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Nonhemolytic and Antibacterial Acrylic Copolymers with Hexamethyleneamine and Poly(ethylene glycol) Side Chains

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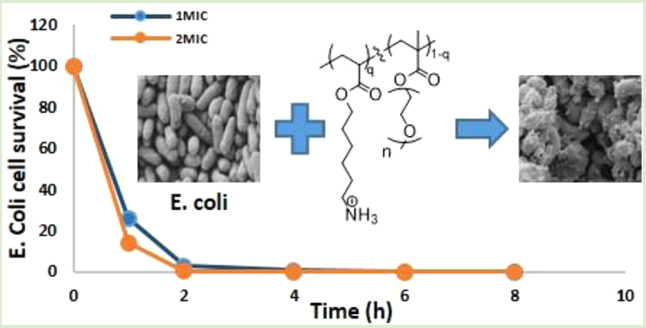
Supporting Information

ABSTRACT: Amphiphilic acrylic copolymers with hexamethyleneamine and poly(ethylene glycol) side chains can show >100-fold selectivity toward *Escherichia coli* over red blood cells. Homopolymer with cationic pendant amine groups is highly hemolytic and antibacterial. Incorporation of approximately 33 mol % of poly(ethylene glycol) methyl ether methacrylate (PEGMA) led to 1300 times reduction in hemolytic activity, while maintaining high levels of antibacterial activity. The hemolytic activity of these PEGylated copolymers depends on the overall content and spatial distribution of the PEGMA units. Higher activity against *Escherichia coli* than *Staphylococcus aureus* was observed for this polymer system, likely due to hydrogen bonding ability of the PEG side chains with polysaccharide cell wall of the bacteria. Field emission scanning electron microscopy analysis confirmed the bacterial membrane rupture activity exerted by these copolymers, whereas time-kill studies revealed significantly different bactericidal kinetics toward the Gram-negative *Escherichia coli* and the Gram-positive *Staphylococcus aureus*.



The undeterred rise in infections involving antibiotic drug-resistant bacteria (superbugs) has led to a serious health threat to the human population.[1a−c] The development of bacterial resistance toward synthetic amphiphilic polymers, based on the design principles of natural antimicrobial peptides (AMPs), is considered to be highly hindered or improbable.[1d,e] Facile synthesis and structural versatility of synthetic amphiphilic polymers can foster their large-scale applications, whereas therapeutic applications of AMPs have been impeded due to their costly and time-consuming synthesis, as well as difficult drug administration.[2] However, toxicity of the synthetic amphiphilic polymers toward mammalian cells has been a challenge for their wide-scale biomedical applications. Thus, amphiphilic polymers with potent antibacterial activity and concomitant low hemolytic activity and toxicity toward mammalian cells are highly desired to combat the threat of superbugs. There have been investigations on the antibacterial and hemolytic activities of synthetic amphiphilic polymers concerning the effects of various structural parameters including: amphiphilic balance,[3] spatial arrangement of cationic and hydrophobic groups,[4] random or block copolymer architecture,[5] and cationic charge density.[6]

We have been investigating polyacrylic amphiphilic copolymer systems,[7] exploiting their adaptability to composition and structure control. We studied their antibacterial and hemolytic activities as influenced by "systematic structural/composition variations" and relative orientation of hydrophobic side groups with respect to the cationic center.[7a] Recently we reported[7b] that copolymers of 6-aminohexyl acrylate, M6, and 2-methylaminoethyl acrylate, M2 (Figure 1a), can show excellent

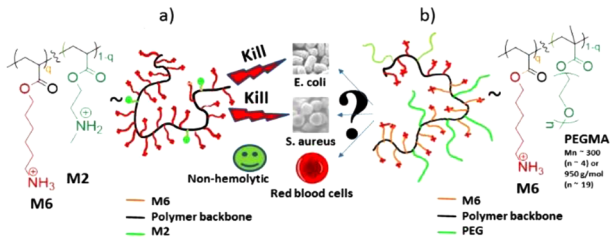


Figure 1. Antibacterial and hemolytic activities of acrylic copolymers with (a) control of spatial charge distribution[7b] and (b) with control of hydrophilic PEG content.

antibacterial activities and selectivities (hemolytic activity ÷ minimum inhibitory concentration). Whereas the homopolymer of M6 is highly hemolytic and antibacterial, a copolymer with just 10 mol % of M2 displayed dramatically lower hemolytic activity, while maintaining comparable levels of antibacterial activity, resulting in a greater than 200-fold selectivity toward *Escherichia coli* (*E. coli*) over red blood cells (RBCs).[7b] This copolymer series represents one of the most

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Table 1. Characterization and Biological Activities of Polymers

					MIC *E. coli*		MIC *S. aureus*		HC_{50} RBCs		selectivity HC_{50}/MIC^b	
polymer	mole % M6	mole % PEGMA	wt % PEGMA	DP[a]	μM	μg/mL	μM	μg/mL	μM	μg/mL	*E. coli*	*S. aureus*
PM6-0-PEG300	0	100	100	31	>215	>2000	>215	>2000	>215	>2000	--	--
PM6-10-PEG300	11	89	93	23	>304	>2000	>304	>2000	>304	>2000	--	--
PM6-20-PEG300	21	79	87	17	>431	>2000	>431	>2000	>431	>2000	--	--
PM6-30-PEG300	29	71	81	21	>327	>1809	>361	>2000	>361	>2000	>1.1	--
PM6-50-PEG300	50	50	64	20	22	104	53	250	>424	>2000	>19	>8
PM6-70-PEG300	67	33	46	21	3.5	16	14	62	>401	>1809	>113	>29
PM6-90-PEG300	90	10	16	20	1.9	7.8	8.4	31	22.4	83	11	2.6
PM6-0-PEG950	0	100	100	14	--	>2000	--	>2000	--	>2000	--	--
PM6-10-PEG950	14	86	97	--	--	>2000	--	>2000	--	>2000	--	--
PM6-20-PEG950	22	78	95	18	>143	>2000	>143	>2000	>143	>2000	--	--
PM6-30-PEG950	34	66	91	19	>154	>2000	>154	>2000	>154	>2000	--	--
PM6-50-PEG950	49	51	85	15	>238	>2000	>238	>2000	>238	>2000	--	--
PM6-70-PEG950	68	32	72	18	32	250	38	295	>258	>2000	>8	>7
PM6-90-PEG950	88	12	43	26	3.8	26	7.6	52	119	809	31	16
PM6-100	100	0	0	36	0.94	6.5	2.4	16	<0.3	<1.9	<0.3	<0.1

[a]Degree of polymerization (DP) calculated from ^{1}H NMR. [b]Calculated from μg/mL values.

promising synthetic polymer antibacterial systems reported. Similar biological activities were indicated by Tew et al.[8a] and Hedrick et al.[8b] for copolymers having small mole % of nonhemolytic comonomer units incorporated with highly hemolytic and antibacterial comonomer units. These systems of copolymers carry a cationic charge on every repeating unit.

Our present investigation involves the copolymerization of a nonionic and biocompatible poly(ethylene glycol) methyl ether methacrylate (PEGMA) comonomer with highly antibacterial and hemolytic comonomer (M6) (Figure 1b). Unlike the short cationic M2, hydrophilic poly(ethylene glycol) (PEG) side chains have highly extended conformation in aqueous environment with the ability of reducing hydrophobic interactions of copolymers with the RBCs. PEG has been widely used in a myriad of polymeric systems based on its properties including the biocompatibility and ability to substantially improve pharmacokinetics of conjugated drugs.[9] Youngblood et al. reported reduction in hemolytic ability of N-hexylated poly(vinylpyridine) copolymers through inclusion of at least 50 mol % of PEGMA.[10] Tew et al. reported that "facially amphiphilic" polynorbornenes with PEG side chains displayed lower hemolytic activity than polynorbornenes.[11] PEGylated polymethacrylates having antibacterial activity toward a Gram-positive bacteria while displaying low hemolytic activity were described by Zhang et al.[12] In this letter, we report the antibacterial and hemolytic activities of the PEG-containing systems with >100 times selectivity toward bacteria over RBCs. The incorporation of the PEGMA monomer to the highly hemolytic M6 homopolymer led to substantial reduction in hemolytic activity of copolymers, while maintaining high levels of activity against both Gram-negative *E. coli* and Gram-positive *Staphylococcus aureus* (S. aureus).

A series of statistical copolymers were synthesized using free radical copolymerization of monomer M6 (Boc protected) with PEGMA, followed by removal of N-Boc protecting groups. The influence of PEG size on antibacterial activity was examined through the incorporation of two PEGMA monomers: PEGMA-300 (300 g/mol, degree of polymerization (DP) of PEG side chain ∼5) and PEGMA-950 (950 g/mol, DP of PEG side chain ∼19). The feed mole ratios of comonomers were varied to map the effect of amphiphilic balance on biological activities. Mole ratios of comonomer units in the copolymers were found to be similar to the feed mole ratios (Supporting Information). The molecular weights of precursor polymers (Boc protected) were estimated using GPC and were found to be similar for all polymers (Supporting Information). Degree of polymerization was found to be ∼21 from end group analysis of ^{1}H NMR spectra. Notation of PM6-X-PEG300 is used to represent a copolymer with "X" feed mole % level of the M6 monomer copolymerized with "100 − *X*" mole % of PEGMA-300, with similar expression used for the PEGMA-950 comonomer system. Branching side reactions are not probable in this polymer system (see Supporting Information).

Antibacterial and hemolytic activities of polymers were obtained following the established procedures[13] (Table 1 and Figure 2). Minimum inhibitory concentrations (MICs) of polymers were obtained as a measure of their antibacterial activity against *E. coli* (TOP 10, ampicillin resistant) and *S. aureus* (ATCC 25923). MIC is defined as the lowest polymer concentration required to inhibit 100% bacterial growth after an incubation period of 18 h. Hemolytic concentration-50% (HC_{50}) of polymers was determined against freshly drawn mouse RBCs to assess the toxicity of polymers toward mammalian cells. HC_{50} is defined as the lowest polymer concentration required to lyse 50% of the RBCs within an incubation period of 1 h. The highest concentration of polymer solution tested was 2000 μg/mL.

Homopolymer PM6-100 is highly antibacterial against both *E. coli* and *S. aureus* but extremely hemolytic (HC_{50} < 1.9 μg/mL or <0.3 μM). Incorporation to the homopolymer PM6-100, with 33 mol % of PEGMA-300, led to low hemolytic activity (HC_{50} >1809 μg/mL or >401 μM) accompanied by high antibacterial activity against both *E. coli* (MIC = 16 μg/mL or 3.5 μM) and *S. aureus* (MIC = 62 μg/mL or 14 μM). The hemolytic activity of PM6-70-PEG300 was 3 orders of magnitude lower than that of homopolymer PM6-100. Thus, addition of 33 mol % of hydrophilic PEG side groups led to >1300 times reduction in hemolytic activity on a molar basis while maintaining high levels of antibacterial activity in polymer. At this copolymer composition of highest selectivity (Figure 2), on an average there are two M6 cationic side chains for each short PEG side chain (DP ∼ 5). For copolymer PM6-

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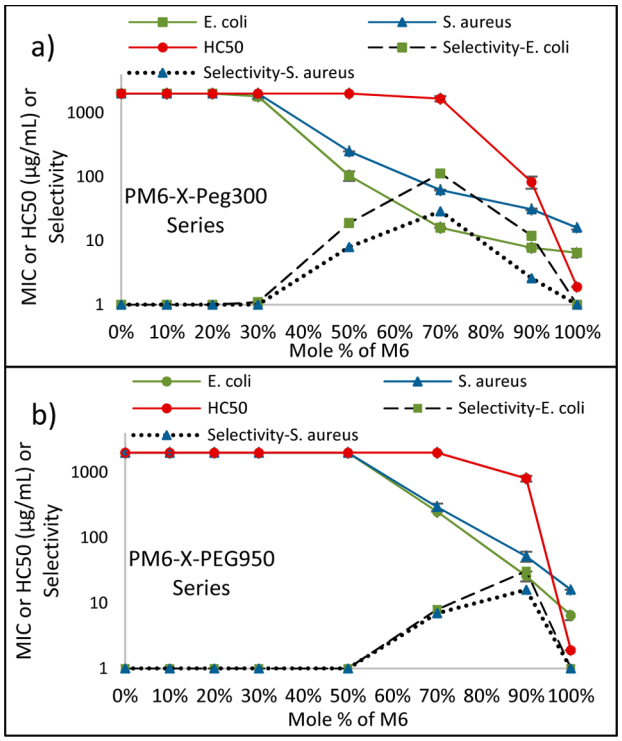

Figure 2. Antibacterial activities (MIC), hemolytic activities (HC_{50}), and selectivities (HC_{50}/MIC) of (a) PM6-X-PEG300 series polymers and (b) PM6-X-PEG950 series polymers. Error bars represent standard deviation of three independent measurements.

90-PEG950, with weight % of PEGMA at the same level as PM6-70-PEG300, incorporation of just 12 mol % PEGMA-950 led to substantially lower hemolytic activity (HC_{50} = 119 μM) without significant loss of high antibacterial activity, resulting in the highest selectivity (HC_{50}/MIC) for this series. Here for every eight charged M6 units there is a long PEG (DP∼ 19) side chain, apparently sufficient to moderate the hemolytic activity significantly. For the case of polymer, PM6-90-PEG300, with one short PEG (DP ∼ 5) for every nine M6 units, the reduction of hemolytic activity is approximately 10 times lower than PM6-90-PEG950. High selectivity (HC_{50}/MIC) of polymers toward bacteria over mammalian cells is required for healthcare applications of synthetic amphiphilic polymers. The PM6-X-PEG300 series shows, in general, higher selectivity and antibacterial activities than the PM6-X-PEG950 series (Table 1). As HC_{50} starts to decrease, the selectivity deteriorates markedly.

The addition of a small mole % of PEGMA led to significant reduction in hemolytic activity. PEG is believed to protect RBCs from the foreign body contact, similar to blood plasma.[10a] PEG may interact with the RBC cell membrane through hydrogen bonds and weakly adsorb on the surface of RBCs, thus enhancing its protecting effects.[14a] Moreover, at the same mole %, longer (DP ∼ 19) PEG side chains have higher ability to deter the insertion of polymers into the hydrophobic domain of RBCs' cell membrane than the case of shorter (DP∼ 5) PEG side chains.

Increasing the mole % of PEGMA-300 units (up to 50 mol %) led to a gradual reduction in antibacterial activity of copolymers, followed by an abrupt loss of antibacterial activity at higher PEGMA-300 mol % (Table 1 and Figure 2a). Similarly, incorporation of PEGMA-950 units (up to 30 mol %) resulted in reduction of antibacterial activity of copolymers, and no antibacterial activity was observed in copolymers with 50 or higher mole % of PEGMA-950 (MIC > 2000 μg/mL). Decrease in number of cationic and hydrophobic side chain, hexamethyleneamine, leads to lower electrostatic and lipophilic interactions between the bacterial cell surface and polymers. At higher mole % of PEGMA, PEG side chains may shield the M6 units from interacting with the bacterial cell surface due to the significant hydrodynamic volume of PEG side groups. At 50 mol %, each cationic center would be on the average, next to PEG side chains.

Incorporation of small mole % of PEGMA units to poly(vinylpyridine) homopolymer was reported[14b] to increase its antibacterial activity. This increase in antibacterial efficacy was attributed to enhanced surface wettability of copolymers due to incorporation of hydrophilic comonomers.[14b] In our case, PM6-100% was readily soluble in water up to the highest concentration tested, and hence the incorporation of PEGMA comonomer units would not have a considerable effect on the aqueous solubility of our copolymers.

At the same mole % of the PEGMA monomer, copolymers with longer PEG side chains (DP ∼ 19) displayed lower antibacterial and hemolytic activities than copolymers with shorter (DP ∼ 5) PEG side chains (Table 1 and Figure 2). To assess the effects of PEG lengths on the biological activities of the copolymer, independent of the overall content of PEG composition, we analyzed the biological activities of PM6-70-PEG300 and PM6-90-PEG950, both having similar wt % (∼43 to 46%) of PEG side groups. We did not observe a significant difference in the antibacterial activities of these two polymers (Table 1). For 80 wt % PEG composition, both PM6-30-PEG300 and PM6-50-PEG950 showed no antibacterial or hemolytic activity. However, we observed a difference in the hemolytic activity of PM6-70-PEG300 and PM6-90-PEG950, even though both polymers have similar wt % of PEG composition. PM6-70-PEG300 with 33 mol % of shorter PEG chains displayed substantially lower hemolytic activity (HC_{50} >401 μM) as compared with PM6-90-PEG950 (HC50 = 119 μM) with 12 mol % of longer PEG chains. Hemolytic activity of amphiphilic polymers involves the hydrophobic interactions of polymers with lipid bilayer of RBCs.[2] In PM6-70-PEG300, shorter but spread out hydrophilic regions throughout the polymer backbone may deter, at a higher level, the permeabilization of polymers into the hydrophobic domain of RBCs' lipid bilayer, whereas in PM6-90-PEG950, with only 12 mol % of longer PEG side chains (DP ∼ 19), wider regions of hemolytic M6 units exist, resulting in higher ability of polymers to lyse the lipid bilayer of RBCs.

Field emission scanning electron microscopy (FE-SEM) analysis of bacterial cell morphology was performed to ascertain the membrane disruption mechanism of antibacterial action of our polymers. *E. coli* treated with PM6-70-PEG300 and PM6-90-PEG950 resulted in severe cell damage and surface rupture, confirming the cell membrane disruption ability of these polymers (Figure 3c and e). Similarly, cell surface damage and rupture were observed in the case of *S. aureus* (Figure 3d and f) with the severity of cell damage to be lower in the case of *S. aureus* as compared with *E. coli*. Time-dependent killing efficiency (time kill) studies showed further details of the bactericidal activity of our polymers in addition to bacteriostatic activity (Figure 4). More than 99.99% killing efficiency was achieved against *E. coli* at both MIC and 2 × MIC concentrations of PM6-70-PEG300 and PM6-90-PEG950,

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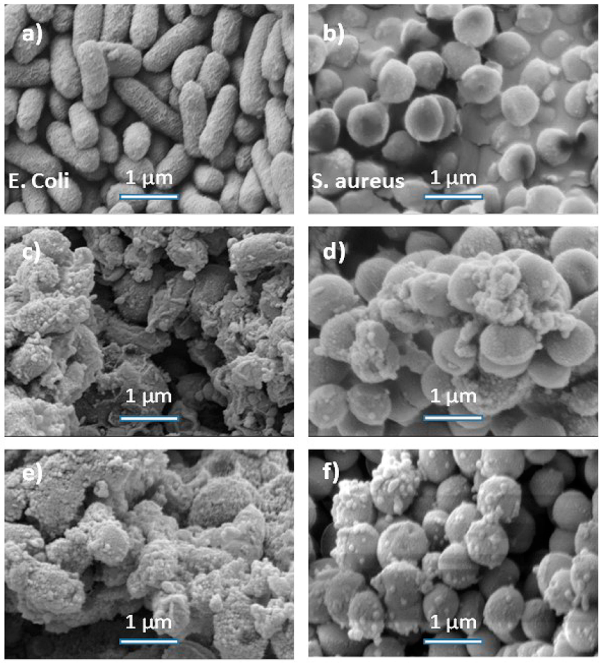

Figure 3. FE-SEM analysis of bacterial cell morphology. (a) Control *E. coli* and (b) control *S. aureus*. (c) *E. coli* and (d) *S. aureus* treated with PM6-70-PEG300. (e) *E. coli* and (f) *S. aureus* treated with PM6-90-PEG950.

within 8 h of incubation. Within 2 h of incubation, greater than 75% of *E. coli* colony forming units (CFUs) were eliminated by both MIC and 2 × MIC polymer concentrations.

However, a substantial fraction of *S. aureus* CFUs survived when challenged with 1 × MIC polymer concentrations for an incubation period of 8 h. These polymers displayed higher MIC values against *S. aureus* than *E. coli* (Table 1). Thus, MIC measurements, FE-SEM analysis, and time kill studies established that PEGylated polymers are more active against *E. coli* than *S. aureus*. Our previously reported M6 with M2 copolymer systems without PEG showed higher activity against *S. aureus*. Higher antibacterial activities of amphiphilic polymers against *S. aureus* than *E. coli* in many cases have been previously reported.[15] The double membrane structure of *E. coli* can be considered more difficult to penetrate and disrupt than the single membrane structure of *S. aureus*.[15a] However, the *S. aureus* has a very thick (20−80 nm) outer cell wall comprised of a negatively charged peptidoglycan layer (polysaccharide with amino acid side chains), whereas *E. coli* has a thin (∼6−8 nm) peptidoglycan layer sandwiched between the outer and inner membrane.[15a] PEG side groups of copolymers can associate, through hydrogen bonding, with the polysaccharides in the bacterial cell wall, thus hindering the progress of the permeation of PEGylated polymers through the thick cell wall, resulting in lowering the activity of PEGylated polymers against *S. aureus*. The *E. coli* cell wall polysaccharide layer is thinner than *S. aureus*, thus presenting less of a hurdle for polymer penetration, resulting in the higher susceptibility. Furthermore, the topological distribution of PEG side chains of different sizes influences the antibacterial activities of the copolymers. Copolymer PM6-90-PEG950 displayed faster killing efficiency against *S. aureus* than PM6-70-PEG300 (Figure 4d). At similar PEG weight levels, PM6-90-PEG950 has 12 mol % of PEGMA-950 units as compared with ∼33 mol % of PEGMA-300 units in PM6-70-PEG300. Thus, in PM6-70-PEG300, PEG short side chains are distributed more densely throughout the polymer backbone than the case of PM6-90-PEG950, leading to a broader level of association to the peptidoglycan layer in *S. aureus*, thus lowering the killing rate (Figure 4d).

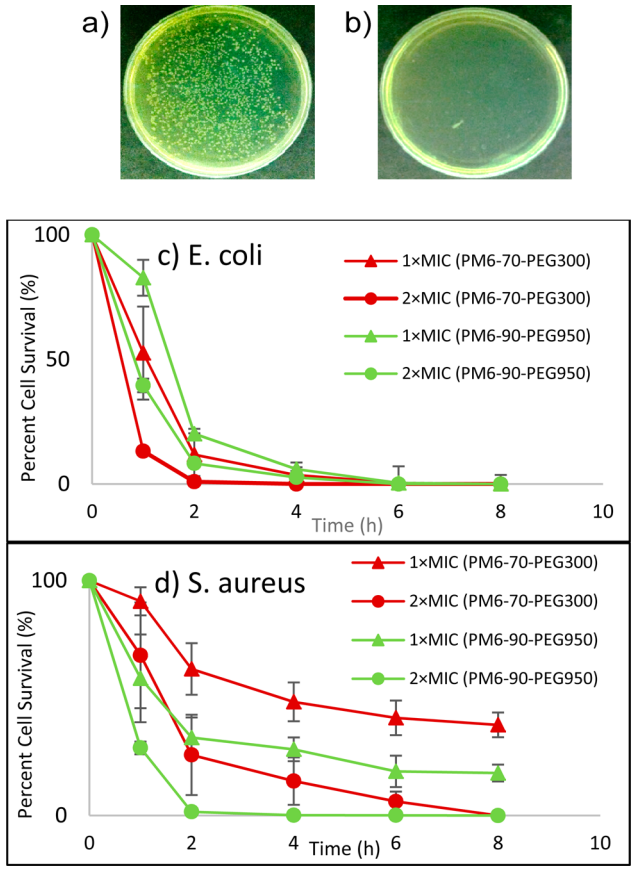

Figure 4. Time kill kinetics of polymers (1 × and 2 × MIC) against bacteria. (a) *E. coli* colony forming units before polymer treatment (0 h) and (b) after 8 h bacterial incubation with PM6-70-PEG300 at 1 × MIC. Colony forming units survival % of (c) *E. coli* and (d) *S. aureus* after treatment with polymers at various time intervals (log reduction data in Supporting Information).

In conclusion, a series of amphiphilic copolymers with hexamethyleneamine and PEG side chains manifesting high antibacterial activity against *E. coli* and *S. aureus* were synthesized. Tuning polymer amphiphilicity through incorporation of hydrophilic PEG side groups led to a dramatic reduction in hemolytic activity while maintaining high antibacterial activity, leading to polymers with over 100-fold selectivity for bacteria over RBCs. At the same weight % composition of PEG, copolymers with smaller and spread out PEG side chains displayed lower hemolytic activity than polymers with longer PEG side groups at lower mole %. It is speculated that H-bonding between long PEG side groups and the polysaccharide cell wall may contribute to lower antibacterial activity of PEGylated copolymers against *S. aureus* than *E. coli*. FE-SEM analysis of treated cells showed severe damage to the bacterial cell surface morphology, confirming the cell membrane disruption mechanism of antibacterial activity. Additional details of bactericidal activity of polymers were obtained by the time kill studies. Thus, a polymer architecture consisting of a controlled distribution of hydrophilic PEG side groups and 6-carbon long lipophilic spacer arms with pendent

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cationic group led to highly antibacterial and nonhemolytic polyacrylates. Such copolymer systems can lead to substantial contributions to a better understanding of the interactions of amphiphilic copolymers with bacterial cells.

■ ASSOCIATED CONTENT

Supporting Information

Experimental procedures, biological assay protocols, GPC results, and NMR spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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