Antibacterial and Hemolytic Activities of Quaternary Pyridinium Functionalized Polynorbornenes[a]

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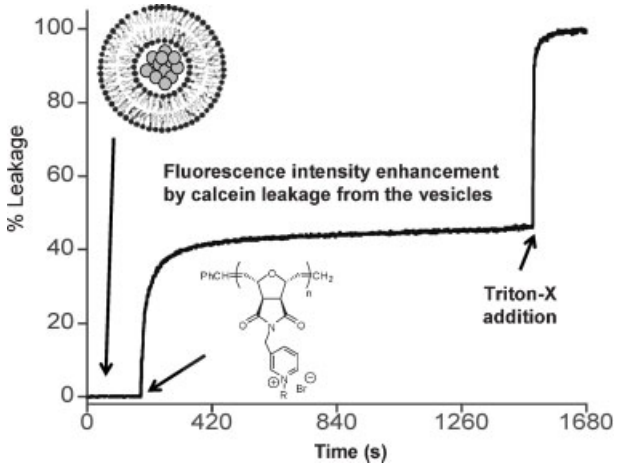
In this study, amphiphilic polyoxanorbornene with different quaternary alkyl pyridinium side chains were synthesized. The biological efficiencies of these polymers, with various alkyl substituents, were determined by bacterial growth inhibition assays and hemolytic activity (HC_{50}) against human red blood cells (RBCs) to provide selectivity of these polymers for bacterial over mammalian cells. A series of polymers with different alkyl substituents (ethyl, butyl, hexyl, octyl, decyl and phenylethyl) and two different molecular weights (3 and 10 kDa) were prepared. The impact of alkyl chain length divided the biological activity into two different cases: those with an alkyl substituent containing four or fewer carbons had a minimum inhibitory concentration (MIC) of 200 $\mu g \cdot mL^{-1}$ and a HC_{50} greater than 1 650 $\mu g \cdot mL^{-1}$, while those with six or more carbons had lower MICs $\leq$ 12.5 $\mu g \cdot mL^{-1}$ and $HC_{50} \leq 250\ \mu g \cdot mL^{-1}$. Using MSI-78, the potent Magainin derivative which has an MIC = 12.0 $\mu g \cdot mL^{-1}$ and HC_{50} = 120 $\mu g \cdot mL^{-1}$, as a comparison, the polymers with alkyl substituents $\leq C_4$ (four carbons) were not very potent, but did show selectivity values greater than or equal to MSI-78. In contrast, those with alkyl substituents $\geq C_6$ were as potent, or more potent, than MSI-78 and in three specific cases demonstrated selectivity values similar to, or better than, MSI-78. To understand if these polymers were membrane active, polymer induced lipid membrane disruption activities were evaluated by dye leakage experiments. Lipid composition and polymer hydrophobicity were found to be important factors for dye release.



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Introduction

Infectious disease has become a growing global concern.[1] Host-defense peptides (HDPs) and their synthetic analogs have been reported to exhibit strong killing activity against bacteria but not mammalian cells.[2–4] Polymers have been used as antimicrobial agents due to their relative ease of synthesis and easy accessibility to a wide range of molecular weights (MW) compared to antimicrobial peptidomimetics.[5] Polymers containing quaternary nitrogen functionalities have been commonly used as biocidal agents.[6–15] A number of polymeric disinfectants based on quaternary pyridinium groups, either in the backbone or as pendant groups, have been prepared, which show good activity against bacteria. Recently, Gao and coworkers synthesized random copolymers of acrylamide and vinyl pyridine of varying MWs and pyridine content, which were subsequently quaternized with dimethyl sulfate.[16] They found that polymers with higher cationic functionality had stronger antibacterial activity. Separately, it was shown that methacrylate based polymers with pendant pyridinium moieties exhibited antibacterial activity depending on the alkyl chain length.[7,8,17] Quaternary pyridinium polymers can also display biological activity when bound to surfaces, as shown by poly-4-vinyl pyridine (PVP) modified glass surfaces containing different alkyl bromide derivatives. This study showed that hexyl units on the backbone of PVP had the highest antibacterial activity on modified glass surfaces.[6,18,19]

An important property of polymers used for antibacterial applications is not just their antibacterial activity, but also their lack of toxicity to human cells (i.e., selectivity). Therefore, interest in novel, selective antibacterial polymers is growing because of their potential use in biomedical devices such as catheters, sutures, indwelling structures, prosthetics, etc. Applications of quaternary pyridinium type materials are limited by their poor solubility in water and low biocompatibility. Specifically, they are limited because they can cause irritation to skin and are well-known biocides.[20] In most cases, only antibacterial activity was reported without any studies on hemolytic activity (or activity against other mammalian cells[21]). Here we report the antibacterial and hemolytic activity of quaternary pyridinium functionalized polynorbornenes.

This study is intended to develop an understanding of the *structure-activity* relationship of synthetic pyridinium based polymers synthesized by ring-opening metathesis polymerization (ROMP) of norbornene. Because these polymers were expected to act directly on the bacterial membrane,[22–24] the interaction of these polymers with model phospholipid membranes was studied using a fluorescent dye, calcein, encapsulated in unilamellar vesicles. The effects of the ionic nature and hydrophobic character on the membrane activity of these polymers were investigated. Results show that by controlling the hydrophobic/hydrophilic balance of the polymers, it is possible to improve their selectivity for bacterial cells over red blood cells (RBCs). It was observed that the antibacterial efficiency depends on the length of the alkyl substituents in the polymer repeat units. As the polymer became more hydrophobic (hexyl and higher alkyl chain lengths in the repeat unit) the integrity of the membrane was more efficiently disrupted. This balance of hydrophobic/hydrophilic interactions leads to polymers with selectivity greater than 10 in six of the thirteen different samples studied.

Experimental Part

Materials

(Tricyclohexylphosphine)(1,3-dimesitylmidazolidine-2-ylidine)benzylideneruthenium dichloride, a Grubbs second generation catalyst, was purchased from Strem Chemicals. The Grubbs third generation catalyst [$(H_2$-Imes$)(3$-Br-py$)_2$-$(Cl)_2$Ru=CHPh] was freshly prepared according to a literature procedure.[25] Furan, maleic anhydride, 3-aminopyridine, bromoethane, 1-bromobutane, 1-bromohexane, 1-bromodecane, 1-bromooctane, bromoethane phenyl, calcein and tetrachloroethylene (TCE) were obtained from Aldrich and used as received. Basic aluminum oxide (Brockmann Activity I, basic particle size 0.05–0.15 mm) was obtained from Aldrich. 1,2-Dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), 1,2- dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE), 1,2-dioleoyl- *sn*-glycero-3-[phospho-*rac*-(1-glycerol)] (sodium salt) (DOPG) and *E. coli* total lipid extracts were purchased from Avanti Polar-Lipids, Inc. All other reagents, including buffers and salts, were obtained from Aldrich.

Instrumentation

^{1}H NMR (300 MHz) and ^{13}C NMR (75 MHz) spectra were recorded using a Bruker DPX-300 MHz spectrometer. MW determination of homopolymer **2** was performed with a Bruker Daltonics Reflects III MALDI-TOF mass spectrometer. The MALDI matrix was dithranol (saturated solution in $CHCl_3$). Fluorescence spectra were recorded with a Perkin-Elmer LS50B luminescence spectrometer. Optical density and absorbance spectra were recorded with a Molecular Devices SpectraMAX 190 plate reader.

Preparation of 2

A literature procedure for the sodium acetate catalyzed maleic anhydride-maleimide transformation[26,27] was adapted for the synthesis of the aminopyridine derivative (4-pyridine-3-ylmethyl-10-oxa-4-aza-tricyclo[5.2.1.$0^{2,6}$]dec-8-ene-3,5-dione, **2**). Pyridin-3-ylmethanamine (2.4 mL, 22 mmol) was added to a furan-maleic anhydride adduct[28] (**1**) (1.92 g, 11 mmol) in DMAc (*N, N*-dimethylacetamide, 6 mL) at 60 °C and stirred for 20 min. A catalytic amount of sodium acetate (0.5 g, 5 mmol), dissolved in

12 mL acetic anhydride, was added to the solution and the reaction mixture was stirred for 2 h at 90 °C. After cooling the reaction mixture to room temperature, the solution was diluted with ethyl acetate, washed with brine and evaporated under reduced pressure. The product was purified by column chromatography using THF:hexane (3:1, v/v) eluent affording **2** in a 50% yield.

^{1}H NMR ($CDCl_3$): $\delta = 8.5$ (2H, m), 7.5 (1H, m), 7.0 (1H, m), 6.5 (2H, s), 5.3 (2H, s), 4.6 (2H, s), 2.8 (2H, s).

^{13}C NMR ($CDCl_3$): $\delta = 176.2$, 148.9, 148.1, 136.7, 136.6, 132.7, 123.3, 81.1, 46.7, 38.9.

HRMS (EI) ($C_{14}H_{12}N_2O_3$): Calcd. 256.080; Found 256.084.

Quaternization of Oxanorbornene Derivatives, 3a–f

Monomer **3a** was obtained upon mixing a solution of **2** (0.13 g, 0.47 mmol) and ethyl bromide (0.5 mL, 6.4 mmol) in dry acetonitrile (5 mL) at room temperature for 48 h. Precipitation from THF resulted in a 90% yield of crude product. Monomer **3b–f** were synthesized using the following similar procedure. In a typical reaction, to a solution of **2** (0.13 g, 0.47 mmol) in 2 mL of dried acetonitrile, bromohexane (0.67 g, 4.7 mmol) was added. The mixture was heated to 60 °C for 48 h. Quaternized products **3a–f** were obtained by precipitation from ether or THF. No further purification was required. The yield was 60% for bromohexane derivative, **3c**.

^{1}H NMR ($CDCl_3$): $\delta = 9.3$ (2H, m), 8.2 (1H, m), 8.0 (1H, m), 6.5 (2H, s), 5.3 (2H, s), 4.6 (2H, s), 3.0, (2H, s), 1.5 (2H, m) 0.8 (3H, t).

^{13}C NMR ($CDCl_3$): $\delta = 176.0$, 144.2, 144.0, 143.5, 136.8, 136.4, 128.3, 81.0, 67.9, 62.1, 48.2, 38.9, 31.7, 31.1, 25.6, 22.3, 13.9.

General Polymerization Procedures for Compound 3a–f

In a typical example, the freshly prepared Grubbs third generation catalyst was dissolved in 0.5 mL of CH_2Cl_2 and added to a solution of **3c** in 2 mL tetrachloroethylene (TCE). The reaction mixture was stirred for 12 h at room temperature. The reaction was then terminated by an injection of 0.5 mL of ethyl vinyl ether. The polymer, **4c**, was then precipitated into 50 mL of THF and recovered by filtration and dried overnight under a vacuum at room temperature. **4c** was dissolved in CD_3OD or DMSO-d_6 for characterization. No GPC data could be collected due to irreversible adhesion to columns in THF and DMF.

^{1}H NMR (CD_3OD): $\delta = 8.8$ (2H, br), 8.5 (1H, br), 8.0 (1H, br), 6.5–6.24 (2H, br), 5.27 (2H, br), 4.64 (2H, br s), 3.2 (2H, br), 2.6 (2H, br), 2.0 (2H, br), 1.2 (2H, br), 0.8 (3H, br).

Homopolymer of 2

0.35 g (0.4 mmol) of Grubbs second generation catalyst was dissolved in 2 mL of CH_2Cl_2 and added to a solution of 2.15 g (8.33 mmol) of **2** in 10 mL of CH_2Cl_2. The reaction mixture was stirred for 3 h at room temperature. The polymerization was terminated with an injection of 0.5 mL of ethyl vinyl ether. Precipitation from pentane resulted in a yellow solid precipitate. The polymer was further purified by precipitation from an ether:THF (2:1) solution to remove unreacted monomer. The polymer was recovered as a yellow solid (1.35 g, yield of 65%). $\overline{M}_{n,obs}$ was 3 kDa from MALDI-TOF analysis.

^{1}H NMR ($CDCl_3$): $\delta = 8.4$ (2H, br), 7.8 (1H, br), 7.1 (1H, br overlapped with solvent signal), 6.0–5.8 (2H, br), 5.0 (2H, br), 4.7 (2H, br), 4.6 (2H, br), 3.2 (2H, br).

Synthesis of 4a′

The homopolymer of **2** ($\overline{M}_{n,obs} = 3\,000$, by MALDI-TOF) (0.11 g, 0.036 mmol) and excess bromoethane (0.2 mL, 2.67 mmol) was mixed in 4 mL of dimethylformamide for 4 d at room temperature. Quaternized polymer, **4a′**, was obtained by precipitation from THF and the conversion was calculated by ^{1}H NMR. ^{1}H NMR peaks at 8.2–9.1 and 0.9–1.1 ppm corresponded to the pyridine ring and the methyl protons of the ethyl chain of the polymer's repeating units, respectively. The integrations of these two regions were compared to calculate the conversion, which was found to be 85%.

^{1}H NMR (DMSO-d_6): $\delta = 9.1$ (2H, br), 8.5 (1H, br), 8.0 (1H, br), 7.8 (1H, br), 7.1 (1H, br) 6.0–5.8 (2H, br), 5.1 (2H, br), 4.8 (2H, br), 3.4 (2H, br), 3.2 (2H, br), 1.5 (3H, br t).

Measurement of Antibacterial Activity

Antibacterial activity measurements were performed with slight modifications to literature procedures.[29–31] Bacterial suspensions (*E. coli* D31 or *B. subtilis* ATCC#8037) were grown in Mueller-Hinton Broth (M-H) overnight at 37 °C and diluted with fresh M-H to an optical density of 0.001 at 600 nm (OD_{600}). This suspension was mixed with different concentrations of freshly prepared polymer solutions in TRIS saline (10×10^{-3} M TRIS, 150×10^{-3} M NaCl, pH 7.2, filtered through a polyethersulfone membrane with 0.22 μm pore size), by serial dilutions in a 96 well plate, and incubated for 6 h at 37 °C under rotary agitation. 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_{90}), the concentration at which 90% inhibition of growth was observed after 6 h. All experiments were done in duplicate.

Measurement of Hemolytic Activity

Fresh human red blood cells (RBCs, 30 μL) were suspended in 10 mL of TRIS saline and washed 3 times by centrifugation (5 min at 1 500 rpm) and resuspended in TRIS saline. Polymer solutions were prepared by dissolution in DMSO-$_{d6}$ at a concentration of 10 mg · mL^{-1} and further diluted into Tris buffer to make a total of 20 μL solution. Freshly prepared polymer solutions with different concentrations were added to 80 μL of the above-prepared RBC suspension to reach a final volume of 100 μL in a 96 well plate. The resulting mixture was kept at 37 °C for 30 min under rotary agitation. Afterwards, the plate was centrifuged (IEC Centra-4B, 5 min at 3 000 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 data was obtained by adding 10 μL of TRITON-X

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solution (20 vol.-% in DMSO-$_{d6}$), a strong surfactant, to the above-prepared RBC 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. All experiments were done in duplicate.

Determination of Polymer-induced Leakage of Vesicle Content

In a typical example, a chloroform solution of lipid (10×10^{-3} M) was evaporated under reduced pressure and subsequently dried under a vacuum for 3 h at room temperature to obtain a dry film. The dried film was hydrated by addition of 1 mL of 10×10^{-3} M Na_2HPO_4, (pH 7.0) containing 40×10^{-3} M calcein dye. The suspension was vortexed for 10 min. Five freeze-thaw cycles were applied to the suspension using warm water and liquid nitrogen. The vesicles were filtered with a nuclepore trac-edge membrane with 400 nm diameter size. The non-encapsulated calcein was removed by eluting through a size exclusion Sephadex G-50 column with 10×10^{-3} M Na_2HPO_4, 90×10^{-3} M NaCl buffer (pH 7.0) as the eluent. Vesicle suspensions were diluted 7-fold with the elution buffer prior to the polymer addition. The polymer induced leakage was monitored by recording the increase of calcein fluorescence intensity at 515 nm (excitation at 490 nm, slit width 3.0). Calcein loaded phospholipid vesicles were stable at physiological pH and no increase of fluorescence was observed before the addition of the polymer. A complete vesicle disruption was achieved by adding a strong surfactant solution of 30 μL of 0.2% TRITON-X 100 (polyoxyethylene (10) isooctylphenylether) and the corresponding fluorescence intensity was used as 100% leakage. The lysis caused by the polymer was reported as "% lysis", which is a fraction of the total lysis caused by TRITON-X.

Results and Discussion

ROMP is an attractive synthetic method that has been widely used to prepare well defined polymers with controlled MWs and low polydispersities (PDI).[32–35] We have previously reported the ROMP of modular facially amphiphilic norbornene monomers to generate polymers that were antimicrobial and non-hemolytic.[22] This was based on designing polymers to mimic the biological activity of the HDPs.[24,36–38] Due to the widespread use of the quaternary pyridinium (or ammonium) group in antimicrobial materials, norbornene derivatives were envisaged that carry this functionality and would undergo ROMP. Here, the quaternary pyridinium functionality was attached to 7-oxanorbornene-5,6-exo-dicarboximide functionalized monomers which were subsequently polymerized using a Grubbs' catalyst, as shown in Figure 1.

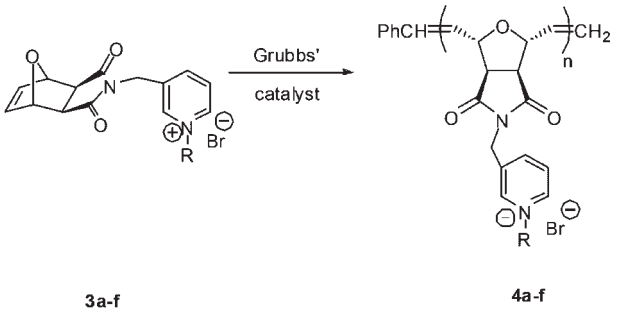

Figure 1. Monomers and polymers based on oxanorbornene derivatives.

Monomer Synthesis

As shown in the supporting information, Scheme S1, monomer synthesis began with a Diels-Alder reaction between maleic anhydride and furan to yield *exo*-7-oxabicyclo[2.2.1]hept-5-ene-2,3-dicarboxylic anhydride, **1**.[28] This anhydride was then reacted with pyridin-3-ylmethanamine in the presence of catalytic sodium acetate (or cobalt acetate) to transform the anhydride into the substituted imide linkage generating **2** (also shown in Figure 2) in a 50% yield. ^{1}H and ^{13}C NMR spectra of **2**, also in the Supporting Information, clearly supported formation of the pyridine substituted oxanorbornene monomer (see Figure S1 and S2). Monomer **2** was subsequently reacted with the six different alkylating agents generating functionalized quaternary monomers, **3a–f**, shown in Figure 1.

Polymer Synthesis

The polymer series, **4a–f**, which was obtained from monomers **3a–f**, respectively, was designed to evaluate the influence of the hydrophobic alkyl substituent on the antibacterial and hemolytic activity of the polymers in this study. Six different alkyl substituents were chosen spanning from two to ten carbons, and they included ethyl, butyl, hexyl, octyl, and decyl, as well as the aromatic phenylethyl. In the first approach, monomers **3a–f** were polymerized using a Grubbs' third generation catalyst in TCE or methanol at room temperature (see Figure 1). Two different targeted $\overline{M}_n$ of 3 000 or 10 000 g·mol^{-1} were achieved and complete conversion was monitored, using ^{1}H NMR, by the total disappearance of the monomer olefin proton peaks at 6.0–6.2 ppm along with the appearance of the broad polymer backbone olefinic proton signals at

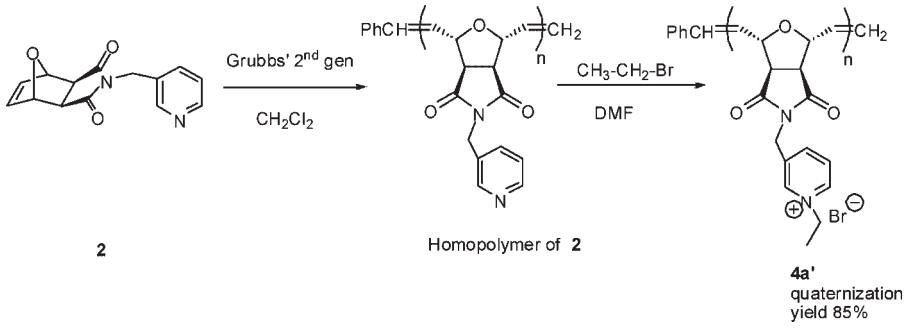

Figure 2. Synthetic pathway for **4a′**.

5.1–5.6 ppm. Unfortunately, GPC measurements of the polymers could not be carried out as the polymers failed to elute from the column, which is well known for cationic polymers,[39] therefore $\overline{M}_n$ was calculated by ^{1}H NMR end group analysis of the resulting purified polymer. The calculation for $\overline{M}_n$ compared integration of the aromatic peaks associated with the styrenic end group from the initiator with the olefinic protons of the polymer backbone and, for example, revealed a degree of polymerization of 8 for the 3 kDa sample of **4a**, which compares well with the theoretical value of 9 obtained from an assumption for full conversion.[40]

Because many approaches in the literature use post-polymerization modification to quaternize the pyridium (or ammonium) functionality, if monomer **2**, in Figure 2, would undergo ROMP, it would provide a direct comparison of polymers made by polymerization of the cationic monomer to those made by post-polymerization modification. We chose one alkyl substituent to study based on ethyl since this was one of the most easily reactive alkylating agents and the high HC_{50} of **4a**. In this second method, the homopolymer of **2** with $\overline{M}_n = 3$ kDa was obtained and its MW confirmed by MALDI-TOF. Although the theoretical $\overline{M}_n$ from the ratio of [M] to [I] was $\overline{M}_n = 10$ kDa, the MALDI-TOF (see Figure 3) showed the $\overline{M}_n$ to be centered around 3 kDa. This discrepancy between the theoretical and observed $\overline{M}_n$ is not surprising since the pyridine functionality was expected to interfere with the polymerization; in fact, it is interesting that the MALDI-TOF confirmed that the obtained polymer chains bear well defined end-groups consisting of the benzylidene and the methylidene, respectively, indicating that the synthesized chains were produced with uniform initiation and termination. Alkylation of this polymer with ethyl bromide resulted in **4a′** with 85% *N*-alkylation, as determined by ^{1}H NMR despite concrete efforts to increase the yield of alkylation. Thus, polymer **4a′** provides a direct comparison to **4a** so that the impact of quaternization on antibacterial activity and selectivity could be studied.

Antibacterial and Hemolytic Activities of Polymers

The activity of all monomers and polymers was probed against *E. coli* (Gram-negative bacteria) and *B. subtilis* (Gram-positive bacteria) as representative bacteria. Monomers, **3a–c** and **f** did not show any activity up to the highest measured concentrations of 200 $\mu g \cdot mL^{-1}$. In contrast, monomers containing the two longest alkyl substituents, octyl and decyl (**3d** and **e**), exhibited antibacterial activities with MIC of 50 and 100 $\mu g \cdot mL^{-1}$ against *E. coli* and *B. subtilis,* respectively, which is not surprising since alkylated pyridiniums are known to be disinfectants.

As the number of carbons in the alkyl substituent increased, more potent polymers were generated. Both the antibacterial and hemolytic activities increased when the alkyl chain length was $\geq C_6$. The activity of each polymer at two different MWs ($\overline{M}_{n,th} = 3$ kDa and 10 kDa) was probed against *E. coli*, *B. subtilis* and human RBCs (Table 1).

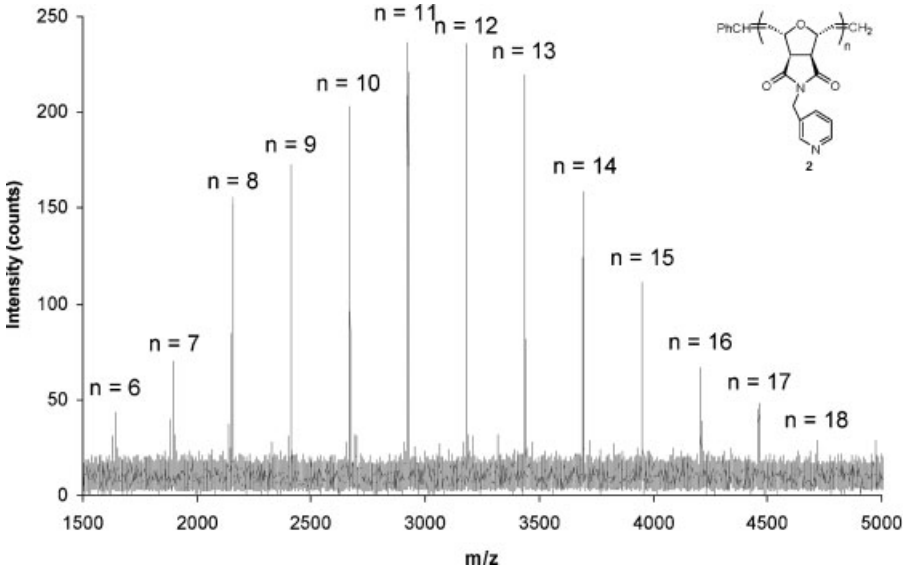

Figure 3. MALDI-TOF spectrum of homopolymer **2**.

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Table 1. Antibacterial and hemolytic activities of polymers. $\overline{M}_n$ values are measured based on full conversion.

$\overline{M}_n$ [a)]	Polymer	R	MIC		HC_{50}	Selectivity (HC_{50}/MIC)	
			$\mu g \cdot mL^{-1}$		$\mu g \cdot mL^{-1}$	*E. coli*	*B. subtilis*
kDa			*E.coli*	*B.subtilis*			
3	**4a**	Ethyl	200	200	4 030	≈20	≈20
10	**4a**	Ethyl	200	200	>2 000	>10	>10
3[a)]	**4a′**	Ethyl	200	200	2 000	10	10
3	**4b**	Butyl	200	200	2 000	10	10
10	**4b**	Butyl	200	200	1 653	8	8
3	**4c**	Hexyl	12.5	4	<50	<4	<12.5
10	**4c**	Hexyl	12.5	4	202	16	50
3	**4d**	Octyl	4	4	8	1.7	1.7
10	**4d**	Octyl	6	4	<50	<8.3	<12.5
3	**4e**	Decyl	12.5	6	7	0.6	1.3
10	**4e**	Decyl	12.5	6	<50	<4	<8.3
3	**4f**	Phenylethyl	12.5	12.5	240	19	19
10	**4f**	Phenylethyl	12.5	12.5	108	8	8
2.4	**MSI-78**		12.5	–	120	10	–

[a)] $\overline{M}_n$ value was observed as 3 kDa by MALDI-TOF and synthetic pathway for **4a** and **a′** are found Figure 1 and 2.

Hemolytic activity was evaluated as HC_{50}, the polymer concentration necessary for 50% lysis of RBC, while the MIC reported was for greater than 90% inhibition and typically was >99% inhibition.

For **4a–b** (ethyl and butyl), the MIC against both *E. coli* and *B. subtilis* was 200 $\mu g \cdot mL^{-1}$, while the HC_{50} was above 2 000 $\mu g \cdot mL^{-1}$ in all cases except one, in which it was 1 653 $\mu g \cdot mL^{-1}$ (**4b**, 10 kDa). For the 3 kDa $\overline{M}_n$ sample of **4a**, the HC_{50} was measured to be 4 030 $\mu g \cdot mL^{-1}$ providing a sample with selectivity of ≈20. To the best of our knowledge, these are the lowest toxicity values reported in the literature for pyridinium based polymers. However, these MIC activities are less potent than most HDPs, which are typically in the range of 10 and 50 $\mu g \cdot mL^{-1}$, but the hemolytic activity of the HPDs is much stronger.[4,29,30,41–44] For example, the magainin derivative, **MSI-78** has an MIC of 12 $\mu g \cdot mL^{-1}$ and a HC_{50} of 120 $\mu g \cdot mL^{-1}$ for a selectivity of 10 (see Table 1).[23]

As the alkyl substituent length increased to six carbons, the MIC and HC_{50} decreased significantly to 12.5 and 240 $\mu g \cdot mL^{-1}$ or less, respectively. An optimum in MIC was observed with the octyl substituent, but these samples were also as hemolytic as any of the polymers. The 10 kDa hexyl polymer, **4c**, had an MIC of 12.5 and 4 $\mu g \cdot mL^{-1}$ against *E. coli* and *B. subtilis*, respectively, and an HC_{50} of 202 $\mu g \cdot mL^{-1}$, giving this polymer potency and non-hemolytic activity on a par with, or slightly better than, **MSI-78**. These MICs appear to be the lowest reported values for pyridinium functionalized polymers.[8,9] Similarly, polymer **4f**, containing the phenylethyl substituent, was antimicrobial (MIC = 12.5 $\mu g \cdot mL^{-1}$) and had selectivities of 19 and 8 for the low and high MW, respectively. The fact that these polymers contain a total of eight carbons with six being confined to the aromatic ring appears to indicate that aromatic units may be less toxic than aliphatic chains.

The observed increase in activity with increasing alkyl substituent chain length suggested that hydrophobicity played a major role in the antibacterial activity of the polymers. Increasing the hydrophobicity enhances the ability of the polymers to bind onto the lipid membrane, increasing the concentration of bound polymer which leads to enhanced membrane perturbation and eventually cell death.[45] Meanwhile, in each polymer series, the HC_{50} decreased with increasing alkyl chain length (i.e., hydrophobicity) for similar reasons.[4,22,41] The bioactivity of the polymer series **4a–f** (Table 1) showed that a balance between hydrophobic and hydrophilic groups is needed in order to increase the antibacterial potency without creating hemolytic polymers. This agrees with reports on native peptides,[4,41,46] as well as previous studies from Tew.[22,45] A comparison of polymers **4b–d** highlights the delicate balance of hydrophobicity needed to generate potently antibacterial yet non-toxic molecules.

The effect of MW on antibacterial and hemolytic activities was also investigated and showed that for polymers **4a–f**, the MW (for the two MWs studied) did not result in significant changes in antibacterial and hemolytic activities when activity was reported in mass/volume rather than molarity. In line with the commonly accepted molecular mechanisms in the literature, it can be expected that high MW polymers, which have lower molar concentrations (the number of molecules per unit volume) for any given weight concentration, are however, more active due to the local concentration of monomer as a result of the large MW. These commonly suggested mechanisms for membrane disruptions by amphiphilic peptides are the toroidal pore (also known as wormhole), barrel stave (also known as helix bundle), and carpet (also known as detergent-like) models.[5,29] All models involve the binding of the active agent to the outer membrane followed by an increase in the population of bound species.

If the membrane disruption activity is associated with the accumulation of macromolecules on the membrane surface, using high MW polymers by definition increases the concentration of monomer units in the local vicinity for a given concentration. These higher MW polymers would cover larger surface areas than lower MW polymers at the same molar concentrations, and thus the polymers can result in an enhancement of the number of electrostatic and hydrophobic interactions at the membrane surface. This is completely consistent with our previous report on facially amphiphilic polynorbornenes in which larger MW polymers were more active on a molar basis.[22] Further support for this idea is found by comparing the above mentioned MICs of the respective monomers. For example, monomers **3a–c** and **3f** were not active at 200 $\mu g \cdot mL^{-1}$ but the polymers synthesized from these monomers were active with **4a–b** having MICs of 200 $\mu g \cdot mL^{-1}$ while polymers **4c** and **f** showed strong antibacterial activity at 12.5 $\mu g \cdot mL^{-1}$. Even the two monomers which showed antibacterial activity (**3d–e**) became more potent when polymerized; the MIC of **3d** against *E. coli* was 50 $\mu g \cdot mL^{-1}$ and the polymer, **4d**, had an MIC of 4–6 $\mu g \cdot mL^{-1}$.

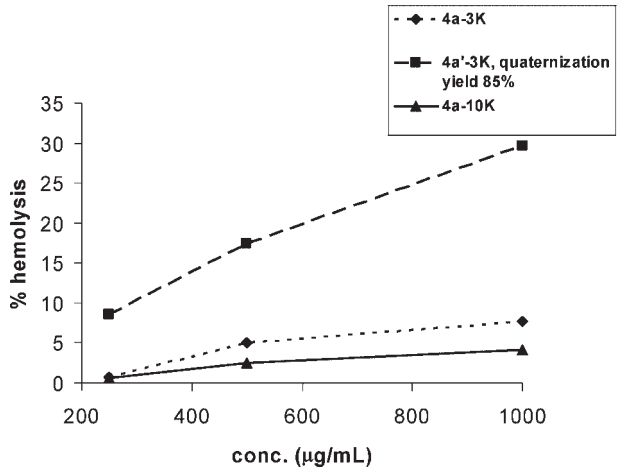

Figure 4. Quaternization yield and MW effect of **4a** on hemolysis activity.

selectivity due to the very high HC_{50} value of **4a**. When these two molecules, **4a** and **4a′**, fully and 85% quaternized, respectively, were evaluated for their biological activity, the MICs were identical at 200 $\mu g \cdot mL^{-1}$ but **4a′** was found to be more hemolytic. As shown in Figure 4, **4a′** caused 30% hemolysis at 1 000 $\mu g \cdot mL^{-1}$ while the fully quaternized polymer **4a** resulted in only 8% hemolysis. This value of 8% hemolysis was consistent regardless of the MW for **4a**, as shown in Figure 4. When the final HC_{50} values were determine, it was found that **4a′** ($HC_{50} = 2\,000$ $\mu g \cdot mL^{-1}$) was twice as hemolytic as **4a** ($HC_{50} = 4\,030$ $\mu g \cdot mL^{-1}$). Previous studies on amphiphilic polynorbornene derivatives also showed that a lack of selectivity arises when hydrophobic interactions begin to dominate.[22] Therefore, the results on quaternization appear to be consistent with this trend since the 85% alkylated polymer is more hydrophobic than the fully alkylated **4a** due to the small ethyl substituent. This has important implications when considering the biological activity of alkylated pyridinium (or ammonium) polymers. Depending on how they are synthesized, they may be more toxic than if they are fully alkylated.

Effect of Quaternization on the Antibacterial and Hemolytic Activities

Hydrophobic interactions have been reported to control hemolytic activities, whereas charge interactions have been suggested to be more important for antibacterial activity.[41,46] The ability to synthesize **4a** by two different methods allowed us to directly compare the 'degree of quaternization' on biological activity. Specifically, the ethyl pendant group was chosen to track the effect of quaternization efficiency on the antibacterial activity and

Disruption of Phospholipid Membranes

The use of dye leakage from model vesicles is a standard method to confirm that antimicrobial molecules act directly on the phospholipid membrane. In addition, the role of specific lipid types on membrane activity can be observed frequently using model membrane assays.[5] In this study, the membrane activity of the synthesized polymers was followed by fluorescence spectroscopy using a fluorescent dye (calcein) encapsulated in phospholipid vesicles that have different lipid compositions. Leakage

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experiments were performed with phosphatidylcholine (PC, zwitterionic, mammalian membrane mimic), phosphatidylethanolamine (PE):phosphatidylglycerol (PG) (3:1, molar ratio, negatively charged bacterial membrane mimic), and *E. coli* total lipid extract (Gram negative bacteria) vesicles. Calcein is entrapped at a concentration that self-quenches so that when it leaks from the vesicle and is diluted into the surrounding media its emission will turn on and can be monitored. In all cases shown in Figure 5, the leakage was measured at 800 s against the same concentration of lipid vesicles and polymer (20 $\mu g \cdot mL^{-1}$).

In all cases, care must be taken when interpreting leakage activities of model membranes as shown in Figure 5. For example, the PE: PG (3:1) leakage experiment does not correlate with the MIC data and shows similar values of leakage for all the polymers, despite a 10-fold variation of the MIC. On the other hand, the use of *E. coli* total lipid extract vesicles showed much stronger correlations between MIC and leakage (see Figure 5). The same break in activity was observed in the leakage assays that was observed in the biological experiments (alkyl substituent $\leq C_4$, % leakage $\leq$20%; alkyl substituent $\geq C_6$, % leakage $\geq$60%). The polymers **4a** and **4b**, with MICs of 200 $\mu g \cdot mL^{-1}$, were the least active against vesicles composed of *E. coli* total lipid extract, while **4c–f** were much more active. It was also observed that leakage was not MW dependent and the activity trend showed almost the same behavior for the series with the $\overline{M}_n = 3$ and 10 kDa. The difference between the PE: PG and *E. coli* total lipid extract data may be related to the presence of other lipids in the *E. coli* total lipid extract that are not present in the PE:PG vesicles. For example, we have shown previously that the concentration of PE present in the lipid vesicles was essential for leakage and membrane re-organization.[47] In addition to PE and PG, *E. coli* total lipid extract contains 10% cardiolipin and 18% other lipids (not specifically identified).

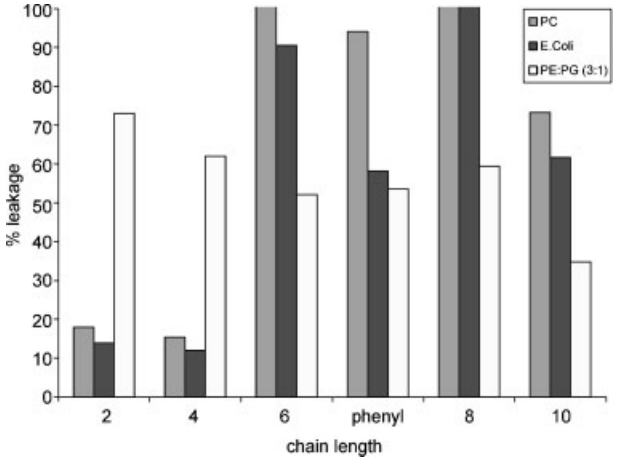

Figure 5. Leakage of neutral vesicles (PC), negatively charged vesicles; *E. coli*, and PE:PG (3:1) at 800 s, caused by 20 $\mu g \cdot mL^{-1}$ 1 of homopolymers **4a–f**, $\overline{M}_{n,th} = 3$ kDa.

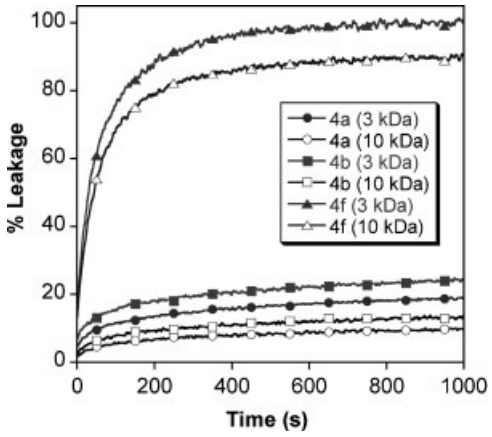

Figure 6. Leakage of neutral vesicles (PC) caused by 20 $\mu g \cdot mL^{-1}$ homopolymers, **4a**, **b** and **f** with $\overline{M}_{n,th} = 3$ and 10 kDa.

The major component of the outer membrane leaflet of RBCs is the zwitterionic phospholipid phosphatidylcholine (PC). As a result, PC vesicles are often used as models for RBC to determine the leakage affinity for antimicrobial peptides. Also shown in Figure 5 are leakage assays against PC vesicle and these also correlate with the HC_{50} experiments discussed earlier in Table 1. Polymer **4a** and **4b** were inactive against these neutral PC vesicles and showed little disruption at the measured concentrations. In contrast, polymers **4c–f**, which were much more hemolytic, showed more activity against PC vesicles. Figure 6 shows a typical example of a hydrophobic polymer, **4f**, which caused 90% leakage along with two polymers, **4a** and **4b**, which showed very little leakage at 20 $\mu g \cdot mL^{-1}$. This 'raw' data was used to create the graph shown in Figure 5 for the vesicles of different lipid composition.

Conclusion

The first synthesis of pyridine functionalized oxanorbornene based monomers and their ROMP was demonstrated. Biological activity (MIC, HC_{50}) and model membrane activities were also investigated and showed that the alkyl substituents have a distinct impact on activity. When the alkyl substituent $\leq C_4$, the polymers are weakly active (and not hemolytic), but when the alkyl substituent $\geq C_6$, the polymers are quite potent (and generally toxic). In addition, the effect of quaternization yield impacted the hemolytic, but not the antibacterial, activities. This implies that the synthetic route can impact the biological activity if quaternization of the polymer is not complete. Several of these pyridinium functionalized polymers were more potent and less toxic than any previously reported

quaternary pyridine based materials. In addition, compared to **MSI-78**, three polymers were as active and two of these were more selective. These results demonstrate the utility of the antimicrobial design based on balancing the hydrophobic/hydrophilic interactions.

Supporting Information Available

Synthesis of monomers and polymers: ^{1}H and ^{13}C NMR spectra of **2** and ^{1}H NMR spectrum of **4c**.

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