Chapter 11

Amphiphilic Polymers with Potent Antibacterial Activity

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A general strategy is summarized for the assembly of polar and nonpolar domains into a modular monomer structure. Living ring-opening metathesis polymerization (ROMP) of these monomers provides access to a large range of molecular weights with narrow molecular weight distributions. The character and size of each domain can be tuned independently and locked into the repeating unit of the amphiphilic polymers. Lipid membrane disruption activities were investigated for amphiphilic polynorbornene derivatives against liposomes. Water-soluble, amphiphilic, cationic polynorbornene derivatives, which exhibited the highest level of activities against liposome membranes, were then probed for their antibacterial activities in growth inhibition assays and hemolytic activities against human red blood cells in order to determine the selectivity of the polymers for bacterial over mammalian cells. By tuning the overall hydrophobicity of the polymer, highly selective, non-hemolytic antibacterial activities were obtained. These simple polymers represent a new approach to the development of nontoxic, broad-spectrum antimicrobials and have significant potential for applications in bio-terrorism defense.

In Antiterrorism and Homeland Defense; Reynolds, J., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2007.

Introduction

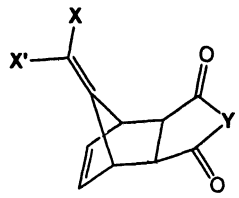
Well-defined amphiphilic macromolecules find important applications in biology and medical sciences. Examples include the use of polymeric materials in drug delivery (*1–5*), gene delivery (*6–9*), tissue engineering (*10–12*), and antibiotic agent applications (*13–19*). Continuing research efforts are focusing on the use of polymeric therapeutics as alternative antibiotic agents in the fight against bacterial diseases. Antibacterial activity of cationic polymers has been known for several decades (*14*). Various polymeric structures carrying cationic moieties have found considerable interest in non-medical use, such as food preservatives, pesticides, and disinfectants (*13*). Very recently, antibacterial activity of relatively simple cationic polymers has started to be considered within the scope of the studies involving naturally occurring host-defense peptides, and their synthetic mimics (*20–22*). Although more complex in their structure, antimicrobial peptides commonly contain cationic and hydrophobic domains (*23*). Successful research efforts that target synthetic mimics of host-defense peptides have typically followed a top-down approach, through modifications of naturally occurring peptide structures, in an effort to establish an understanding of structure-property relationships (*24*). In the development stage, synthetic mimics of host-defense peptides require elaborate and extensive techniques (*25–28*). Relatively simple synthetic cationic polymers offer an inexpensive alternative; however, they suffer from their high cytotoxicity if considered for therapeutic applications (*13*). Encouraged by the synthetic abilities for the controlled preparation of amphiphilic polymers, and inspired both by antimicrobial peptide research and synthetic biocidal polymers, we seek the determination of macromolecular properties that allows for antibacterial activity, while suppressing cytotoxicity. This chapter is a compilation of our efforts, including the design and synthesis of novel amphiphilic polymers (*29*), probing their structure-biological activity relationship patterns, and, finally, optimization of their amphiphilic character for selective antibacterial activity (*30*).

The initial focus of the current study is the preparation and homopolymerization of a novel class of monomers with amphiphilic character, where the amphiphilicity of the resulting polymer is tuned at the repeating unit level, giving rise to a polymer backbone structure with regularly spaced hydrophilic and hydrophobic groups. The molecular weight of the amphiphilic polymer is independently controlled through the choice of polymerization procedure. Ring-opening metathesis polymerization (ROMP) of amphiphilic modular monomers will be described as a synthetic tool for the facile probing of the effect of basic macromolecular variables on the interactions of polymers with living cells, prokaryotes and eukaryotes (*29*).

The starting point for monomer design is based on widely used norbornene derivatives. Norbornene derivatives having 2-mono or 2,3-difunctionalization

are known to be excellent monomers for ROMP. They have been used in the preparation of a wide range of polymeric structures (*31*). Using various norbornene derivatives, polymers bearing a variety of side groups have been prepared via ROMP. Functionalized norbornene derivatives are readily prepared via Diels-Alder cycloaddition of a diene, most generally furan or cyclopentadiene, to a dienophile possessing a desired functional group (*31*). This procedure affords an endo or exo 2- or 2,3-functionalized norbornene derivative. Endo isomers are known to be poor monomers for ROMP, presumably because of the increased steric crowd around the polymerization-active carbon-carbon double bond.

The task of preparing a monomer structure with dual functionality, in this case a hydrophilic and a hydrophobic group, lead us to investigate the preparation and polymerization of modular norbornene derivatives with additional functionality at the 7 position of the ring (Figure 1). Using this general strategy, two complementary functionalities can be introduced into the monomer structure and the properties of the resulting amphiphilic polymer can thus be fine-tuned.



X, X' = hydrophobic
Y = hydrophilic

Figure 1. General structures of amphiphilic modular norbornene derivatives.

Monomer Synthesis

Fulvene derivatives were used as functionalized dienes for the Diels-Alder cycloaddition reaction with an appropriate dienophile to obtain the modular norbornene structures (Figure 2). Three different fulvene derivatives, 6,6'-dimethyl fulvene, 6-isopropyl fulvene, and 6,6'-di-*n*-propyl fulvene, were prepared through a simple, synthetic methodology involving pyrrolidine-catalyzed condensation of cyclopentadiene with the appropriate aldehyde or ketone, resulting in high yields (*32*). The hydrophobic character of the monomer and the resulting polymer can be tuned by the choice of fulvene derivative. The modular approach to the monomer preparation allows for a variety of different alkyl groups to be readily incorporated. This allows for facile increase, or decrease, of the hydrophobic character of the monomer and, thus the resultant polymer.

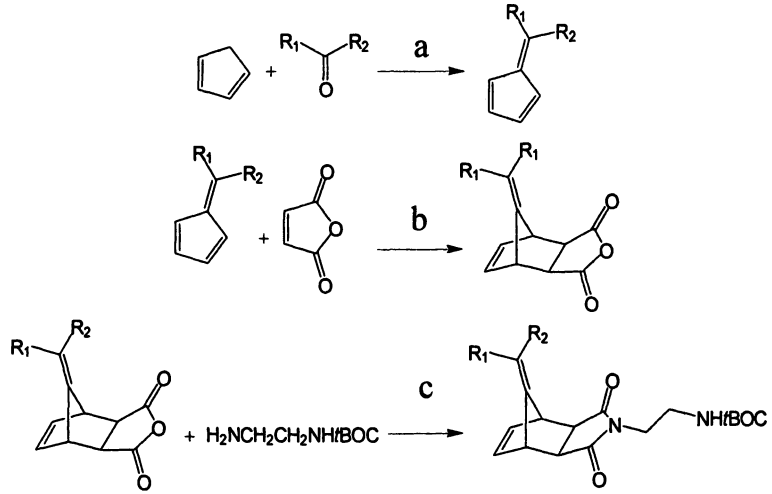
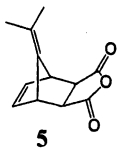
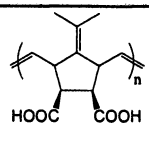
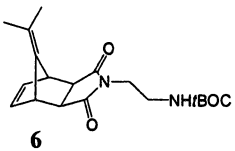
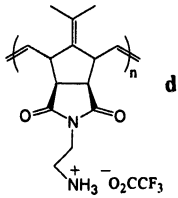
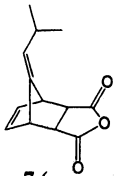
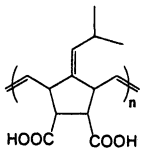
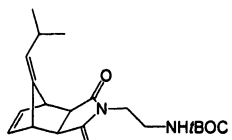
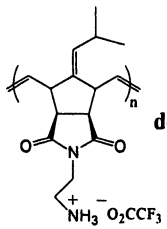
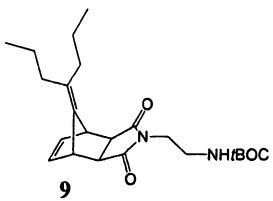
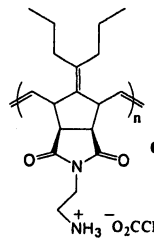


Figure 2. Representative preparation of modular norbornene derivatives. (a) pyrrolidine, CH_3OH (b) EtOAc, 80 °C (c) $CoAc_2$, Ac_2O, DMAc, 80 °C, 4 h.

Maleic anhydride was used as the dienophile, allowing for further functionalization following the assembly of the norbornene skeleton. Diels-Alder cycloadditions of the above-mentioned fulvene derivatives with maleic anhydride at elevated temperatures, between 80 °C and 120 °C, and moderate concentrations, 0.2 to 0.5 M, afforded quantitative yields of the corresponding norbornene derivatives **5**, **7**, and anhydride precursor of **9** (see Table I) (*29*). At total adduct concentrations above 1.5 M or temperatures above 130 °C, a solid oligomeric side product, presumably a copolymer of the reactants, was obtained. Two isomers, endo or exo, can be obtained from cycloaddition reactions, depending on the nature of adducts or the reaction temperature. These isomers exhibit different polymerization kinetics, where, in most cases, endo adducts polymerize very slowly, and result in low conversions.

To achieve a high-level of control over polymerizations, and resulting polymer microstructures, the preparation of pure exo isomers of the monomers were targeted. Exo-endo mixtures that were obtained as the cycloaddition adducts were not always separable by selective recrystallizations. Compounds **5**, **6**, **8**, and **9** were separated from their endo isomers through selective recrystallization to give white crystalline solids. Cobalt-catalyzed transformation of the anhydride into a substituted imide linkage resulted in the protected amine functionalized monomer structure in excellent yield. For monomers **6**, **8** and **9**, pure exo isomer was isolated by successive recrystallizations from cold ether; overall yields were 40 to 56%.

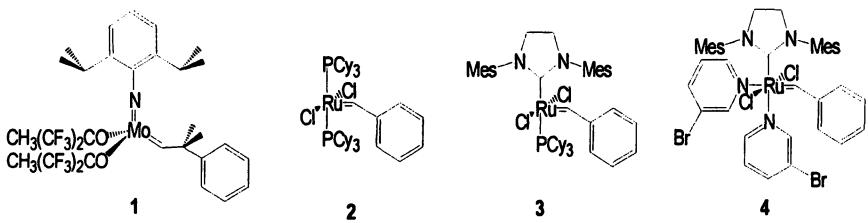
Table I. Examples of modular norbornene derivatives and amphiphilic polymers resulting from corresponding polymerizations[a] and deprotections

Monomer	*Deprotected Polymer*		*Theo. M_n[b] (g/mol)*	*Obs. M_n[c] (g/mol)*	*PDI[c]*
 **5**		**dep-poly5**	2,000	2,900[d]	1.15[d]
			5,100	7,000[d]	1.14[d]
			10,200	10,000[e]	1.17[e]
 **6**		**dep-poly6**	2,400	1,950	1.13
			5,900	7,000	1.08
			19,800	17,900	1.11
			29,900	24,100	1.13
 **7 (*exo-endo*)**		**dep-poly7**	10,900	9,500	1.63
			21,900	19,500	1.49
 **8**		**dep-poly8**	1,900	1,800	1.20
			8,800	8,600	1.10
			31,100	27,000	1.13
			63,300	57,200	1.70
 **9**		**dep-poly9**	4,900	5,300	1.09
			14,600	14,500	1.24
			32,300	32,200	1.13
			60,500	57,000	1.19

[a]Polymers were prepared using catalyst **4** (see Figure 3). [b]Theoretical molecular weights calculated based on catalyst to monomer ratio, assuming full conversion. [c]Determined by THF GPC relative to polystyrene standards prior to deprotection of polymer.[d]Determined by water GPC relative to poly(ethylene oxide) standards. [e]Determined by DMF GPC relative to polystyrene standards prior to the hydrolysis of polymer.

Homopolymerization Studies

The initial target of the current study was to prepare amphiphilic polymers with well-defined architectures. Because the amphiphilic character was already dictated in the monomer unit, the target in the polymerization study of modular norbornene derivatives was to achieve controlled polymerization and obtain narrow polydispersities. The polymerization of a model monomer, **8**, was tested using four different metathesis catalysts, **1–4** (see Figure 3), in order to screen the polymerizability and the effect of catalyst on the resulting polydispersities. The polymerization of **8** using catalysts **1–3** required elevated temperatures between 40–55 °C; whereas, catalyst **4** allowed for polymerization at room temperature. This result was in accordance with the reported high reactivity of catalyst **4** (*33*). Desired molecular weights ranging between 1,600 g/mol to 75,000 g/mol (M_n) could be obtained by adjusting the catalyst to monomer ratio for all four catalysts. For a targeted number average molecular weight of 8,800 g/mol at complete conversion, the polymerization of **8** using catalysts **1–4** resulted in polydispersity values of 1.23, 1.27, 1.96, and 1.10, respectively. Based on these results, the homopolymerizations, and subsequent random and block copolymerizations, involving monomers **5–9** were studied using catalyst **4** (Table I). Polymer obtained from monomer **5** (poly**5**) precipitated from the polymerization solution. Despite the early precipitation during polymerization, 88 to 90% yield of poly**5** was isolated, with polydispersity values ranging between 1.14 and 1.17 (M_n ranging from 2,900 to 10,000 g/mol). From the polymerization of monomer **6** using catalyst **4**, poly**6** was obtained in 85 to 90% yield, with polydispersity values ranging between 1.08 and 1.13.



1 2 3 4

Figure 3. Catalysts 1–4.

For all monomers, the obtained molecular weights were in agreement with the targeted molecular weights, as observed from GPC results. The slight discrepancy between the targeted and observed molecular weights of the polymers in Table I was expected due to the differences in hydrodynamic volume of these polymers versus narrow polydispersity polystyrene and

poly(ethylene oxide) GPC standards. ^{1}H NMR end-group analysis was performed to confirm the match between the targeted and observed number average molecular weights for samples with M_n values less than 9000 g/mol. The relative integrations of the resonances from the repeat units versus the multiplet from styrenic end-group at 7.32 ppm were in good agreement with the targeted molecular weight.

The polymerizations of exo-endo mixtures resulted in very low yields, where only a fraction of the exo isomer was polymerized. Despite the presence of endo isomer in the case of monomer **7**, the exo-endo mixture was polymerized in good yields into high molecular weight polymers using catalysts **3** and **4**; however, the resulting polydispersities were broader when compared to the other monomers (Table I).

Polymer Deprotection to Form Polyelectrolytes

The *t*-BOC protected pendant primary amine groups of poly**6**, poly**8**, poly**9**, poly**10**, and the anhydride functionalities of poly**5** and poly**7** provide a non-ionic and hydrophobic character to these polymers that allows for controlled ROMP and subsequent characterization of the polymers in a wide range of organic solvents. To obtain the final amphiphilic nature of the polymers, these groups were deprotected into their ionic forms, resulting in water-soluble polymers. Protected primary amine functionalities of different molecular weight samples of polymers were deprotected, quantitatively, by dissolution in warm (45 °C) trifluoroacetic acid (TFA) to obtain dep-poly**6**, dep-poly**8**, dep-poly**9**, and dep-poly**10**, as observed by ^{1}H NMR recorded in D_2O solutions. ^{1}H NMR spectra of these polymers also showed that carbon-carbon double bonds on the polymer backbone remained unaffected after treatment with TFA. Anhydride functionalities of poly**5**, and poly**7** were hydrolyzed by dissolution of polymers in NaOH solutions to obtain dep-poly**5**, and dep-poly**7**. After these processes, well-defined amphiphilic polymers with a desired anionic or cationic character and hydrophobic character were obtained.

Study of Phospholipid Membrane Disruption Activities

One interesting aspect of amphiphilic macromolecules is their interactions with phospholipid membranes, natural or artificial. Also amphiphilic in their chemical nature, phospholipid building blocks change their supramolecular ordering by incorporating amphiphilic polymers within their membrane assemblies. Depending on structural and compositional factors, various membrane deformations such as pore or tube formation, or complete disruption have been reported (*34–40*). In this respect, biological activities of amphiphilic

polymers are often associated with their ability to permeate cell membranes. Because phospholipid-based cell membranes are a principal structural component of living organisms that exclude the interior from the outside environment, their disruption by amphiphilic polymers and oligomers has attracted attention in the biomedical field. Applications, which are based on polymer-induced transport through or disruption of cell membranes, include drug delivery (*1–3*), gene delivery (*6–8*) and antibacterial agents (*20, 27, 41–44*). The antibacterial activity of cationic amphiphilic macromolecules, which will be discussed in the following sections, has been suggested to be through perturbation of bacterial cell membranes (*23, 34, 45, 46*).

Similarly, toxicity against mammalian cells can also be induced by the disruption of cell membranes, often measured as hemolytic activity against red blood cells (*24, 25, 27, 44*). The difference in the lipid compositions of cell membranes from different organisms has been widely suggested to be one of the likely causes for the selective activities of certain membrane disrupting antibacterial agents (*24, 34, 41*). Bacterial cell membranes are known to contain an excess of negative charge on the polar outer surface of their cell membranes. Mammalian cell membranes, on the other hand, possess a neutral zwitterionic outer surface, and contain cholesterol that stiffens the membrane. The outcome from the exposure of phospholipid membranes to amphiphilic macromolecules is dictated by the detailed physiochemical properties of both parties (*24, 47, 48*).

These scientific findings and suggestions, summarized above, were elucidated by a large number of studies that commonly utilize artificial liposomes as model membranes (*28, 34, 40, 44, 47, 49–52*). Liposomes consist of a phospholipid bilayer envelope isolating an inner volume (*53, 54*). They are available through well-established preparative techniques that allow strict control over molecular components of the membrane and the environment. Depending on the preparation details, the average diameters of vesicles typically range between 0.1–5 μm, with a lipid bilayer thickness of several nanometers. With these structural features, liposomes have also been widely studied as microcapsules for drug and gene delivery applications (*55–58*). Liposomes make it possible to monitor the dynamics of membrane perturbations, either by observing deformations using microscopy, if applicable, or by using an appropriate fluorescent dye encapsulated within, or excluded from, the liposome (*59–61*). The leakage of the fluorescent dye can be monitored as an indication of increased permeability or disruption of the membrane (Figure 4). In a typical experiment, liposomes are loaded with a self-quenching concentration of fluorescent dye. Following the addition of membrane-disrupting agent, the disruption of vesicles can be monitored by quantitatively measuring the increasing fluorescence arising from the leakage and dilution of the dye in the larger outer volume.

The disruption of neutral or anionic liposomes, with respect to their total lipid content and surface charge, has been commonly correlated to the selective activities of certain antibacterial agents against bacterial versus mammalian cells

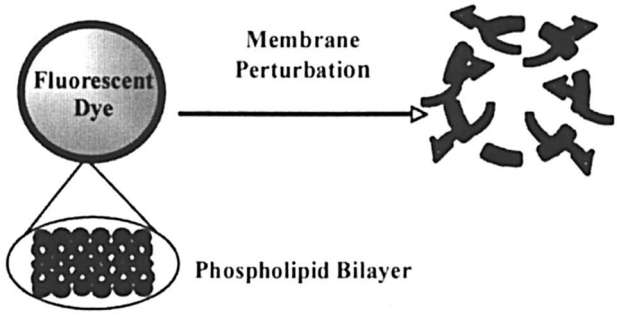


Figure 4. Representative illustrations of liposome and dye leakage experiment.

(*28, 40, 44, 47, 49–52*). These assays are well-documented in the literature and provide useful insight about the structure-property relationships of membrane-disruptive agents (*20, 27, 44*). Neutral zwitterionic liposomes, as mimics for mammalian cell membranes, are typically prepared from mixtures of 1-stearoyl-2-oleoyl phosphatidylcholine (SOPC) and cholesterol (CL) as a minor component (Figure 5) (*44*). Anionic liposomes, on the other hand, are prepared from SOPC and anionic phospholipid 1-stearoyl-2-oleoyl phosphatidylserine (SOPS), as mimics for bacterial cell membranes (*34, 39, 40*). These are simplified abiogenic models and, therefore, in our study, these tests were used to evaluate the overall membrane disruption activities of polymers. We do not make direct comparisons of these results to activity against biological cells. Selective disruption activity against anionic liposomes or neutral liposomes often depends on very subtle structural details of the amphiphilic macromolecule. The level of structural control over amphiphilic polynorbornene derivatives is used to control lipid membrane disruption activities. The effects of hydrophobicity and molecular weights for these amphiphilic polymers are probed against liposomes of different lipid content (*29*).

The current study was conducted side by side with the synthetic efforts presented above. Dep-poly**8**, which possesses an intermediate hydrophobicity compared to dep-poly**6** and dep-poly**9**, was the first to be probed for its membrane disruption activities against a series of neutral and anionic liposomes. The effects of lipid content, polymer concentration, and molecular weight on the outcome of membrane disruption activities were elucidated using dep-poly**8**. Then, the effect of polymer hydrophobicity was probed by testing dep-poly**6** and dep-poly**9**, with decreased and increased hydrophobicity, respectively.

When anionic liposomes prepared from 1:9 (molar ratio) mixtures of phosphatidylserine (anionic) and phosphatidylcholine (zwitterionic) were exposed to dep-poly**8**, a 13,500 g/mol (M_n) sample caused 100% lysis at concentrations as low as 5 μg/mL. Approximately 50% lysis was observed at a

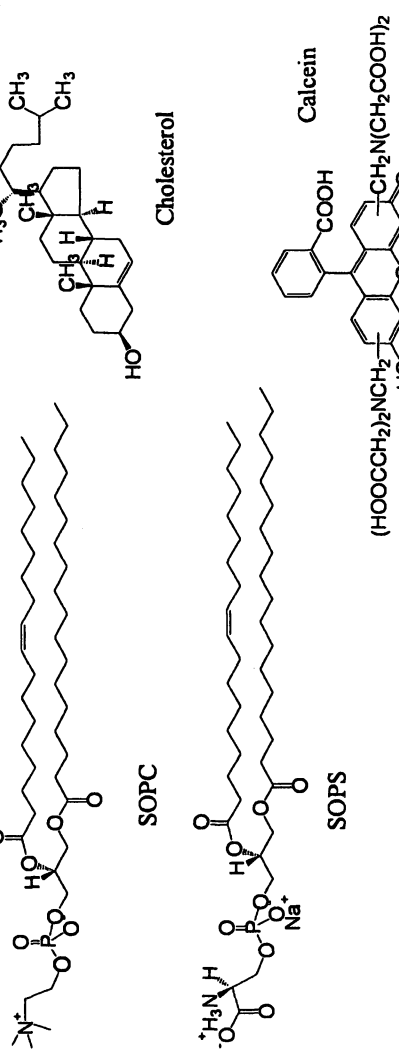


Figure 5. Structures of stearoyl-oleoyl-phosphatidylcholine (SOPC), stearoyl-oleoyl-phosphatidylserine (SOPS), cholesterol, and calcein used in the preparations of liposomes.

concentration of 1.25 μg/mL. Lysis was dose and molecular weight dependent. Figure 6, shows the increase of fluorescence from calcein release in the first 3 minutes after polymer addition, marked as percent lysis, indicating the disruption of vesicles caused by different molecular weights of dep-poly**8**. When a series of molecular weights of dep-poly**8** ranging between monomer and 64,000 g/mol (M_n) were studied, it was observed that the membrane disruption activity was lower for the monomer and oligomers with molecular weights less than 4,500 g/mol. Dep-poly**8** of molecular weights above 4,500 g/mol and up to 64,000 g/mol showed very high activities independent of molecular weight in this range. This result suggests that the membrane disruption activity of dep-poly**8** increases with molecular weight until it reaches a critical molecular weight necessary to obtain maximum membrane disruption activity. The living nature of ROMP allows for the precise targeting of the desired molecular weight and hence allows for tuning the membrane activity of dep-poly**8**s.

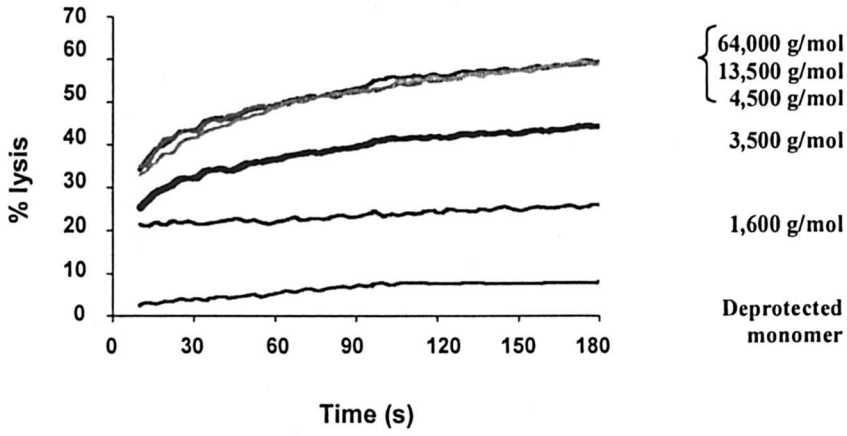


*Figure 6. Lysis of anionic liposomes caused by different number average molecular weight (M_n) samples of dep-poly**8** at the concentration of 1.25μg/mL.*

It should be noted that while probing the molecular weight effect, the concentration of the polymer added into the liposome suspension was calculated in terms of mass/volume. If the corresponding molar concentrations were to be calculated, the dep-poly**8** sample with a number average molecular weight of 64,000 g/mol would have 14 times fewer, but longer, chains than the dep-poly**8** sample of 4,500 g/mol at the same mass/volume concentration. With this idea in mind, very similar activities obtained from different molecular weights of dep-poly**8** at the same mass/volume concentration may suggest a cooperative action of polymer chains being responsible for membrane disruption.

Effect of Membrane Composition of Liposomes

In order to observe the effect of lipid composition of the membranes on the activity of the dep-poly**8**, neutral, zwitterionic liposomes with a 9:1 SOPC to cholesterol molar ratio, and anionic liposomes with 9:1 and 1:1 SOPC to SOPS molar ratios were prepared. Batches of liposomes with different ionic character were tested within the same experiment, using the same reagents and equipment in order to minimize experimental errors. The results were also confirmed in a second set of experiments.

When a solution of dep-poly**8** (M_n = 27,000, PDI = 1.13) in TRIS saline buffer (pH 6.5) was added to each of three different liposome suspensions, the membrane disruption activity was observed to increase with increasing anionic lipid content of liposome from 0 mol % to 50 mol %. 20 μg/mL of dep-poly**8** caused 90% lysis in 3 min against anionic liposomes with 1:1 SOPC to SOPS ratio. The percent lysis values at the same experimental conditions decreased to 67% as anionic lipid content decreased to a 9:1 ratio, and 24% for neutral vesicles with no anionic lipid content, but 10% cholesterol content. These results show increasing affinity of dep-poly**8** for negatively charged liposome membranes. This trend is consistent with the cationic nature of the polymer causing stronger interactions between phospholipid membrane and the polymer. More than two-fold selectivity against anionic liposomes can be induced by introducing 10% or more anionic lipid content.

Effect of Polymer Hydrophobicity

The previous sections have shown that high disruption activities against phospholipid membranes can be obtained from dep-poly**8**, depending on polymer molecular weight and membrane composition. The activities of dep-poly**6** and dep-poly**9** with relatively lower and higher degrees of hydrophobicity were tested against neutral (SOPC: CL = 9:1) and two different anionic (SOPC: SOPS = 9:1 and 1:1) liposomes. Similar molecular weight samples of dep-poly**6** (M_n = 24,100, PDI = 1.10), dep-poly**8** (M_n = 25,500, PDI = 1.17), and dep-poly**9** (M_n = 32,200, PDI = 1.17) were compared within the same experiment (Figure 7). The membrane disruption activities of all polymers were observed to increase with increasing anionic strength of the membrane. However, all three types of membranes were less vulnerable to both dep-poly**6** and dep-poly**9** when compared to dep-poly**8**. Increasing or decreasing the hydrophobicity in reference to dep-poly**8** resulted in diminished membrane disruption activities.

When the effect of molecular weight was probed for dep-poly**6** against anionic liposomes, increased molecular weights were shown to have increased activities, in accordance with the result obtained from dep-poly**8**. However, for dep-poly**6**, a plateau of maximum membrane disruption activity was reached

over approximately 50,000 g/mol. Finally, when the hydrophobicity was totally removed, in the case of dep-poly**10** (M_n = 25,000 g/mol), with an oxygen atom replacing the alkylidene group, the activity against anionic vesicles (SOPC: SOPS = 9:1) was no more than 8% lysis, up to a sufficiently high polymer concentration, 200 μg/mL, within 3 min. These results reveal that a specific hydrophilic/hydrophobic balance is crucial to obtain the highest membrane disruption activities from amphiphilic cationic polynorbornene derivatives.

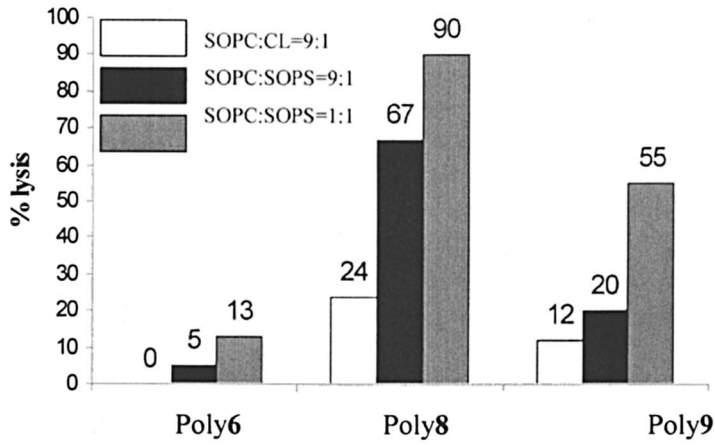


*Figure 7. Lysis values 3 min after the addition of 40 μg/mL of dep-poly**6** (M_n = 24,100, PDI = 1.10), dep-poly**8** (M_n = 25,500, PDI = 1.17), and dep-poly**9** (M_n = 32,200, PDI = 1.17) into suspensions of neutral (left, SOPC: CL = 9:1)and anionic liposomes (middle, SOPC: SOPS = 9:1 and right, SOPC: SOPS = 1:1).*

Control Experiments

In a control experiment, the anionic dep-poly**7** (M_n = 22,000 g/mol) was tested against the liposome suspensions and no lysis was observed at comparable concentrations. It is remarkable that dep-poly**7**, which has the same hydrophobicity as the cationic dep-poly**8**, caused no lysis. A change from cationic to anionic character of the polymer resulted in a dramatic decrease of membrane disruption activity. Three commercially available cationic polymers, polyallylamine (M_n = 25,000 g/mol), polyethyleneimine (M_n = 400,000 g/mol), and poly(dimethyldiallyl ammonium dichloride) (M_n = 75,000 g/mol), were also tested as control experiments. These polymer samples provide models for primary amine, secondary and tertiary amine (hyperbranched PEI), and

quaternary amine containing polymers, within a large range of high molecular weights. These polymers were observed to be far less active in the lysis of the liposomes when compared to dep-poly**8**, where percent lysis caused by this set of polymers remained below 10% over 3 minutes at a concentration of 15 μg/mL. These results confirmed once again that cationic amphiphilic polymer structures with a specific hydrophobicity have the highest activity for disruption of phospholipid membranes amongst the polymers studied, thus confirming the overall design principles.

Liposomes provide simplified models for bacterial and mammalian cell membranes, although they underestimate several factors such as cell walls and lipopolysaccharides in bacterial cell membranes. However, our results from membrane disruption activities of amphiphilic polymers built a strong foundation for structure-property relationships of these materials and warrant further exploration of antibacterial activities as well as any other relevant biomedical application of these polymeric materials.

Applications for Well-Defined Amphiphilic Polymers: Antibacterial Activity

Antibacterial activities of macromolecules, including oligomeric compounds, have been studied under two separate thrusts. One group of studies has focused on the structure-property relationships of natural host-defense peptides derived from multicellular organisms (*23, 24, 41, 62*). Despite their structural diversity, most are cationic peptides with a certain degree of hydrophobicity. Extensive studies on the mechanism of action suggest that antimicrobial peptides act by rendering permeable the cell membranes of microorganisms through favorable interactions with negatively charged and hydrophobic components of the membranes followed by aggregation and subsequent disruption (*23, 34, 42, 46*). Host-defense peptides and their synthetic analogs are reported to exhibit varying degrees of activity against wide spectrum of bacteria and mammalian cells (*23*). 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 (*23, 41, 62*). Hemolytic activity against highly susceptible human red blood cells, as representatives of normal mammalian cells, is conventionally used as a measure of cytotoxicity (*24, 34*). 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 (*34, 63*), α-peptides (*47*), β-peptides (*27, 42, 64, 65*), cyclic α-peptides (*26*), peptoids (*28*), and poly-arylamides (*49*), all of which are oligomeric with molecular weight below 3,000

g/mol. Many of these examples target an amphiphilic secondary structure, typically helical, in addition to their cationic nature. Depending 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 (*34, 47, 65*).

Independent from the antimicrobial peptide research, a second thrust involves studies of synthetic cationic polymers that exhibit varying degrees of antibacterial activities (*13–19*). This class of polymeric compounds is relatively inexpensive and less cumbersome to prepare when compared to peptide mimics. 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 (*13, 14*). Consistent with the targeted 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 (*66*). 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 (*67–71*). To the best of our knowledge, a direct comparison of antibacterial and hemolytic action has not been reported for either of these classes 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 (*21*). In their initial study, a crossover between the research on antimicrobial peptide mimics and polymer disinfectants, cationic polystyrene derivatives have shown similar antibacterial activities as the magainin derivative, but were highly hemolytic. As a part of very recent efforts in the area, selective activities of facially amphiphilic low molecular weight polyphenyleneethynylenes were reported, with activities and selectivities similar to a magainin derivative (*22*). The successful design of non-hemolytic, antibacterial, and high molecular weight polymers has not been achieved thus far.

Here we present the antibacterial and hemolytic activities of narrow polydispersity homopolymers of modular norbornene derivatives, spanning a large range of molecular weights. The 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. The results show that it is possible to design simple polymers that are both potent against bacteria, but non-hemolytic.

Antibacterial and Hemolytic Activities

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 homopolymers 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 II). The upper limit of polymer concentration that was required to cause 50% hemolysis is reported as HC_{50}. Antibacterial activity was expressed as minimal inhibitory concentration (MIC), the concentration at which 90% inhibition of growth was observed after 8 h (*30*). Dep-poly**10**, a cationic polymer with no substantial hydrophobic group, did not show any significant antibacterial or hemolytic activity within the measured concentrations. At the highest concentration measured for hemolytic activity, 1000 μg/mL, dep-poly**10** caused 5% hemolysis.

Table II. Antibacterial and hemolytic activities of homopolymers

Polymer	*M_n (g/mol)*	*PDI*	*MIC [μg/mL, (μM)]*		*HC_{50} [μg/mL, (μM)]*	*Selectivity*	
			E. coli	*B. subtilis*		*E. coli*	*B. subtilis*
Dep-Poly**10**	10,250	1.07	>500, (>49)	>500, (>49)	>1000, (>98)	-	-
Dep-Poly**6**	9,950	1.10	200, (20)	300, (30)	>4000, (>400)	>20	>13
Dep-Poly**8**	10,050	1.13	25, (2.5)	25, (2.5)	<1, (<0.1)	<0.04	<0.04
Dep-Poly**9**	10,300	1.08	200, (19)	200, (19)	<1, (<0.1)	<0.005	<0.005

This result is consistent with the lack of activity against phospholipid membranes. 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. Dep-poly**6**, with an isopropylidene pendant group, exhibited antibacterial activity with MIC of 200 μg/mL against *E. coli*, which is less efficacious than most antimicrobial peptides, and their mimic, that have MICs typically ranging between 1–50 μg/mL (*23, 25–28, 47, 62–65*). However, the hemolytic activity of dep-poly**6** remained below 5% up to 3000 μg/mL, a value well above its MIC. Above 3000 μg/mL the hemolytic activity of this polymer increase more rapidly with increasing concentration. Increase in hemolysis, to 25%, at 4000 μg/mL could be induced through different

mechanisms, such as increased osmotic pressure at high polymer concentration, rather than a local membrane perturbation. However HC_{50} value remained above the measured concentration of 4000 μg/mL, thus giving a selectivity, defined as the ratio of HC_{50} to MIC (*49*), greater than 20.

Dep-poly**8**, with an additional carbon atom per repeat unit, is more hydrophobic than dep-poly**6**, and has additional mobility of the pendant alkyl group. Dep-poly**8** exhibited a substantial increase in antibacterial activity, with MIC of 25 μg/mL for both *E. coli* and *B. subtilis* as well as hemolytic activity, HC_{50} less than 1 μg/mL, with an 80% hemolysis at 1 μg/mL. 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 (*23, 24, 41, 62*). In the case of dep-poly**9**, when the hydrophobic size was further increased, the hemolytic activity was retained with a 100% hemolysis at 1 μg/mL; however, the antibacterial activity decreased to a MIC of 200 μg/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 (*23, 47*). These results show that the presence, and balance, of hydrophobic and hydrophilic groups dictate the antibacterial and hemolytic activities of the amphiphilic, non-natural polymer, in agreement with natural peptide studies. Moreover, copolymers of **6** and **8** generated highly active agents, with MIC of 40 μg/mL and selectivity greater than 100 (*30*).

Table III. Percent hemolysis values at the lower and upper limits of HC_{50} measurements

Polymer	*M_n (g/mol)*	*PDI*	*% Hemolysis at*	
			4000 μg/mL	*1 μg/mL*
Dep-Poly**6**	1,600	1.15	24	-
	137,500	1.27	22	-
Dep-Poly**8**	1,650	1.26	-	78
	57,200	1.70	-	70
Dep-Poly**9**	5,300	1.09	-	100
	57,000	1.19	-	100

It was previously suggested in the literature that a comparison between new compounds and a reference peptide would be the best indicator for clinical cytotoxicity, and allow a better comparison between different antibacterial agents from different laboratories (*72*). In a control experiment, the activity of a Magainin derivative (MSI-78), a well-known antimicrobial peptide, was measured against the same *E. coli* strain. In comparison to above described

homopolymers, MSI-78 exhibited a selectivity of 9.6 that was calculated from an MIC of 12.5 μg/mL and HC_{50} of 120 μg/mL.

Experimental Considerations: Effect of Blood Freshness

All hemolysis results that are reported in this work were obtained using freshly drawn blood from one individual. During the course of this study, the hemolytic activities of polymers were observed to be dependent on the freshness of the blood. Differences were also noted for blood obtained from different individuals. It was determined that blood stored for more than 7 d was more susceptible to hemolysis than freshly drawn blood. These observations were in accordance with previous literature that reported higher susceptibility to hemolysis, caused by a series of cationic antimicrobial peptides, in the case of blood stored for 21 d in 4 °C, as opposed to fresh blood (*72*). Non-hemolytic polymers, dep-poly**6**, and dep-poly**10** remained non-hemolytic against old blood that was stored for 3 weeks at 4 °C, and blood from different individuals, with HC_{50} values above 4000 μg/mL.

Advantages of ROMP-Based Synthetic Strategy

As mentioned in the introduction section, amphiphilic polymers have attracted attention for a number of biomedical and therapeutic applications. A variety of amphiphilic polymer architecture was considered either as delivery agents for drugs (*73–75*) and genes (*6, 9, 76*), structural components in tissue engineering, or active therapeutics, such as antibacterial agents (*15–17, 24*). A number of reports described the use of ROMP for the preparation of polymers decorated with biologically active agents, including peptides (*77*), carbohydrates (*78*), oligonucleotides (*79*), and anti-cancer drugs (*80*). These unique materials with high local density of the active groups in the vicinity of the polymer chains warrant further evaluations in therapeutic applications. These synthetic approaches can easily be combined with our approach through copolymerizations, in order to incorporate various polymer segments with distinct, and complementary biological activities. A proper choice of membrane disrupting amphiphilic block, based on modular norbornene derivatives, could provide selective antibacterial activity, as well as facilitate delivery of these multi-component polymeric agents through mammalian cell membranes. With a powerful set of ROMP-based synthetic approaches available, and careful monomer design, the potency of polymeric therapeutic agents can thus be fine-tuned.

Conclusions

Our initial motivation was to develop amenable synthetic approaches for the preparation of amphiphilic polymers with well-controlled structures that would broaden the interface between macromolecular science and biological sciences. Following those efforts, amphiphilic polymers based on modular norbornene derivatives were shown to exhibit good antibacterial activities and high selectivity for bacteria versus red blood cells. Small modifications to the hydrophobic character of the cationic amphiphilic polymer were shown to change dramatically the antibacterial and hemolytic activities. Tuning the hydrophilic/hydrophobic balance and molecular weights of these copolymers allowed preparation of highly selective, antibacterial, non-hemolytic 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. Equally important, we have extended the antimicrobial activity of these designed polymers to include efficient killing against all category A organisms (*B. anthracis*, *F. tularensis*, and *Y. pestis*) as well as other serious pathogens (*L. monocytogenes*, *P. aeruginosa*, and *MRSA*). The overall data strongly suggest this approach will lead to new materials for homeland defense applications, as well as important contributions to the prevention and treatment of bacterial infections.

References

1. Thomas, J. L.; Tirrell, D. A. *Acc. Chem. Res.* **1992**, *25*, 336-342.
2. Stayton, P. S.; Hoffman, A. S.; Murthy, N.; Lackey, C.; Cheung, C.; Tan, P.; Klumb, L. A.; Chilkoti, A.; Wilbur, F. S.; Press, O. W. *J. Controlled Release* **2000**, *65*, 203-220.
3. Kyriakides, T. R.; Cheung, C. Y.; Murthy, N.; Bornstein, P.; Stayton, P. S.; Hoffman, A. S. *J. Controlled Release* **2002**, *78*, 295-303.
4. Gonzalez, H.; Hwang, S. J.; Davis, M. E. *Bioconjugate Chemistry* **1999**, *10*, 1068-1074.
5. Wen, J.; Kim, G. J. A.; Leong, K. W. *J. Controlled Release* **2003**, *92*, 39-48.
6. Hwang, S. J.; Davis, M. E. *Current Opinion in Molecular Therapeutics* **2001**, *3*, 183-191.
7. Cheung, C. Y.; Murthy, N.; Stayton, P. S.; Hoffman, A. S. *Bioconjugate Chemistry* **2001**, *12*, 906-910.

8. Wolfert, M. A.; Dash, P. R.; Nazarova, O.; Oupicky, D.; Seymour, L. W.; Smart, S.; Strohalm, J.; Ulbrich, K. *Bioconjugate Chemistry* **1999**, *10*, 993-1004.
9. Zhao, Z.; Wang, J.; Mao, H. Q.; Leong, K. W. *Advanced Drug Delivery Reviews* **2003**, *55*, 483-499.
10. Wan, A. C. A.; Mao, H. Q.; Wang, S.; Phua, S. H.; Lee, G. P.; Pan, J. S.; Lu, S.; Wang, J.; Leong, K. W. *Journal of Biomedical Materials Research Part B-Applied Biomaterials* **2004**, *70B*, 91-102.
11. Wang, J.; Sun, D. D. N.; Shin-ya, Y.; Leong, K. W. *Macromolecules* **2004**, *37*, 670-672.
12. Wan, A. C. A.; Mao, H. Q.; Wang, S.; Leong, K. W.; Ong, L.; Yu, H. *Biomaterials* **2001**, *22*, 1147-1156.
13. Tashiro, T. *Macromolecular Materials and Engineering* **2001**, *286*, 63-87.
14. Worley, S. D.; Sun, G. *Trends in Polymer Science* **1996**, *4*, 364-370.
15. Stiriba, S. E.; Frey, H.; Haag, R. *Angew. Chem.-Int. Edit.* **2002**, *41*, 1329-1334.
16. Lim, S. H.; Hudson, S. M. *Journal of Macromolecular Science-Polymer Reviews* **2003**, *C43*, 223-269.
17. Thorsteinsson, T.; Loftsson, T.; Masson, M. *Current Medicinal Chemistry* **2003**, *10*, 1129-1136.
18. Kenawy, E. R.; Mahmoud, Y. A. G. *Macromolecular Bioscience* **2003**, *3*, 107-116.
19. Pavlikova, M.; Lacko, I.; Devinsky, F.; Mlynarcik, D. *Collect. Czech. Chem. Commun.* **1995**, *60*, 1213-1228.
20. Tew, G. N.; Liu, D. H.; Chen, B.; Doerksen, R. J.; Kaplan, J.; Carroll, P. J.; Klein, M. L.; DeGrado, W. F. *Proc. Natl. Acad. Sci. U. S. A.* **2002**, *99*, 5110-5114.
21. Gelman, M. A.; Weisblum, B.; Lynn, D. M.; Gellman, S. H. *Organic Letters* **2004**, *6*, 557-560.
22. Arnt, L.; Nusslein, K.; Tew, G. N. *Journal of Polymer Science Part A-Polymer Chemistry* **2004**, *42*, 3860-3864.
23. Andreu, D.; Rivas, L. *Biopolymers* **1998**, *47*, 415-433.
24. van 't Hof, W.; Veerman, E. C. I.; Helmerhorst, E. J.; Amerongen, A. V. N. *Biological Chemistry* **2001**, *382*, 597-619.
25. Porter, E. A.; Wang, X.; Lee, H. S.; Weisblum, B.; Gellman, S. H. *Nature* **2000**, *405*, 298-298.
26. Fernandez-Lopez, S.; Kim, H. S.; Choi, E. C.; Delgado, M.; Granja, J. R.; Khasanov, A.; Kraehenbuehl, K.; Long, G.; Weinberger, D. A.; Wilcoxen, K. M.; Ghadiri, M. R. *Nature* **2001**, *412*, 452-455.
27. Liu, D. H.; DeGrado, W. F. *J. Am. Chem. Soc.* **2001**, *123*, 7553-7559.
28. Patch, J. A.; Barron, A. E. *J. Am. Chem. Soc.* **2003**, *125*, 12092-12093.
29. Ilker, M. F.; Schule, H.; Coughlin, E. B. *Macromolecules* **2004**, *37*, 694-700.

30. Ilker, M. F.; Nusslein, K.; Tew, G. N.; Coughlin, E. B. *J. Am. Chem. Soc.* **2004**, *126*, 15870-15875.
31. Ivin, K. J., Mol, J. C. *Olefin Metathesis and Metathesis Polymerization*; Academic Press: San Diego, CA, 1997.
32. Stone, K. J.; Little, R. D. *J. Org. Chem.* **1984**, *49*, 1849-1853.
33. Choi, T. L.; Grubbs, R. H. *Angew. Chem.-Int. Edit.* **2003**, *42*, 1743-1746.
34. Oren, Z.; Shai, Y. *Biopolymers* **1998**, *47*, 451-463.
35. Menger, F. M.; Seredyuk, V. A.; Kitaeva, M. V.; Yaroslavov, A. A.; Melik-Nubarov, N. S. *J. Am. Chem. Soc.* **2003**, *125*, 2846-2847.
36. Murthy, N.; Robichaud, J. R.; Tirrell, D. A.; Stayton, P. S.; Hoffman, A. S. *J. Controlled Release* **1999**, *61*, 137-143.
37. Helander, I. M.; Latva-Kala, K.; Lounatmaa, K. *Microbiology* **1998**, *144*, 385-390.
38. Arnt, L.; Tew, G. N. *J. Am. Chem. Soc.* **2002**, *124*, 7664-7665.
39. Yaroslavov, A. A.; Yaroslavova, E. G.; Rakhnyanskaya, A. A.; Menger, F. M.; Kabanov, V. A. *Colloids and Surfaces B-Biointerfaces* **1999**, *16*, 29-43.
40. Oku, N.; Yamaguchi, N.; Shibamoto, S.; Ito, F.; Nango, M. *Journal of Biochemistry* **1986**, *100*, 935-944.
41. Zasloff, M. *Nature* **2002**, *415*, 389-395.
42. Hamuro, Y.; Schneider, J. P.; DeGrado, W. F. *J. Am. Chem. Soc.* **1999**, *121*, 12200-12201.
43. Porter, E. A.; Wang, X. F.; Lee, H. S.; Weisblum, B.; Gellman, S. H. *Nature* **2000**, *404*, 565-565.
44. Oren, Z.; Shai, Y. *Biochemistry* **1997**, *36*, 1826-1835.
45. Hancock, R. E. W.; Chapple, D. S. *Antimicrob. Agents Chemother.* **1999**, *43*, 1317-1323.
46. Huang, H. W. *Biochemistry* **2000**, *39*, 8347-8352.
47. Dathe, M.; Schumann, M.; Wieprecht, T.; Winkler, A.; Beyermann, M.; Krause, E.; Matsuzaki, K.; Murase, O.; Bienert, M. *Biochemistry* **1996**, *35*, 12612-12622.
48. Tytler, E. M.; Anantharamaiah, G. M.; Walker, D. E.; Mishra, V. K.; Palgunachari, M. N.; Segrest, J. P. *Biochemistry* **1995**, *34*, 4393-4401.
49. Liu, D. H.; Choi, S.; Chen, B.; Doerksen, R. J.; Clements, D. J.; Winkler, J. D.; Klein, M. L.; DeGrado, W. F. *Angew. Chem.-Int. Edit.* **2004**, *43*, 1158-1162.
50. Zhao, H. X.; Bose, S.; Tuominen, E. K. J.; Kinnunen, P. K. J. *Biochemistry* **2004**, *43*, 10192-10202.
51. Pokorny, A.; Almeida, P. F. F. *Biochemistry* **2004**, *43*, 8846-8857.
52. Juvvadi, P.; Vunnam, S.; Merrifield, R. B. *J. Am. Chem. Soc.* **1996**, *118*, 8989-8997.
53. Almeida, P. F. F.; Vaz, W. L. C.; Thompson, T. E. *Biochemistry* **1992**, *31*, 6739-6747.

54. Hotani, H.; Nomura, F.; Suzuki, Y. *Current Opinion in Colloid & Interface Science* **1999**, *4*, 358-368.
55. Kisak, E. T.; Coldren, B.; Evans, C. A.; Boyer, C.; Zasadzinski, J. A. *Current Medicinal Chemistry* **2004**, *11*, 199-219.
56. Drummond, D. C.; Zignani, M.; Leroux, J. C. *Progress in Lipid Research* **2000**, *39*, 409-460.
57. Noble, C. O.; Kirpotin, D. B.; Hayes, M. E.; Mamot, C.; Hong, K.; Park, J. W.; Benz, C. C.; Marks, J. D.; Drummond, D. C. *Expert Opinion on Therapeutic Targets* **2004**, *8*, 335-353.
58. Santos, N. C.; Castanho, M. *Quimica Nova* **2002**, *25*, 1181-1185.
59. Holopainen, J. M.; Angelova, M.; Kinnunen, P. K. J. *Liposomes, Pt A* **2003**, *367*, 15-23.
60. Antonietti, M.; Forster, S. *Advanced Materials* **2003**, *15*, 1323-1333.
61. Vaz, W. L. C.; Almeida, P. F. F. *Current Opinion in Structural Biology* **1993**, *3*, 482-488.
62. Hancock, R. E. W. *Drugs* **1999**, *57*, 469-473.
63. Wade, D.; Boman, A.; Wahlin, B.; Drain, C. M.; Andreu, D.; Boman, H. G.; Merrifield, R. B. *Proc. Natl. Acad. Sci. U. S. A.* **1990**, *87*, 4761-4765.
64. Raguse, T. L.; Porter, E. A.; Weisblum, B.; Gellman, S. H. *J. Am. Chem. Soc.* **2002**, *124*, 12774-12785.
65. Schmitt, M. A.; Weisblum, B.; Gellman, S. H. *J. Am. Chem. Soc.* **2004**, *126*, 6848-6849.
66. Li, G. J.; Shen, J. R.; Zhu, Y. L. *J. Appl. Polym. Sci.* **1998**, *67*, 1761-1768.
67. Rowden, A.; Cutarelli, P. E.; Cavanaugh, T. B.; Sellner, P. A. *Investigative Ophthalmology & Visual Science* **1997**, *38*, 5135-5135.
68. Liu, N. H.; Khong, D.; Chung, S. K.; Hwang, D. G. *Investigative Ophthalmology & Visual Science* **1996**, *37*, 4058-4058.
69. Vogelberg, K.; Boehnke, M. *Investigative Ophthalmology & Visual Science* **1994**, *35*, 1337-1337.
70. Albert, M.; Feiertag, P.; Hayn, G.; Saf, R.; Honig, H. *Biomacromolecules* **2003**, *4*, 1811-1817.
71. Messick, C. R.; Pendland, S. L.; Moshirfar, M.; Fiscella, R. G.; Losnedahl, K. J.; Schriever, C. A.; Schreckenberger, P. C. *J. Antimicrob. Chemother.* **1999**, *44*, 297-298.
72. Helmerhorst, E. J.; Reijnders, I. M.; van't Hof, W.; Veerman, E. C. I.; Amerongen, A. V. N. *FEBS Lett.* **1999**, *449*, 105-110.
73. D'Souza, A. J. M.; Topp, E. M. *J. Pharm. Sci.* **2004**, *93*, 1962-1979.
74. Pawar, R.; Ben-Ari, A.; Domb, A. J. *Expert Opinion on Biological Therapy* **2004**, *4*, 1203-1212.
75. Ghosh, S. *Journal of Chemical Research-S* **2004**, 241-246.
76. Pannier, A. K.; Shea, L. D. *Molecular Therapy* **2004**, *10*, 19-26.
77. Maynard, H. D.; Okada, S. Y.; Grubbs, R. H. *Macromolecules* **2000**, *33*, 6239-6248.

78. Mortell, K. H.; Gingras, M.; Kiessling, L. L. *J. Am. Chem. Soc.* **1994**, *116*, 12053-12054.
79. Watson, K. J.; Park, S. J.; Im, J. H.; Nguyen, S. T.; Mirkin, C. A. *J. Am. Chem. Soc.* **2001**, *123*, 5592-5593.
80. Watson, K. J.; Anderson, D. R.; Nguyen, S. T. *Macromolecules* **2001**, *34*, 3507-3509.