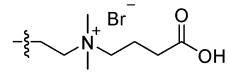
It is well-known that GPC of charged polymers is challenging due to formation of ionic aggregation or interactions with the stationary phase of the chromatography column.[42] Therefore, instead of performing GPC analysis on the charged polymers, ^{1}H NMR end group analysis was used for the molecular weight determination of **Poly3$_n$-*co*-Poly2$_m$** copolymers. The ratio of the phenyl end group integral value to the methyl protons of the isopropylfulvene backbone yielded the degree of polymerization, which was used to calculate the total molecular weight of the polymers.

It has been previously reported that PEG containing norbornenes polymerized via ROMP yielded polymers with very broad PDIs (~1.6) and multimodal molecular weight distributions.[36,43] To prevent these complications and to obtain well-defined systems for the biological activity analysis, an alternative approach was employed for the synthesis of the **Poly3$_n$-*co*-PolyP$_m$** copolymers. Prior to any functionalization, **M3** was polymerized to yield *Boc*-protected **Poly3**, which was then deprotected with TFA to yield the antimicrobially active polymer according to the previously published procedure.[34] Positively charged ammonium groups were neutralized to yield the amine functionalities, which were then used to react with the acrylate end functionalized PEG via Michael addition (Scheme 3).[44] In the ^{1}H NMR spectra of the purified polymers, no residual acrylate peaks were observed, and the appearance of a peak at 4.07 ppm corresponding to the methylene peaks next to the ester linkage of the PEG-acrylate indicated the successful addition. Due to relatively lower yields obtained in this specific reaction, only copolymers containing up to 50% PEG functionality were synthesized.

Antimicrobial and Hemolytic Activity Analyses. To assess the biological activities of the polymers, the minimum inhibitory concentrations (MIC) against *Eschericia coli* (*E. coli*) and *Staphylococcus aureus* (*S. aureus*) and hemolytic activity (HC_{50}) were determined according to standard procedures.[45] All of the analyses were performed using the deprotected polymer samples. Figure 4 shows the results of the **Poly3$_n$-*co*-Poly1$_m$** series. As the sugar content of the copolymer was increased from 0 to 100% (referring to the **Poly1** homopolymer), a linear decrease in the biological activity was observed; MIC values increased from 50 to 200 μg/mL. This loss of activity might be attributed to the fact that, as the number of repeat units containing sugar residues is increased, the overall positive charge of the polymer is being reduced. It should also be noted that the sugar functional homopolymer, **Poly1**, is less active toward bacteria. However, due to its biocompatible nature and overall neutral charge, its toxicity toward mammalian cells, $HC_{50} = 2000$ μg/mL, is much lower than that of **Poly3**, $HC_{50} < 1$ μg/mL. Thus, while increasing the sugar content of the **Poly3$_n$-*co*-Poly1$_m$** copoly-

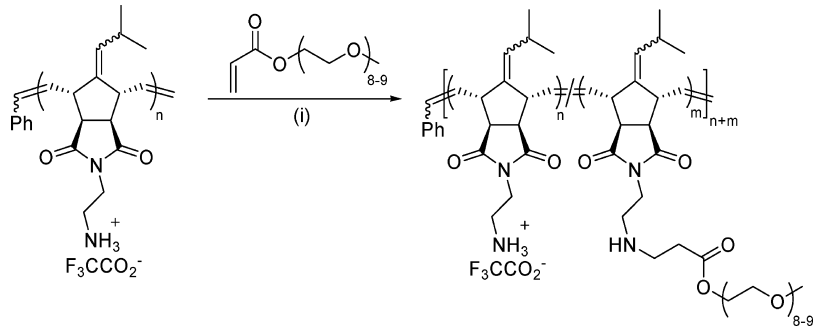
Scheme 2. Copolymerization of **M1** and **M2** with **M3** and Deprotection of the Resulting Polymers[a]



X	R_x	R_x'
1		
2		

[a] Reagents and conditions: (i) X = 1: THF, 60 °C, 2 h; X = 2: THF, CH_3OH, 60 °C, 2 h. (ii) X = 1: TFA, RT, 24 h; X = 2: TFA/H_2O (9/1), RT, 24 h.

Scheme 3. Synthesis of **Poly3$_n$**-*co*-**PolyP$_m$** Copolymers[a]



[a] Reagents and conditions: (i) triethylamine, DMSO, RT, 24 h.

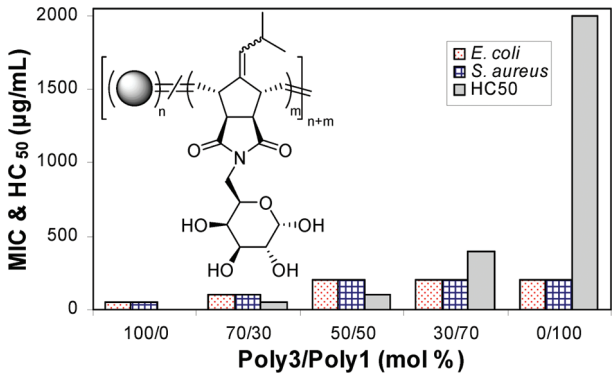


Figure 4. MIC and HC_{50} data of **Poly3$_n$**-*co*-**Poly1$_m$**.

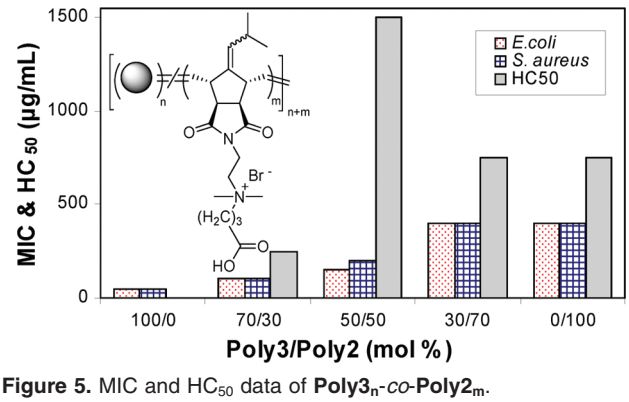


Figure 5. MIC and HC_{50} data of **Poly3$_n$**-*co*-**Poly2$_m$**.

mers improved their hemolytic properties, it caused a reduction of the antibacterial activities.

The same behavior was observed for the **Poly3$_n$**-*co*-**Poly2$_m$** copolymer series until equal comonomer composition was reached (**Poly3$_{0.5}$**-*co*-**Poly2$_{0.5}$**; Figure 5). As the number of zwitterionic residues was further increased, loss of antimicrobial activity along with higher hemolytic activity was observed. A reasonable explanation for the loss of activity with higher zwitterionic content could be the increased formation of intra- or interchain associations, causing significant conformational changes in the polymer backbone, thus affecting overall facial amphiphilicity.

The highest biological activity was observed for **Poly3$_n$**-*co*-**PolyP$_m$** samples (Figure 6). Upon incorporation of PEG groups up to 30%, lower MIC values were obtained compared to **Poly3**, accompanied with a slight improvement in the hemolytic

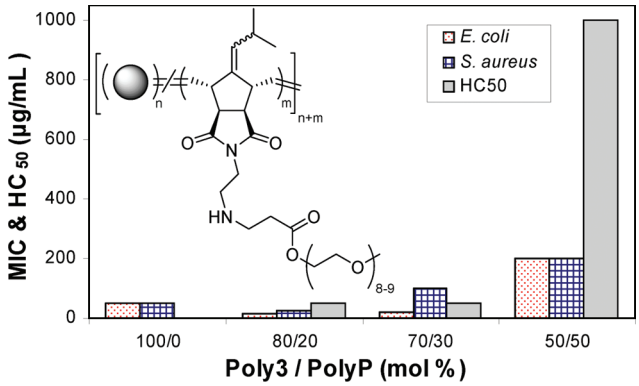


Figure 6. MIC and HC_{50} data of **Poly3$_n$-*co*-PolyP$_m$**.

activities. Compared to the sugar or zwitterionic functionalized copolymers, the presence of an amine (primary or secondary) on every repeat unit in the **Poly3$_n$-*co*-PolyP$_m$** structures indicates that the overall charge remained constant even as PEG content increased. However, after 50% functionalization the same loss of activity was observed as for the other polymers.

Table 1 summarizes the resulting MIC and HC_{50} values of all the studied SMAMPs, as well as the selectivity values, calculated taking the ratio of HC_{50} and MIC. When the results are compared to that of **Poly3**, generally the same behavior is observed for all the samples; with increasing hydrophilicity, biological activity is reduced. However, due to the biocompatible nature of these hydrophilic groups, improvements of the selectivities were observed (from <0.02 up to 7.5 μg/mL for **Poly3$_{0.5}$-*co*-Poly2$_{0.5}$**).

Conclusions

An amphiphilic polynorbornene derivative, **Poly3**, was modified by successful incorporation of hydrophilic, biocompatible groups via copolymerization of the corresponding monomers or postpolymerization functionalization. Biological activities of these SMAMPs were studied and it was observed that increasing the hydrophilicity reduced the antibacterial properties, which could be interpreted as resulting from the overall charge reduction. However, the hydrophilicity of the biocompatible groups reduces the hemolytic activities of the new polymers, thus, improved selectivities were obtained. The obtained selectivities remained modest compared to other recent norbornene-based polymers reported from our laboratory, which in general gave selectivities of $\sim$250[32]$-$500.[23] Those polymers were significantly more potent with single μg/mL MICs.

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Supporting Information Available. Representative GPC data and a table including the M/I ratios for all the synthesized homopolymers and copolymers. This material is available free of charge via the Internet at http://pubs.acs.org.

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