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Acrylic copolymers with appropriate compositions of counits having cationic charge with 2-carbon and 6-carbon spacer arms can show superior antibacterial activities with concomitant very low hemolytic effect. These amphiphilic copolymers represent one of the most promising synthetic polymer antibacterial systems reported.

There is a pressing need to develop new antibacterial agents to combat the serious public health threat caused by the emergence of antibiotic resistant bacteria (superbugs). The development of conventional antibiotics involves huge costs, and rapid increase in bacterial resistance renders them ineffective within a few years.^{1a} Compared to traditional antibiotics, natural host defense antimicrobial peptides (AMPs) are known to involve much lower levels of bacterial resistance development.^{1b,c} Challenging synthesis and proteolytic degradation of AMPs have hindered their therapeutic applications.^{2a,b} Synthetic amphiphilic polymers based on the design principles of AMPs have generated enormous research interest in the last decade due to their cost effective synthesis and structural versatility.^{2c} A variety of synthetic amphiphilic polymers including derivatives based on polymethacrylates,^{3a} polynorbornenes,^{3b} nylon,^{3c} and poly(vinyl pyridine)s^{3d} have displayed similar level of antibacterial activities as AMPs. The inability of bacteria to gain resistance toward synthetic amphiphilic polymers as opposed to conventional antibiotics has been demonstrated.^{4a}

One of the major challenges toward therapeutic applications of synthetic amphiphilic polymers is their toxicity to mammalian cells.^{3a} The biocompatibility of synthetic polymers has been widely assessed in terms of hemolytic activity toward red blood cells (RBCs). Some of the factors affecting the hemolytic activity of synthetic amphiphilic polymers have been recently explored.

Cationic amphiphilic non-hemolytic polyacrylates with superior antibacterial activity[†]

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Compared with random copolymers, block copolymer architecture can show lower toxicity toward RBCs.^{4b} "Same centered" polymers, having alkyl tail attached to their cationic center, demonstrated lower hemolytic activity in comparison with "separate center" copolymers.^{4c} Incorporation of hydrophilic poly(ethylene glycol) side groups reduced hemolytic activity in *N*-hexylated poly(vinyl pyridine)s.^{3d} Hemolytic activity of synthetic amphiphilic polymers primarily arises from their hydrophobic interactions with the lipid bilayer of RBCs.^{3a,b} Mole ratios and size of hydrophobic groups were varied to optimize the antibacterial and hemolytic activity of polymers.^{3b,5a}

In this communication, we report the synthesis of highly antibacterial but selective (bacteria over RBCs) polyacrylates by copolymerization of a monomer (M2) having 2-carbon spacer arm (distance from polymer backbone to cationic center) with a 6-carbon spacer arm monomer (M6), in varying mole ratios (Scheme 1). All copolymers in the range of 10 to 90 mole% of M6 displayed highly selective activity toward bacteria over RBCs. A copolymer having 90 mole% of M6 demonstrated 208 times more selectivity toward *Escherichia coli* (*E. coli*) over RBCs (Table 1 and Fig. 1).

We have previously found that amphiphilic polyacrylates with 2-carbon spacer arms and various lengths of alkyl tail attached to cationic center have low hemolytic activity and high



Scheme 1 Synthesis of polymers from comonomers with 2- and 6-carbon spacer arm lengths for charge center.

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Table 1 Characterization and biological activities of polymers

Polymer	$f_{ m M6}{}^a$	$f_{\mathrm{M2}}{}^a$	$M_{ m w}(m kDa)$	$M_{ m n}~(m kDa)$	PDI	DP^b	MIC (μg mL ⁻¹) <i>E. coli</i>	MIC $(\mu g m L^{-1})$ S. aureus	$\mathrm{HC}_{50}\ (\mathrm{\mu g}\ \mathrm{mL}^{-1})\ \mathrm{RBCs}$	Selectivity (HC ₅₀ /MIC)	
										E. coli	S. aureus
PM6-0%	0	100	6.2	4.3	1.45	30	1428	104	>2000	>1.4	>19
PM6-10%	11	89	6.6	4.5	1.47	39	809	62	> 2000	>2.5	>32
PM6-20%	21	79	6.3	4.3	1.46	33	250	62	> 2000	>8	>32
PM6-30%	30	70	6.6	4.5	1.46	33	125	52	1667	13	32
PM6-40%	40	60	6.8	4.6	1.46	30	62	52	1619	26	31
PM6-50%	52	48	6.9	4.6	1.49	25	52	52	> 2000	>38	>38
PM6-60%	61	39	6.7	4.6	1.48	30	41	31	>2000	>49	>64
PM6-70%	71	29	7.1	4.8	1.47	25	26	31	>2000	>80	>64
PM6-80%	81	19	7.8	5.4	1.46	31	16	26	>2000	>125	>80
PM6-90%	91	9	7.2	5.1	1.42	29	7.8	16	1619	208	101
PM6-100%	100	0	7.5	5.8	1.28	36	5.8	16	<1.9	< 0.33	< 0.12



Fig. 1 MIC values of polymers against (a) *E. coli*, and (b) *S. aureus*. (c) Hemolytic activity of polymers against mouse RBCs. (d) Biological activity of polymers (MIC and HC₅₀).

activity against Staphylococcus aureus (S. aureus).^{5b} Kuroda and coworkers recently reported an amine functionalized polymethacrylate homopolymer with 6-carbon linear spacer arm to be highly active against both *E. coli* and *S. aureus*.^{5a} However, the hemolytic activity of this polymer was also extremely high, and it did not display selectivity toward bacteria over RBCs.^{5a} Thus, by copolymerizing a longer spacer arm monomer with a short spacer arm monomer, copolymers with high antibacterial activity and lower hemolytic activity could be expected. The "snorkel effect" from longer spacer arms in which the cationic charge attach to bacterial cell membrane and long alkyl spacer arm can extend through the hydrophobic core of lipid bilayer may lead to high antibacterial activity,^{5a} whereas the high cationic charge density and lower hydrophobicity of short spacer arms may lower the hemolytic ability of polymers.

We synthesized a series of polyacrylate random copolymers *via* free radical copolymerization of a monomer having 2-carbon spacer arm (M2), with a 6-carbon spacer arm monomer (M6). The mole% (in feed) of M6 was increased in steps of 10%,

giving polymers with compositions that closely match the feed mole% (Table 1). The molecular weights of precursor copolymers, before deprotection with TFA, were estimated using gel permeation chromatography. Average degree of polymerization (DP) of polymers was estimated to be close to 30 for all preparations based on ¹H NMR, using end group analysis and assuming chain transfer dominating kinetics (ESI,[†] p. 5). PM6-*x*%, denotes the cationic amphiphilic copolymer having *x* mole% (in feed) of M6 monomer.

The antibacterial activities of polymers, in terms of Minimum Inhibitory Concentration (MIC), were determined against gram negative *E. coli* (TOP 10, ampicillin resistant) and Gram positive *S. aureus* (ATCC 25923), following established literature procedure.^{5c} MIC is expressed as the minimum polymer concentration that resulted in 100% inhibition of bacterial growth after an incubation period of 18 hours. Hemolytic activities of polymers were determined in terms of hemolytic concentration-50% (HC₅₀), the minimum polymer concentration resulting in 50% lyses of mouse RBCs within an incubation period of 1 hour.^{5c} Each experiment was done in triplicate, and the values reported here are the averages of three sets of independent experiments performed on different days.

Antibacterial activity of polymers toward E. coli was substantially increased as the mole% of counit M6 was increased in the copolymer (Fig. 1a and Table 1). Whereas, PM6-0% is inactive against E. coli; adding just 20% of M6 monomer (PM6-20%) resulted in significant reduction in MIC value. PM6-90% and PM6-100% displayed highest antibacterial activity toward E. coli in this series of polymers. The effect of increasing monomer M6 mole% on the antibacterial activity of polymers toward S. aureus was less pronounced as compared with E. coli (Fig. 1b and Table 1). Compared with the MIC values of PM6-0%, PM6-90% showed 6 times lower MIC value against S. aureus, and 183 times lower MIC value against E. coli. Increase in the separation of side chain attach points along the homopolymers backbone has been shown to result in high antibacterial activity.^{5d} In the present study, copolymerization of a 6-carbon spacer arm monomer (M6) with a 2-carbon spacer arm monomer (M2) changes the spatial distribution of cationic charges, even though the attach point separation distance remained the same.

Longer cationic spacer arms can interact with anionic cell membrane of bacteria *via* "snorkel effect" resulting in high antibacterial activity.^{5a}

Significantly, all polymers, except PM6-100%, displayed very low hemolytic activity against RBCs (Fig. 1c and Table 1). In comparison with extremely hemolytic PM6-100%, polymer PM6-90% was found to be >850 times less hemolytic toward RBCs, even though its antibacterial activities are similar to PM6-100%. We found that majority of our polymers have HC₅₀ value in excess of 2000 µg mL⁻¹. RBC cell membrane has zwitterionic phospholipid head groups and thus lacks net negative charge on its outer surface. Amphiphilic polymers can penetrate the RBCs' cytoplasmic membrane through hydrophobic interactions.^{3a} The observation that all of our polymers in the range of PM6-0% to PM6-90% are non-hemolytic but PM6-100% is highly hemolytic, indicates that even a small mole% of the shorter spacer arm counits can prevent the insertion of these polymer into lipid bilayer of RBCs. Monomer M6 has a long hydrophobic spacer arm, and increasing the mole% of M6 should have inevitably led to rapid increase in hemolytic activity. Incorporation of 20-30 mole% of hydrophobic monomer in polymers was reported to drastically increase the hemolytic activity of polymers.^{3a,5c} In our polymers, the presence of positive charge on each counit and a combination of longer and shorter cationic spacer arms have led to high antibacterial but low hemolytic activity. Recently, Hedrick et al. reported that the copolymerization of smaller spacer arm monomers with longer spacer arm monomers had resulted in reduction of hemolytic activity without significant effect on antibacterial activity of amphiphilic polycarbonates.^{6a} Tew et al. also reported earlier the highly selective antibacterial activity in amphiphilic polynorbornenes obtained through the copolymerization of highly antibacterial and hemolytic monomers with non-hemolytic monomers.^{6b,c}

The selectivity (HC₅₀/MIC) of our polymers toward bacteria over RBCs is apparent from Fig. 1d and Table 1. PM6-100% was highly antibacterial as well as hemolytic, whereas PM6-90% was found to be 208 times more selective toward E. coli over RBCs, and 101 times selective toward S. aureus over RBCs. PM6-80% is >125 times selective toward *E. coli* over RBCs and >80 times more selective toward S. aureus over RBCs. Both polymers displayed high antibacterial activity similar to PM6-100%. All copolymers in the range of 0 to 90 mole% of M6 monomer manifested highly selective antibacterial activity. Moreover, polymers containing 0 to 60% of M6 monomer, displayed selective antibacterial activity against S. aureus over E. coli. PM6-0%, a homopolymers of M2, is inactive against E. coli, but it displayed high activity against S. aureus. The double membrane structure of E. coli is more difficult to penetrate than the single membrane structure of S. aureus. Also, S. aureus has a 15-80 nm thick negatively charged murein layer (peptidoglycans) covering the lipid bilayer, whereas E. coli has a thin 6 nm thick peptidoglycan layer, sandwiched between the outer and inner membranes. This may result in higher coulombic interactions between PM6-0% and S. aureus, as compared with E. coli.6d

The membrane rupturing mechanism of antibacterial action of our polymers was confirmed by field-emission scanning electron microscope analysis of bacteria cells (ESI,† Fig. S4). The membranes of control bacteria cells without polymer treatment were intact, whereas the PM6-90% treated bacteria cells were found to be severely damaged.

In conclusion, we have synthesized cationic amphiphilic acrylic random copolymers having 2- and 6-carbon spacer arm counits as antibacterial agents. These amphiphilic copolymers (DP \sim 30) displayed superior antibacterial activities with concomitant low hemolytic effect. The homopolymer with 6-carbon spacer arm (PM6-100%) has high cell membrane disruption ability and is non-selective. Incorporation of 2-carbon cationic spacer arm counits in the copolymers resulted in highly selective antibacterial activity. We found that with just 10 mole% of M2 counits, PM6-90% polymer displayed drastically reduced hemolytic activity, by a factor of 850, without serious deterioration of antibacterial activity, in comparison with highly hemolytic PM6-100% homopolymer. Unlike the case of strong electrostatic interactions between negatively charged cell surface of bacteria and cationic polymers, the selectivity of cationic amphiphilic polymers toward bacteria over RBCs may arise from the weaker electrostatic interactions between cationic polymers and zwitterionic lipid head groups of RBC membrane. Incorporation of only three M2 counits into a chain with DP of 30 can significantly impact the conformation of the polymer during the process of membrane rupture. M2 with four carbon shorter hydrophobic spacer arm than M6, can lead to a level of polymer conformation with less degree of freedom, thus substantially reducing the "snorkel effect" of longer spacer arm of the copolymer. This copolymer system represents one of the most promising systems in synthetic polymeric antibacterial agents. The control of spacer arm lengths and copolymer composition can serve as one of the effective structural parameters in the synthesis of polymers with highly selective (bacteria over mammalian cells) antibacterial activity.

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