Antimicrobial Activity of Statistical Polymethacrylic Sulfopropylbetaines Against Gram-Positive and Gram-Negative Bacteria

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ABSTRACT: A series of statistical copolymers derived from 2-(dimethylamino)ethyl methacrylate with four different hydrophobic comonomers (ethyl, butyl, cyclohexyl, and octyl methacrylates) have been prepared via conventional free radical copolymerization under bulk conditions. The copolymers have been subsequently modified, with 1,3-propanesultone to yield the corresponding polysulfoproylbetaine derivatives. Those copolymers exhibiting the requisite aqueous solubility have been screened with respect to their antimicrobial activity against two common and notorious pathogens, namely *Staphylococcus aureus (S. aureus)* and *Escherichia coli (E. coli)*. We show that certain copolymers do indeed exhibit antimicrobial activity. The extent of activity is related to the molecular characteristics of the materials such as the molar composition and structure of the hydrophobic comonomer. © 2006 Wiley Periodicals, Inc. J Appl Polym Sci 101: 1036–1041, 2006

Key words: biological applications of polymers; copolymerization; radical polymerization; structure-property relations

INTRODUCTION

Polymeric betaines are a subclass of polyzwitterion in which both the cationic and anionic residues are present on the same repeat unit.[1] The cationic moiety is most commonly a quaternary ammonium species while the anionic segment may be, for example, a phosphonate (phosphobetaines), a sulfonate (sulfobetaines), a carboxylate (carboxybetaine), or a dicyanoethenolate species. In the parlance of biochemistry, these can be considered to be the synthetic polymeric analogs of phospho- or sulfolipids. Synthetic polymeric betaines have been known since the 1950s[2,3] with most of the examples to date having been synthesized via conventional free radical chemistry, although in recent years several research groups have reported the synthesis of well-defined polymeric betaines employing a number of controlled/"living" polymerization methodologies.[4–9] Such materials, which are mostly examples of statistical copolymers, are interesting for a variety of reasons. For example, in aqueous solution polymeric betaines typically exhibit so-called antipolyelectrolyte[10] behavior—a feature which is important in both biomedical and nonbiomedical applications. For example, such behavior renders certain polymeric betaines effective viscosifying agents for enhanced oil recovery.

Synthetic polymeric betaines have attracted a significant amount of interest from the biomedical community because of their biomimetic characteristics. In particular, synthetic phosphobetaines[11] have been studied extensively because of their bio- or hemocompatible properties.[12–17] Specifically, materials based on 2-(methacryloyloxy)ethyl phosphorylcholine (MEPC) have been widely evaluated. Much of the research on these materials has focused on applications in which the antibioadherent characteristics of these materials play a crucial role. For example, Sugiyama et al.[18] and Ishihara et al.[19] have demonstrated that polymers containing MEPC coated onto surfaces are extremely effective at reducing the occurrence of protein adsorption from human plasma. This particular attribute has, for example, led to the evaluation of MEPC-containing polymers as coatings for blood filtration devices.[20] Additionally, other classes of betaines, and specifically the sulfobetaines, have also been evaluated with respect to their antiadherent properties, and although they do not generally perform as well as the synthetic phosphobetaine analogs, they do possess the same general characteristics and activity.[21,22]

The development of new antimicrobials is currently an important issue in the biomedical community due,

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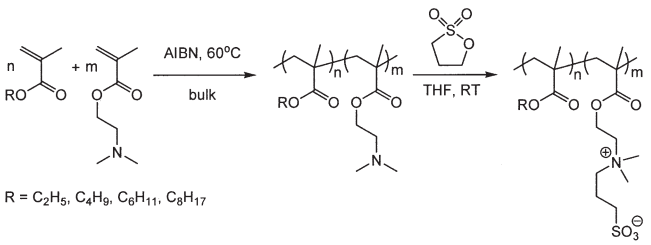
in part, to the alarming increase in antibiotic resistance.[23] In some instances, infections that have been routinely treated with conventional antibiotics no longer respond to treatment. In addition, because of an aging population that requires more health care, there has been an increase in the number of surgical procedures and interventions and in the number of implants used. Such procedures are inevitably hampered by infections that develop as a direct result of contaminated surgical instruments or implants. Use of new antimicrobial and or antiadherent agents to coat medical instruments will greatly improve the success rate of surgery and reduce the incidence of nosocomial disease (infections originating in hospitals).

It is well known that certain quaternary ammonium-containing compounds and polymers possess antimicrobial properties.[24–27] Much less studied are the antimicrobial characteristics of polymeric betaines, even though some have been shown to possess such characteristics. For example, Sawada et al.[28] described the synthesis and properties of fluoroalkylated telechelic carboxybetaine polymers based on 2-(3-acrylamidopropyldimethylammonio)ethanoate (APDMAE). Although the nonfluorinated polyAPDMAE homopolymer did not exhibit any antibacterial properties, the fluoroalkylated species did possess some activity against bacteria. The observed antimicrobial activity was attributed to the interaction of the ammonium functional group of the betaine species with the negatively charged cell membranes. Although this facilitates binding via simple covalent interactions, presumably, the hydrophobic fluoroalkylated end-groups also play an important role in cell-membrane disruption. This highlights the need for a hydrophobic component in these materials to render them effective bactericides.

As part of a larger collaborative program aimed at developing novel betaine-based materials for biomedical applications, we report herein our preliminary observations regarding the antimicrobial properties of a series of statistical methacrylic-based polysulfopropylbetaines. Precursor AB random copolymers comprised of four different alkyl methacrylates with 2-(dimethylamino)ethyl methacrylate (DMAEMA) were prepared via conventional free radical copolymerization under bulk conditions employing 2,2′-Azobis(isobutyronitrile) (AIBN) as the source of primary radicals. These precursor copolymers were subsequently derivatized via the tertiary amine residues on DMAEMA with 1,3-propanesultone to yield the corresponding polymeric sulfobetaines, according to literature procedures[4] (Scheme 1). The statistical betaine copolymers were then evaluated with respect to their antimicrobial activity against *Escherichia coli* (*E. coli*) and *Staphylococcus aureus* (*S. aureus*). These two notorious pathogens are intimately associated with nosocomial infections. Furthermore, they represent



Scheme 1 Reaction pathway outlining the preparation of the target sulfopropylbetaine copolymers.

both gram-negative (*E. coli*) and gram-positive (*S. aureus*) bacteria, which have distinct cell wall structures and physiology. These two groups of bacteria can respond differently to antimicrobial agents, and therefore it is critical to test the efficacy of novel materials against both.

EXPERIMENTAL

All chemicals were purchased from Aldrich Chemical Co. (Milwaukee, WI) or Polysciences Inc. (Warrington, PA) and were used as received unless stated otherwise. Ethyl, butyl, cyclohexyl, octyl, and 2-(dimethylamino)ethyl methacrylates were passed through a column of basic alumina to remove inhibitor and were stored in a refrigerator until needed. 2,2′-Azobis(isobutyronitrile) (AIBN) was recrystallized from methanol and stored in the freezer until needed.

Bacterial strains were obtained from well-characterized stocks and were frozen at −80°C. *E. coli* strain TOP10 was purchased from Invitrogen, and *S. aureus* RN6390 was obtained from Richard Novick's laboratory. Bacteria were cultured in broth using tryptic soy broth (TSB) or in solid medium using tryptic soy agar. Culture media were obtained from Difco Laboratories. Bacterial inocula were incubated at 37°C with shaking. Optical density was used to estimate the number of bacteria. A spectrophotometer (Biomate 3) from Thermo Spectronic was used to measure optical density. Aseptic techniques were used for all microbiological experiments.

Synthesis of statistical copolymers

A typical procedure for preparation of a statistical copolymer of 2-(dimethylamino)ethyl methacrylate (DMAEMA) with an alkyl methacrylate comonomer is discussed here. To a scintillation vial (20.0 mL capacity) equipped with a magnetic stir bar was added butyl methacrylate (5.02 g, 0.035 mol), 2-(dimethylamino)ethyl methacrylate (3.70 g, 0.024 mol), and AIBN (9.0 mg, 5.5×10^{-5} mol). The vial was sealed with a rubber septa and the solution purged with Ar for ~15 min. Subsequently, the vial was immersed in a preheated-oil bath at 60°C. Polymerizations were left

for ~2 h prior to termination by cooling and exposure to air. The highly viscous material was then diluted with THF. The copolymer was isolated by precipitation into a large excess of hexane. The hexane was decantered and the copolymer samples were dried overnight *in vacuo* at room temperature.

Conversion of statistical copolymers to corresponding polysulfopropylbetaines

Conversion of the alkyl methacrylate-*stat*-DMAEMA copolymers to the corresponding betaines was accomplished by reaction of the tertiary amine residues on DMAEMA with 1,3-propanesultone, according to published procedures.[4,5] Where possible, (co)polymers were purified by Soxhlet extraction with THF to remove unreacted 1,3-propanesultone and then dried at room temperature, overnight *in vacuo*.

(Co)polymer analysis

Copolymer compositions were determined by ^{1}H nuclear magnetic resonance (NMR) spectroscopy on a Bruker 300 MHz/53 mm spectrometer in either deuterated chloroform ($CDCl_3$) or deuterium oxide (D_2O). Confirmation of successful derivatization to the polymeric sulfobetaines was also accomplished by ^{1}H NMR spectroscopy. The molecular weights and polydispersity indices were determined by size exclusion chromatography (SEC) in DMF/NEt_3 at a flow rate of 1.0 mL/min at 40°C. The SEC comprised of a Waters 515 HPLC pump, Waters 2410 RI detector, Waters 2457 Dual λ absorbance detector, column oven, and a PolymerLabs PLgel 5 μm MIXED-C 300 × 7.5 mm^2 column. The column was calibrated with a series of narrow molecular weight distribution poly(methyl methacrylate) standards (PolymerLabs). Data was manipulated with the Waters Empower software package.

Evaluation of the antimicrobial properties

The antimicrobial properties of the statistical sulfopropylbetaine copolymers were tested using the broth dilution method as described previously by Dizman et al.[29] Briefly, the minimum inhibitory concentration (MIC) and the minimum bactericidal concentration were determined for each copolymer sample. Each copolymer was first dissolved in 2,2,2-trifluoroethanol (TFE) at a concentration of 20 mg/mL. In a 96-well microtiter plate, the copolymer solution was diluted 10-fold in TSB yielding a starting solution at a copolymer concentration of 2000 μg/mL. Twofold serial dilutions of the starting solution were then prepared in TSB. The lowest concentration tested was 31.25 μg/mL. One hundred and five colony-forming units of the test organism (*E. coli* or *S. aureus*) were then added to each well. The plates were incubated at 37°C for 24 h. The MIC was defined as the lowest concentration of copolymer with no visible bacterial growth (no turbidity). Experiments were repeated at least four times. A control experiment to test the effect of TFE was also conducted.

RESULTS AND DISCUSSION

As part of a larger concerted effort to develop new biomimetic polymeric betaines for a variety of biomedical applications, here we describe initial observations relating to the antimicrobial properties of simple statistical sulfopropylbetaine copolymers. Copolymers of 2-(dimethylamino)ethyl methacrylate (DMAEMA) with four different hydrophobic alkyl methacrylate comonomers, namely the ethyl, butyl, cyclohexyl, and octyl derivatives were prepared via conventional free radical polymerization under bulk conditions (see Scheme 1).

A wide range of copolymers of varying composition were prepared ranging from DMAEMA-rich to alkyl methacrylate-rich species. Table I summarizes the copolymers prepared along with their theoretical and calculated molar compositions, polymerization yields, and in some cases the number average molecular weights (M_n) and polydispersity indices (M_w/M_n). These particular statistical copolymers were prepared as part of a larger screening study aimed at identifying the key structural characteristics conferring the desired biological response, in this case antimicrobial activity.

Copolymers of varying composition ranging from typical values of 90 : 10 to 10 : 90 (mol %, theoretical) alkyl methacrylate/DMAEMA were prepared. Polymerizations were conducted under bulk conditions under an Ar atmosphere and were typically left for ~2 h at 60°C. Conversions were intentionally kept below ~70%, and were more typically in the range 20–40%, to avoid gelation.

A typical ^{1}H NMR spectrum, recorded in $CDCl_3$, of the precursor alkyl methacrylate-*stat*-DMAEMA copolymers is shown in Figure 1, with relevant peak assignments.

Copolymer compositions were determined according to eq. (1), where I_A = O—CH_2—, that is, the methylene adjacent to oxygen of the ester group in DMAEMA (δ ~ 4.1 ppm) and I_B = O—CH_2— of the alkyl methacrylate comonomer (δ ~ 4.6 ppm).

$$\text{mol \% DMAEMA} = \frac{I_A}{(I_A + I_B)} \quad (1)$$

This equation is slightly modified in the case of the cyclohexyl copolymers:

$$\text{mol \% DMAEMA} = \frac{I_A/2}{[(I_A/2) + I_B]} \quad (2)$$

TABLE I
Summary of Molar Compositions and Polymerization Yields for the Statistical Copolymers

Sample ID	Alkyl comonomer	Theoretical composition alkyl: DMAEMA	Observed composition[a]	Yield (%)
Homo	Ethyl	100 : 0	—	36
B_1	Ethyl	90 : 10	95 : 05	29
B_2	Ethyl	80 : 20	85 : 15	41
B_3	Ethyl	60 : 40	60 : 40	31
B_4	Ethyl	50 : 50	41 : 59	23
B_5	Ethyl	40 : 60	45 : 55	46
B_6	Ethyl	20 : 80	28 : 72	21
B_7	Ethyl	10 : 90	12 : 88	47
Homo	Butyl	100 : 0	—	32
A_1	Butyl	90 : 10	89 : 11	21
A_2	Butyl	80 : 20	80 : 20	8
A_3	Butyl	60 : 40	63 : 37	37
A_4	Butyl	50 : 50	50 : 50	25
A_5	Butyl	40 : 60	36 : 64	44
A_6	Butyl	20 : 80	16 : 84	56
A_7	Butyl	10 : 90	11 : 89	39
Homo	Cyclohexyl	100 : 0	—	
C_1	Cyclohexyl	90 : 10	75 : 25	22
C_2	Cyclohexyl	80 : 20	82 : 18	50
C_3	Cyclohexyl	60 : 40	57 : 43	32
C_4	Cyclohexyl	50 : 50	50 : 50	23
C_5	Cyclohexyl	40 : 60	44 : 56	22
C_6	Cyclohexyl	20 : 80	19 : 81	23
C_7	Cyclohexyl	10 : 90	13 : 87	30
Homo	Octyl	100 : 0	—	
D_1	Octyl	90 : 10	90 : 10	55
D_2	Octyl	80 : 20	85 : 15	79
D_3	Octyl	60 : 40	64 : 36	66
D_4	Octyl	50 : 50	53 : 47	57
D_5	Octyl	40 : 60	44 : 56	58
D_6	Octyl	20 : 80	22 : 78	52
D_7	Octyl	10 : 90	11 : 89	21

[a] As determined by ^{1}H NMR spectroscopy in $CDCl_3$.

Unfortunatley, due to a lack of solubility of the copolymers in DMF, the molecular weights and molecular weight distributions for the statistical copolymers could not be determined by GPC. Of those samples exhibiting antimicrobial activity only B_7 (a DMAEMA/ethyl methacrylate copolymer with ~88 mol % DMAEMA) showed sufficient solubility to be analyzed by GPC. The experimentally determined M_n was ~235,000 with a polydispersity index (M_w/M_n) of 2.23. This is entirely consistent with copolymers prepared under these conditions.

All these precursor copolymers were subsequently converted to the corresponding polysulfopropylbetaine species by reaction of the tertiary amine functional group on the DMAEMA residues with 1,3-propanesultone in THF at room temperature, according to literature procedures[4,5] (see Scheme 1). The resulting betaine materials are typically easily isolated and purified, since many precipitate from THF at near-quantitative degrees of modification. The final degree of derivatization was determined by NMR spectroscopy and was ≥95% in all instances.

Biological activity

Testing for antimicrobial activity requires that the copolymers be molecularly dissolved in the TSB medium. Given the amphiphilic nature of the statistical copolymers, it was important that a suitable solvent was employed to ensure unimeric dissolution prior to dilution with TSB. The presence of the hydrophobic alkyl methacrylate comonomer demanded the use of an organic solvent to facilitate molecular dissolution. Polymeric betaines however, and especially sulfobetaine derivatives, exhibit very limited solubility characteristics in organic media. Besides water and aqueous salt solution, polysulfobetaines are generally soluble only in organic solvents such as 2,2,2-trifluoroethanol TFE, trifluoroacetic acid (TFA), and in some instances formamide. Given that TFE has previously been shown to be a thermodynamically better solvent than salt solution,[30] and that Lowe et al.[5] have also demonstrated that it is a suitable solvent for the molecular dissolution of low molecular weight DMAEMA-methyl methacrylate block copolymers, we opted to use this as the organic cosolvent. Given the need for dissolution in TSB, clearly not all the copolymers reported in Table I were anticipated to be viable test materials given the hydrophilic and hydrophobic compositions of the materials. Indeed a critical molar content of the betaine residues is required to confer or maintain solubility in the TSB solution, thus enabling screening. In all instances, ~70 mol % of the betaine residues was found to be necessary to enable

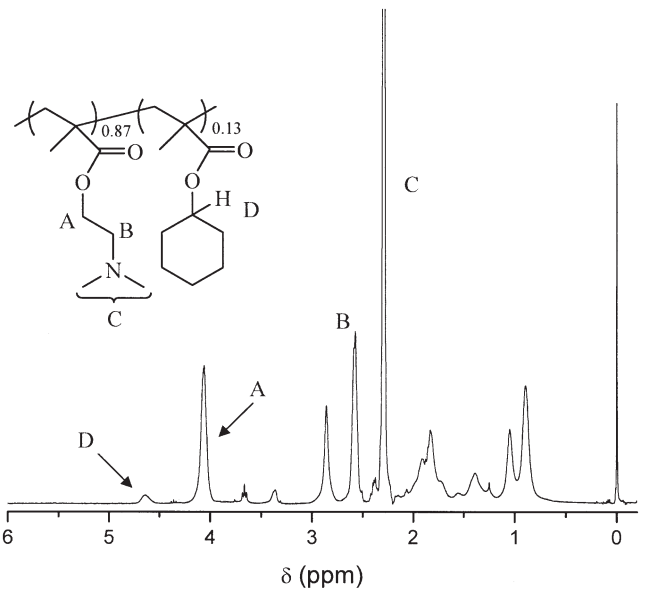


Figure 1 The ^{1}H NMR spectrum of C_7 recorded in $CDCl_3$.

TABLE II
MIC Values for the Copolymers Exhibiting Antimicrobial Activity

Copolymer	MIC[a] for *E. coli* (μg/ml)	Standard error	MIC[a] for *S. aureus* (μg/ml)	Standard error
$A_{5,1}$[b]	2000	0	2000	0
$A_{6,1}$	1750	250	1750	250
$A_{7,1}$	1750	250	1750	250
$B_{6,1}$	1750	250	1750	250
$B_{7,1}$	1500	289	1500	289
$C_{6,1}$	2000	0	1250	250
$C_{7,1}$	1125	315	1500	289
$D_{6,1}$	2000	0	1333	289
$D_{7,1}$	2000	0	1667	289
Ampicillin[c]	18.75	0	15.63	0
Erythromycin[c]	62.5	0	6.25	0

[a] Values represent the mean minimum inhibitory concentration (MIC) from four experiments.
[b] $X_{n,1}$ denotes the betaine derivative of X_n.
[c] Ampicillin and erythromycin were used as controls.

testing. Copolymers with betaine contents less than this approximate critical value either phase separated upon addition of the TSB dilutant or appeared cloudy.

To ensure that the small volume of residual TFE was not responsible for any observed bacteriostatic activity, a control experiment was performed in which a mixture of TSB and the equivalent amount of TFE were inoculated with the test organisms (*E. coli* or *S. aureus*). Both organisms were capable of growing in the presence of TFE, and as such any observed antimicrobial activity for the copolymers can be solely attributed to the action of the macromolecules.

Of those copolymers which exhibited the necessary solubility characteristics, antimicrobial activities were observed for the nine statistical copolymers listed in Table II.

Of these, all exhibited bacteriostatic activity against both the test organisms. These two species were chosen because they represent two structurally different types of bacteria, gram-negatives and gram-positives, and they are also two of the most common pathogens that contaminate medical devices and implants.

Although these specific statistical copolymers do exhibit antimicrobial activity against both *E. coli* and *S. aureus*, their effectiveness appears to show some dependence on the test organism, copolymer composition, and the nature of the hydrophobic comonomer. The MIC values for both organisms lie in the range 1125–2000 μg/mL. These are approximately two orders of magnitude higher than the established low molecular weight antibiotics ampicillin and erythromycin, (Table II) while they are only approximately one order of magnitude less active (based on the MIC values against the same test organisms) than the more established low molecular weight antimicrobial polycations based on poly(trialkylvinylbenzylammonium chlorides).[31]

Broadly, the polymeric betaines appear to be more active against the gram-positive *S. aureus*, although there is clearly an effect on the nature of the hydrophobic comonomer. In the case of the ethyl and butyl methacrylate comonomer derivatives there appears to be little or no effect on the antibacterial activity against either *E. coli* or *S. aureus* with MICs in the range 1500–2000 μg/mL. However, we did observe distinct differences in the case of the copolymers with the more hydrophobic cyclohexyl and octyl methacrylate comonomers. In the case of the gram-negative *E. coli*, we observed little difference in the MIC values. The one exception appears to be $C_{7,1}$, which exhibits a significant activity. At this point we have no explanation for this apparent 'anomaly'. In contrast, these copolymers do exhibit clearly distinct activity against the gram-positive *S. aureus*. A comparison of the cyclohexyl versus the octyl methacrylate (C versus D) derivatives indicated that the cyclohexyl species are slightly more active for a given copolymer composition. For example, a direct comparison of $C_{6,1}$ with $D_{6,1}$ with ~10 and 22 mol % hydrophobic comonomer, respectively, yields MICs of 1250 and 1333 μg/mL. Additionally, we noted that copolymers with a higher mol % of the hydrophobic comonomer are more active. This is highlighted in a comparison of $D_{6,1}$ with $D_{7,1}$, which possess 22 and 11 mol % octyl methacrylate respectively. For $D_{6,1}$, the measured MIC is 1333 μg/mL whereas it is 1666 μg/mL in the case of $D_{7,1}$.

The difference in activity might be related to the difference in the cell wall structure of the gram-negative versus the gram-positive bacteria. Gram-positive bacteria have a significantly simpler cell wall structure in which the cytoplasmic membrane is surrounded only by a rigid peptidoglycan layer. Although this layer is capable of protecting the cell interior, it is reasonably porous and thus foreign molecules can fairly easily traverse this outer layer. In contrast, gram-negative bacteria have considerably more complicated cell-wall structures. Beyond the peptidogly-

can layer is a second outer membrane that is structurally similar to the cytoplasmic membrane. This outer layer is less porous and thus offers an additional layer of protection against foreign molecule migration to the inner cytoplasmic membrane. As such, we would predict that the betaine materials described here (if possessing biocidal properties) would likely be more active against *S. aureus*, which is indeed the case.

The observed effect of the hydrophobic comonomer is related to the accepted elementary steps associated with the kill (biocidal) process. According to Ikeda and Tazuke this involves (a) adsorption onto the bacterial cell surface, (b) diffusion through the cell wall, (c) adsorption onto the cytoplasmic membrane, (d) disruption of the cytoplasmic membrane, (e) leakage of the cytoplasmic constituents, and (f) cell death.[31] The adsorption process in step a) is electrostatic in nature in which the cationic functionality of the betaine interacts with the negatively charged constituents on the cell surface. This interaction is often enhanced in the case of polymeric species by virtue of multivalent interactions. However, diffusion through the cell wall and disruption of the cytoplasmic membrane, and thus cell death, requires hydrophobic interactions.[32] The larger and longer hydrophobic alkyl methacrylate derivatives containing the cyclohexyl and octyl functionalities are more effective in this capacity and are thus expected to possess enhanced biocidal properties.

CONCLUSIONS

Here we have reported the first systematic evaluation regarding the antimicrobial activity of polysulfopropylbetaine copolymers against both gram-negative and gram-positive bacteria. High molecular weight statistical copolymers, prepared under bulk free radical conditions, of a hydrophobic alkyl methacrylate with a hydrophilic sulfopropylbetaine comonomer derived from the modification of 2-(dimethylamino)ethyl methacrylate residues exhibit antimicrobial activity against both *E. coli* and *S. aureus*. The extent of activity is related to the copolymer composition, the nature of the hydrophobic alkyl methacrylate comonomer, as well as the test organism.

We are using these preliminary results as a guide to prepare more well-defined sulfopropylbetaine copolymer structures via controlled/"living" free radical polymerization to more closely evaluate the effect of composition, polydispersity, comonomer, and molecular weight on the antimicrobial properties.

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