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Synthesis and Antibacterial Study of Star-shaped Poly[2-(dimethylamino)ethyl methacrylate]-based copolymers with an Inorganic Core

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ABSTRACT: Star polymers with poly[2-(dimethylamino)ethyl methacrylate] as the arms and POSS as the core (POSS-*g*-PDMA) were synthesized by atom transfer radical polymerization (ATRP). The effect of molecular weight on the antibacterial activity was studied and lower molecular weight POSS-g-PDMA has better bactericidal activity as measured by the minimum inhibition concentration. POSS-*g*-PDMA was further modified by various techniques to increase hydrophilicity in attempting to improve their antifouling activity without compromising bactericidal activity. POSS-g-PDMA was quaternized to different degrees and the antibacterial activities of the obtained quaternary polymers were studied; the antibacterial activity decreased as the degree of quaternization increased. Finally, cationic-zwitterionic polymers with both random and block structures, where PDMA and poly(sulfobetaine) were cationic and zwitterionic blocks respectively, were synthesized. The random cationic-zwitterionic polymers showed poor antibacterial activity while the block polymers retained the antibacterial activity of the pristine POSS-*g*-PDMA. The block copolymers of POSS-g-(PDMA-*b*-polysulfobetaine) showed enhanced antifouling property and serum stability as seen by their nanoparticle size stability in the presence of serum and reduced red blood cell aggregation. The antibacterial kinetics showed that *E. coli* can be killed within 30 min by both random and block polymers. Finally, block polymers showed low toxicity to zebrafish embryo and could be potentially used in aquaculture antibacterial applications.

INTRODUCTION

Antimicrobial peptides(AMPs) from plants and animals have been extensively studied because of their broad spectrum killing efficiency and also the rarity of evolution of bacterial resistance to them.[1] Their cationic and hydrophobic structures are believed to be critical for disrupting bacterial membranes with concomitant cell death. However, therapeutic application has been limited partly because of the high cost of synthesizing specified peptide sequences by solid peptide synthesis, which hinders large scale preparation of AMPs. Inspired by the cationic and hydrophobic properties of AMPs, amphiphilic synthetic antimicrobial polymers which mimic natural AMPs have been designed and synthesized. [2-3] These include synthetic polypeptides[4-5] and their mimics[6] like poly(vinyl-N –hexylpyridinum)[7], polynorbornene[8], polymethacrylates[9-10], polycarbonates[11], polyoxetanes[12] , and polymethacrylamide[13] *etc*. Of these, polymethacrylates can be easily synthesized in large scale by atom transfer radical polymerization (ATRP).[14] In addition, the ester linkage of the side chain could be degraded by hydrolysis. Poly[2-(dimethylamino)ethyl methacrylate] (PDMA here after), one of the polymethacrylates, has been extensively studied in antimicrobial applications because of its ease of synthesis in large scale and good antibacterial activities.[10, 15-18] Degrado *et al*. developed polymethacrylates antimicrobials with primary amine as cationic blocks and hydrophobic carbon side chains as hydrophobic segments.[10, 16] Benoit *et al* developed pH-responsive nanoparticles by using PDMA based polymer to deliver farnesol as an anti-oral-biofilm agent.[17]

Recent research on antibacterial polymers has been mainly focused on linear copolymers[6, 8, 10], graft polymers[5, 19-20], and dendrimers[21-23] . On the other hand, star shape polymers have been comparatively neglected although there are few reports on antibacterial star polymers based on cyclodextrin (CD) [24] and tetraphenylethylene[25] cores. Here, we used a nontoxic and three-

dimensional polyhedral oligomeric silsesquioxane (POSS) as a core, and PDMA as arms to construct a star polymer POSS-*graft*-PDMA (Scheme 1, also shorted as PPDMA). POSS was used for the polymer core because it has eight functional groups in three-dimension and is much easier to quantitatively modify than cyclodextrin. PDMAs of various designed molecular weights were grafted to the POSS core and the antibacterial activities of the resulting POSS-*g*-PDMA star polymers were studied. Quaternary ammonium polymers have been widely used as polycationic antibacterials and post-quaternization of PDMA could achieve the permanently cationic polyammonium antibacterials easily. Herein, POSS-*g*-PDMA was successively quaternized by reaction with CH_3I (Scheme 1, path 1) to obtain quaternized PDMA (specifically POSS-graft-PDMA-Qx, where x is the mole percent of quaternization). The antibacterial and hemolytic activities of these quaternized PDMA were also investigated.

Although the main structures of AMPs are composed of cationic and hydrophobic segments, there are usually hydrophilic residues which are not cationic (such as serine and glutamic acid). PEG has been used as a hydrophilic segment in the synthesis of non-hemolytic polymethacrylates. [26-27] Poly(sulfobetaine) which is zwitterionic and hydrophilic and has antifouling properties,[28-29] has been explored in many biological applications such as gene delivery[30]. Herein, we also introduced a zwitterionic segment to replace the traditional hydrophobic segment and studied the antibacterial and hemolytic activity of PDMA-Poly(sulfobetaine) copolymers with both random (Scheme 1, path 2) and block (Scheme 1, path 3) structures. The serum stability and red blood cell aggregation behavior of POSS-*graft*-(PDMA-*random*-Poly(sulfobetaine)) and POSS-*graft*-(PDMA-*block*-Poly(sulfobetaine)) were also studied. Finally, the compatibility of block poly(sulfobetaine) polymers with zebrafish embryo was evaluated.

MATERIALS AND METHODS

Materials. (3-aminopropyl)triethoxysilane (APTES, 99%), concentrated HCl, methanol, tetrahydrofuran (THF), α-bromoisobutyryl bromide (BiBB, 98%), triethylamine (TEA), N,N-dimethylformamide (DMF, anhydrous), ethyl acetate, anhydrous sodium sulfate, 2-(dimethylamino)ethyl methacrylate (DMAEMA, 98%), *N,N,N′,N′′,N′′*-Pentamethyldiethylenetriamine (PMDETA, 99%), CuBr(I) (99.999%), iodomethane, isopropanol, 1,3-propanesultone (>99%), [2-(Methacryloyloxy)ethyl]dimethyl-(3-sulfopropyl)ammonium hydroxide (97%) , aluminum oxide (activated, basic), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich. NIH 3T3 fibroblasts were purchased from Millipore, Singapore. Bacteria (*E. coli* ATCC 8739, *P. aeruginosa* ATCC 27853, and *S. aureus* ATCC 29213) were purchased from ATCC. Dulbecco's modified Eagle medium (DMEM), fetal bovine serum (FBS), antibiotic-antimycotic solution, LB broth, and MHB broth were purchased from Becton Dickinson Company (Franklin Lakes, US). Dialysis tubes were purchased from Spectra/Por, Singapore.

Synthesis of star shape initiator POSS-Br$_8$. The synthesis of POSS-$(NH_2{\cdot}HCl)_8$ has been described previously[31]. Briefly, POSS-$(NH_2{\cdot}HCl)_8$ (1.0 g, 0.85 mmol) was dispersed in 20 mL anhydrous DMF (10 mL) and TEA (2.1 mL, 15.4 mmol) was added. BiBB (1.05 mL, 8.52 mmol) in DMF (10 mL) was added dropwise in an ice bath over a 1h-period. The solution was then stirred at room temperature for 24 h. DMF was removed under vacuum and ethyl acetate was added. The filtrate was washed with 5% citric acid, 5% $NaHCO_3$, and brine, and then dried with anhydrous Na_2SO_4. The filtrate was concentrated and dried under vacuum overnight (0.61 g, 33.8%). ^{1}H NMR in DMSO-d_6 (Figure S2). MALDI-TOF MS (Calculated 2073, Observed 2073.21. DHAC matrix substance, Figure S3).

Synthesis of POSS-*g*-PDMA (PPDMA). Table 1 lists the different PPDMAs obtained by varying the reaction time and ratio of the DMAEMA monomer to the POSS-Br_8 initiator. Taking PPDMA1 which has the lowest DMAEMA to initiator ratio as an example, POSS-Br_8 (50 mg, 193 μmol Br), PMDETA (40 μL, 193 μmol), and DMAEMA (606 mg, 3.86 mmol) were dissolved in methanol (5 mL) and purged with argon for 15 min. CuBr (28 mg, 193 μmol) was added and the mixture was purged with argon for another 15 min. The solution was sealed and stirred for 24 h at room temperature. The reaction mixture was diluted with THF and passed through a short Al_2O_3 column (basic, activated) to remove copper catalyst and concentrated and precipitated into excess hexane twice. The white precipitate was dried under vacuum overnight at 40 °C. PPDMAs with other feed ratios were similarly prepared.

Synthesis of POSS-*g*-PDMA-Q (Q-X%, X means the designed quaternization degree). Taking Q-25% as an example, PPDMA6 (50 mg, 318 μmol repeat units) and CH_3I (5 μL, 79.5 μmol) were added in 1 mL isopropanol and stirred for 24 h. The solvent and CH_3I was removed under vacuum and the residue was dissolved in DI water and dialyzed against DI water (3.5 kDa molecular weight cut off (MWCO)) for three days. White solid was obtained by freeze-drying. Other quaternized polymers were prepared similarly.

Synthesis of POSS-*g*-(PDMA-*r*-Psulfo-Y%) (shortened as Psulfo-Y%, Y means the designed zwitterionization degree). To a solution of PPDMA6 (112 mg) in DMF (5 mL), 1,3-propanesultone (21.8 mg, 0.25 eq PDMA repeat units) was added. The solution was stirred at room temperature for 1 h and at 60 °C overnight. The solution was then diluted with DI water (10 mL) and dialyzed against DI water in a dialysis tube (3.5 kDa MWCO) for three days. The DI water outside the dialysis tube was exchanged with fresh DI water every two hours for the

first 10 hours and then every six hours. Psulfo-25% was obtained after lyophilization. The polymers with other degrees of random zwitterionization were synthesized similarly.

Synthesis of POSS-*g*-(PDMA-*b*-Psulfo) (Psulfo #). In order not to avoid shielding the chain end initiator, PPDMA5 with not too high molecular weight (M_n 36.1 kDa , PDI 1.37) was used to initiate another block by atom transfer radical polymerization. Typically, to a 250 mg PPDMA5 in methanol (3 mL) and DI water (6 mL) mixed solution, [2-(Methacryloyloxy)ethyl]dimethyl-(3-sulfopropyl)ammonium hydroxide (186 mg, 0.4 eq PDMA repeat units; 372 mg , 0.8 eq; 558 mg, 1.2 eq) and PMDETA (13 μL) was added. The mixture was purged with argon for 30 min. CuBr (9 mg) was added and the mixtures were purged with argon for 20 minutes. The mixture was sealed and stirred at 40 °C for 24 h. The solution was then dialyzed against DI water for three days (3.5 kDa MWCO) and Psulfo 1 was obtained after freeze-drying.

Spectroscopy Characterization. ^{1}H NMR spectra were recorded on a Bruker Avance DPX 300 instrument at 298 K with solvents $CDCl_3$, DMSO-d_6, and D_2O. The molecular weights and polydispersities were measured using a Waters' gel permeation chromatography (GPC) system equipped with a 2410 refractive index detector (RID), using two ultrahydrogel columns and sodium acetate buffer (0.5 M of NaAc and 0.5 M of HAc, pH ~4.5) as mobile phase at 40 °C with a flow rate of 1.0 mL min^{-1}. Monodisperse pullulan was used as the standard to generate the calibration curve. Dynamic light scattering (DLS) and Zeta potential measurements were performed on a Malvern Zetasizer Nano-ZS90 apparatus equipped with a He–Ne laser operated at 633 nm. All samples were measured at a scattering angle of 163^o.

Minimum inhibitory concentrations (MICs). Bacteria cells were grown overnight at 37 ºC in MHB broth to a mid-log phase and diluted to 10^5 CFU mL^{-1} in MHB broth. A two-fold

dilution series of 100 μL product solution in the broth was made on 96-well microplate, followed by the addition of 100 μL bacterial suspension (10^5 CFU mL^{-1}). The plates were incubated at 37 ºC for 18-24 h, and the absorbance at 600 nm was measured with a microplate reader (BIO-RAD Benchmark Plus, US). Positive control was without product, and negative control was without bacteria inoculum. MICs were determined as the lowest concentration that inhibited cell growth by more than 90%. For the MICs in blood, MHB were replaced by fresh blood. The bacterial concentrations were quantified by plating method.

Time-kill assay. *E. coli* ATCC 8739 were grown overnight in MHB at 37 ºC. Cells were diluted to 5 x 10^5 CFU/mL and 100 μL of this suspension was then added to 100 μL MHB broth or broth containing polymers (concentration 2 and 4x MIC). 20 μL sampling aliquots were withdrawn from all cultures immediately after polymer addition (t =0 min) and then again at 15, 30, and 60 min after polymer addition. The diluted aliquots were plated on solid LB plate and incubated 37 ºC overnight before colony numbers were counted for determination of surviving CFU. Data from triplicate plate counts were averaged, and the resulting values were plotted on a log scale against time.

Hemolysis test. Fresh human blood (5 mL) was collected from a healthy donor (age 26, male) (NTU IRB-2015-03-040). Erythrocytes were separated by centrifugation at 1000 ×g for 10 min, washed three times with Tris buffer (10 mM Tris, 150 mM NaCl, pH 7.2) and diluted to final concentration (5%, v/v). 50 μL of antimicrobial solution at different concentrations mixed with 50 μL erythrocytes stock were added to a 96-well microplate and incubated for 1 h at 37 °C with 150 rpm shaking. The microplate was centrifuged at 1,000 ×g for 10 min. 80 μL aliquots of the supernatant were then transferred to a new 96-well microplate and diluted with another 80 μL of

Tris buffer. Hemolytic activity was determined at 540 nm with a 96-well plate spectrophotometer (Benchmark Plus, BIO-RAD). Triton X-100 (0.1% in Tris buffer) which is able to lyse RBCs completely was used as positive control, while Tris buffer was used as negative control. The hemolysis percentage (H) was calculated from the following equation:

$$\text{Hemolysis (\%)} = [(O_p - O_b)/(O_t - O_b)] \times 100\%,$$

where O_p is the absorbance with the antimicrobial agent, O_b is the absorbance for the negative control (Tris buffer), and O_t is the absorbance for the positive control (Triton X-100).

Cytotoxicity. NIH 3T3 fibroblasts were cultured in DMEM media supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic-antimycotic at 37 °C under an atmosphere with 5% CO_2.The cells were seeded in 96-well plates at a density of 1×10^4 cells per well for 24 hours before treatment. The cells were then treated with polymers at a concentration of 100, and 200 μg/mL for additional 1 or 2 hours incubation. At the end of the treatment, the cell culture medium was removed and the wells were rinsed with PBS (pH=7.4). 100 μL PBS (pH=7.4) containing 10 μL of 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, 5 mg/mL) solution was added to each well. After the cells were incubated for additional 4 h, the medium containing unreacted MTT was removed carefully and 100 μL DMSO was added into each well to dissolve the internalized purple formazan crystal. The optical density (OD) of the solution in each well was measured against a blank (a well with 100 μL DMSO) at 492 nm with a microplate reader (BIO-RAD Benchmark Plus, US).

Serum stability. Because polymers can't be separated by centrifugation like some metal nanoparticles and quantified the protein adsorption by UV method, we used hydrodynamic size change to characterize the polymer-protein interactions. FBS (Fetal Bovine Serum) were co-

incubated with polymer at a shaking speed of 100 rpm at 37 ºC. The sizes were measured by Malvern Nano-ZS Particle Sizer.

Polymers and Red blood cells interactions. Ethylenediaminetetraacetic acid (EDTA) anticoagulated whole blood was mixed with polymers stock solutions in PBS buffer to yield final concentrations of 10, 20, 50, 100, and 200 μg/ml. PBS buffer was used as a normal control. The samples were incubated for 1 h at 37 °C and the red blood cells morphology and aggregation behavior was analyzed using an Olympus light microscope. The suspension was gently centrifuged and re-suspended in supernatant plasma on the microscopic slides before examining under the light microscope. A minimum of five images were taken from different parts of the slides and were visually examined.

Zebrafish test. Zebrafish (*Danio rerio*) embryos used for experiments were obtained from natural crosses and were maintained at 28 ºC. Stocks of high molecular weight polymers were prepared in water and were diluted to final assay concentrations with zebrafish E3 embryo medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, 0.33 mM $MgSO_4$; pH 7.2). Zebrafish embryos at 1 day post fertilization (dpf) (n=30) were transferred into 6 well plates and exposed to 5 mL of polymers at defined concentrations (10 μg/mL, 100 μg/mL). Control fish embryos were incubated in corresponding solvent concentration (E3 medium with or without DMSO at final concentration of 0.01%). Zebrafish embryos were monitored at 18 hpe (hours post exposure), 24 hpe and 48 hpe for mortality using Leica MZ16FA microscope, to determine the survival rate of the fish embryos.

RESULTS AND DISCUSSION

Synthesis and antibacterial activities of star polymer POSS-*g*-PDMA (PPDMA). Star polymers with a polyhedral oligomeric silsesquioxane (POSS) core and poly[2-(dimethylamino)ethyl methacrylate] (PDMA) arms were synthesized via atom transfer radical polymerization (ATRP). POSS is a nanocage molecule with a diameter of around 1 nm and has been shown to have good biocompatibility[32-33]. Firstly, the star shape initiator POSS-Br_8 was synthesized through the amidation reaction between POSS-$(NH_2{\cdot}HCl)_8$ and BiBB in the presence of triethylamine (Figure S1). The chemical structure of POSS-Br_8 was confirmed by ^{1}H NMR (Figure S2). The appearance of the peak at 2.0 ppm is attributed to the protons (-C($C\underline{H}_3)_2$Br) close to the bromine atom. The peak at 0.6 ppm corresponds to the proton adjacent to the POSS backbone (-SiC$\underline{H}_2$). The integration ratio of these two peaks is 3:1, consistent with the theoretical composition ratio (6 protons to 2 protons). This result suggests that a bromine atom was successfully immobilized onto each branch of the POSS core. Secondly, DMAEMA (2-(dimethylamino)ethyl methacrylate) was used as a monomer for the synthesis of the star polymer POSS-*g*-PDMA by a typical ATRP procedure. Different feed ratios of monomer (DMAEMA) to bromine on initiator (20:1, 120:1 and 240:1, DMAEMA to bromine atoms) and reaction times (10 min, 20 min, 30 min, and 24 h) were employed; the properties of the resulting polymers are summarized in Table 1 (PPDMA1-6). The polymer molecular weight was mostly affected by the feed ratio and when the monomer to initiator ratio was higher, the molecular weight of the obtained polymer was correspondingly higher. The number average molecular weights (M_n) of these polymers, determined by GPC, are in the range 9.90 to 64.1 kDa which was (PPDMA1, 5, and 6, Table 1). The variation in reaction time, 10 min to 24 h (PPDMA2 to 5), had very little effect on the molecular weight of polymer in our study, in contrast to the previous report by Xu *et al*[34]: at the monomer to initiator feed ratio of 120:1, the molecular weights of our polymers

(PPDMA2-5) produced by10, 20, 30 min and 24 h reactions were very similar (Table 1), suggesting that the polymerization speed was very fast. This is also suggested by our observation that the reaction solution turned very viscous or even gel-like in the first minutes of polymerization. Typical ^{1}H NMR spectra of PPDMA6 in D_2O and $CDCl_3$ are shown in Figure 1A and Figure S3, respectively. The peaks at 2.0 ppm, which are attributed to the protons on the polymer backbone, were very weak in the D_2O spectrum but strong in the $CDCl_3$ spectrum, suggesting that PPDMA6 self-assembles in water and the hydrophobic backbone signals are almost shielded in D_2O. The signals of the POSS core were not detected due to the small fraction of the POSS portions in the star polymers statistically. The size and zeta potential of these polymers were studied by DLS (Figure S5). The polymers were most probably monodisperse and the sizes were in the range 6-10 nm. The zeta potentials were in the range 30 to 40 mV. The zeta potential increased with increasing molecular weight.

The antibacterial activities of the polymers were studied using the broth dilution method. Two Gram-negative bacteria (*E. coli* and *P. aeruginosa*) and one Gram-positive bacterium (*S. aureus*) were tested as model bacteria. The MIC results are summarized in Table 1. As has been reported by many researchers, [3] we find that the variation of antibacterial activity varies with polymer molecular weight. The lowest molecular weight polymer, PPDMA1, is much less effective than the intermediate molecular weight PPDMA2-5 against two of the three pathogens (*P. aeruginosa* and *S. aureus*). Among the similar intermediate molecular weight polymers PPDMA2-5, there is a distinct but pathogen-dependent trend of increasing efficacy with molecular weight. It seems that PPDMA5 is the most effective against all three pathogens. The highest molecular weight polymer, PPDMA6, has higher MICs than any of the intermediate molecular weight polymers. The reduced efficacy of higher molecular weight polymers has also been reported by other

groups[35] and may be due to poor permeability through the cell wall of ultra-high molecular weight polymers.

Synthesis and antibacterial activities of quaternized star polymer POSS-*g*-PDMA-Q (Q-X%). Quaternary ammonium salts are one of the most widely studied antibacterial materials.[36] Here we synthesized the quaternized polymer of POSS-*g*-PDMA-Q-x% (where x% is the design quaternization degrees) by reaction of PPDMA6 with CH_3I (Path 1 in Scheme 1) in varying feed ratios in isopropanol; the resulting polymers are denoted Q1-theoretical quaternization degree except Q1-100% in which feed ratio of CH_3I to DMA repeat unit was 500%, Table 2). The ^1H NMR spectra of these polymers are shown in Figure 1. As the feed ratio of CH_3I increased, the intensity of peaks at 3.3 ppm, corresponding to the quaternary ammonium protons (e′ in Figure 1, $-N^+(C\underline{H}_3)_2I^-$), increased. This result suggests that the quaternization degree increased with the increased feed ratio of CH_3I to the DMA repeat units. The quaternization degree (Table 2) was calculated from the ratio of peak area of protons methyl group of DMA and quaternized DMA, (e and e′, Figure 1) and it ranged from 18% to 100%. The molecular weights (M_n) of these polymers were characterized by GPC and ranged from 72.3 kDa to 90.3 kDa (PDI 1.81-2.23, Table 2). The size and zeta potential of these quaternized polymers were studied by DLS (Figure S5). The sizes increased with the degree of quaternization and ranged from 15 to 50 nm, several fold larger than the input PPDMA6 (11 nm), indicating a significant degree of self-assembly into nanoparticles. After quaternization, the zeta potentials increased and were in the range 40-50 mV compared to 37 mV for PPDMA6.

The antibacterial activities of the quaternized polymers are summarized in Table 2. There is a distinct trend of decreasing antibacterial activity with increasing quaternization degree, with a large increase in MIC against *E. coli* and *P. aeruginosa* for the fully quaternized polymer (Q1-

100%). Quaternization increases polymer hydrophilicity, which may reduce the ability to penetrate the hydrophobic bacterial membrane. We also found that the antibacterial activity of the quaternary polymers was greatly affected by the testing medium. The results in Table 2 were obtained using full MHB medium which is a complex medium with anionic proteins that is typically used for antibiotics screening. When the testing medium was 50% MHB (with 50% DI water), the quaternary ammonium polymers showed MICs comparable to the unquaternized PPDMA6 (Table S1). The ionic strength or medium concentration may affect the self-assembly behaviors of the quaternary ammonium polymers, which may in turn affect their antibacterial properties.

Synthesis and antibacterial activities of star polymer POSS-*g*-(PDMA-*r*-Psulfo-Y%) (Psulfo-Y%). We also studied the effect of zwitterion functionalization on the antibacterial activity of POSS-*g*-PDMA. We used PPDMA6 as the starting material and prepared random POSS-*graft*-(PDMA-*random*-poly(sulfobetaine)-Y%) polymers by reactions of 1,3-propanesultone (Path 2, Scheme 1). Polymers with different design zwitterionization degrees (denoted by Y%) were obtained by tuning the feed ratio of 1,3-propanesultone and the DMAEMA repeat units (and the label of the resulting polymers are shortened as Psulfo-Y%). The ^{1}H NMR spectra of these polymers are shown in Figure 2. Similar to quaternary ammonium polymers POSS-*g*-PDMA-Q-X%, the peaks at 2.3 ppm which are attributed to the protons adjacent to the nitrogen atom shifted to 3.3 ppm after zwitterionization. The actual zwitterionization degree was calculated by ^{1}H NMR and varied from 10%, 36%, to 44% for the design Psulfo-25%, 50%, and 75% respectively. Molecular weights of these polymers were characterized by GPC and summarized in Table 3. The molecular weights were in the range 65.8 to 87.8 kDa and the PDI increased after zwitterionization (PDI 1.89-2.05). Nanoparticles self-

assembled from the random zwitterionic-cationic polymers were measured to have sizes in the range 30-120 nm (Figure S6). The sizes increased a lot compared with PDMA6 (11 nm). The zeta potentials were in the range 40-45 mV.

The antibacterial activities of these polymers are summarized in Table 3. After random zwitterionization, the MIC against *E. coli* increased rapidly from16 μg/mL to more than 512 μg/mL. Even only after actual 10% zwitterionization (with the design Psulfo-25%), the MICs increased two to four times. When the actual random zwitterionization degree was 36% or more (for Psulfo-50% and Psulfo-75%), the MIC was above the upper limit of concentration we tested. Like quaternary modification we discussed before, random zwitterionization increased the hydrophilicity of polymers and in turn decreased its antibacterial activity.

Synthesis and antibacterial activities of star polymer POSS-*g*-(PDMA-*b*-Psulfo). POSS-PDMA-*block*-poly(sulfobetaine) polymers were also synthesized by a successive ATRP after purification of PDMA5. PDMA5 was used as a macro-initiator and zwitterionic sulfobetaine methacrylate as monomer to build star shape cationic-*block*-zwitterionic copolymers (Path 3, Scheme 1). We chose PDMA5 as a macro-initiator because it has a moderate molecular weight in which bromine atoms at the chain ends were probably not shielded due to a too long polymer chain and so could initiate the next ATRP. By varying feed ratios of monomer to macro-initiator, three star shape cationic-*block*-zwitterionic copolymers POSS-*g*-(PDMA-*b*-Psulfo 1-3) were obtained (denoted Psulfo 1-3). The ^{1}H NMR spectra of the polymers are shown in Figure 3. Similar to the random PDMA-poly(sulfobetaine) polymers, the zwitterionization degree was calculated by NMR; for PSulfo 1, 2, and 3, it is 16%, 31%, and 44%, respectively. The molecular weights were characterized by GPC (Table 4). The M_n didn't show great change after block

zwitterionization but M_w increased a lot and thus the PDIs increased from 1.4 to about 2.0. The sizes and zeta potentials were measured by DLS and the results were quite different from the random zwitterionic polymers (Figure S5D). The polymers had no apparent change in sizes and zeta potentials compared with pristine PDMA polymers.

The antibacterial activities of these block cationic-zwitterionic polymers were also investigated and the MICs were much lower than those of the random polymers (Table 4). After block zwitterionization, the MICs were comparable to those of PDMA5. Even for the highest zwitterion degree polymer Psulfo 3, the MICs were only twice those of PDMA5. For Psulfo 1 and 2, the MICs against *E. coli* and *S. aureus* were the same as those of PDMA5 (10 μg/mL). These results suggest that cationic-zwitterionic block structure didn't interfere with the antibacterial activity much. This could be explained by that the cationic charge and partial charge density didn't change significantly after block copolymerization, which preserved its antibacterial activity.

Antibacterial kinetics. We evaluated the antibacterial kinetics against *E. coli* of PDMA and their random and block zwitterionic polymers by time-kill assay. Bacteria were suspended in MHB (10^5 CFU/mL), treated with polymers sampled at various time intervals and colony-counted after plating the samples on LB agar. The results are shown in Figure 4. PPDMA5 and 6 showed fast time-kill efficiency; bacteria were killed in 15 mins with treatments of 4 times MIC polymer concentration. The random and block zwitterionic polymers were not as fast but still eliminated (>5 log reduction) the bacteria in 30 min. Hence, star PDDMA and PDMA-poly(sulfobetaine) random and block polymers exhibit fast killing kinetics, killing *E. coli* in short time (15-30 min).

Hemolysis study. Hemolysis behaviors of these polymers against human red blood cells were studied to evaluate their cytotoxicity of against mammalian cells. The hemolytic behaviors are summarized in Figure 5. For the PPDMA series, the hemolysis curves exhibit an omega (Ω) trend with a maximum hemolytic activity at intermediate concentrations (Figure 5A). They are non-hemolytic at both low and high polymer concentration, but somehow hemolytic, with hemolysis of 10% - 50%, in the concentration range of 128-2048 μg/mL. This could be explained by the amphiphilicity of PPDMA: the backbone of PPDMA is hydrophobic and the side chains are not 100% protonated. This amphiphilicity is also demonstrated in the ^{1}H NMR spectra as previously mentioned. At low polymer concentration, the hemolysis is low because the hydrophobic segment concentration is low. At high concentrations, PPDMA self-assembles into nanoparticles (NPs) with the hydrophobic backbones in the NP cores surrounded by hydrophilic shells, which is then non-destructive to cell membranes of RBCs. At intermediate concentrations, there is enough less-assembled PPDMA that the higher concentration of exposed hydrophobic backbone with charged side groups can be cytotoxic. At all concentrations, the hemolysis declines with increasing PPDMA molecular weight (MW), which is compatible with the self-assembly hypothesis; the longer chains of higher MW individual PDMA molecules can assume configurations which partially hide the hydrophobic backbones. Self-assembly into nanoparticles by POSS based cationic star polymers has also been reported by He Chaobin *et al*[37] in which the polymers were used in gene and drug delivery.

The quaternary ammonium polymers showed different hemolytic activities and the results are shown in Figure 5B. When the quaternization degree was low (Q-25% and Q-50%), the polymer showed very low hemolytic activity even at the concentration of 10 000 μg/mL. With increasing degree of quaternization, the polymer showed higher hemolytic activity; for example with

polymer Q-75%, the HC_{50} (polymer concentration at which 50% hemolysis took place) is 9000 μg/mL. With 100% quaternization, the polymer (Q-100%) becomes less hemolytic again.

The random and block PDMA-poly(sulfobetaine) polymers show different hemolysis behaviors (Figure 5C and 5D). The random polymers series were non-hemolytic at all concentrations. This is because of interspersing hydrophobic PPDMA chain with the zwitterionic fraction so that the average hydrophilicity increased. On the other hand, the block polymers (Fig. 5D) showed hemolysis behaviors that are similar to that of PPDMA5, with maximum hemolysis below 50% and occurring at intermediate concentration. But the hemolysis curves showed a rightward shift, suggesting that the Psulfo 1-3 series have lower propensity than PPDMA5 to aggregate into NPs. The HC_{10} of the block polymers of Psulfo-1, -2, and -3 were about 250, 700, and 700 μg/mL, respectively, slightly higher than that of the PPDMA5 (100 μg/mL).

Together with the antibacterial activity results, the random PPDMA-poly(sulfobetaine) polymers showed rapid decrease in both antibacterial activity and hemolytic activity when the degree of zwitterionization was increased. However, the block polymers showed similar antibacterial and hemolytic behaviors when compared with the pristine POSS-*g*-PDMA. The reason why the biological results were different could be explained that the random addition of sulfobetaine monomers changes the polymer structures cationicity and the charge density and the hydrophobic/hydrophilic balance of the resulting copolymer while the block polymer structures retain these properties of the PDMA block which is the killing block. This difference can also be explained by size differences of zwitterionic random and block polymers. MIC was found to have a correlation with its size for the quaternary and zwitterionic polymers (Figure S6). With increasing nanoparticle sizes, the MIC generally increased and hemolysis decreased. This MIC and hemolysis-size trend was observed with the Q1 series in which after quaternization, MIC

increases and hemolysis decreased while the size increased. For the zwitterionic random polymers (Figure 7), polymer sizes increased with higher degree of zwitterionization, along with increasing MICs. For block polymers, polymer sizes were almost the same as PDMA5 and their antibacterial activities and hemolysis behaviors are comparable.

Antifouling test in serum and MBC in blood. Zwitterionic polymers have been applied in antifouling surfaces and hydrogels because of their excellent antifouling and hydrophilic properties. The antifouling properties of both random and block zwitterionic polymers in solution were tested by their interaction with FBS (Fetal Bovine Serum). The hydrodynamic diameters of the polymer nanoparticles in the presence of the FBS protein were determined by DLS (Figure S7). PPDMA5 showed the most significant size increase after 24 h co-incubation, suggesting that the cationic PPDMA5 had the most intense interaction with FBS. The zwitterionic block polymer (Psulfo-3) showed negligible size change in the same conditions, suggesting better antifouling property and stability in serum.

Polymers and red blood cell interactions. We studied the aggregation behavior of RBCs in the presence of varying concentrations (10, 20, 50, 100, and 200 μg/mL) of our polymers. RBCs treated with PPDMA5 showed some aggregation (aggregates are highlighted with arrows in Figure 7B, polymer concentration 200 μg/mL). However, no obvious aggregation was observed when RBCs were treated with Psulfo 1 (200 μg/mL, Figure 7C). The reduction of RBC aggregation after block zwitterionization was ascribed to the improved antifouling property.

Zebrafish cytotoxicity test. We also studied the toxicity of our polymers using the zebra fish model. PPDMA5 and its block zwitterionizaiton polymers Psulfo 1-3 were tested and 1dpf (day post fertilization) zebrafish embryo was used as the model. Zebrafish survival percentage was

measured after various exposure times (18, 24, and 48 h) and polymer concentrations (10 and 100 μg/mL). The zebrafish survival results show that our block polymers as well as PPDMA5 are biocompatible with zebrafish embryos (survival rate>80%, Table 6). These results suggested that these polymers are not acutely toxic to zebrafish embryos and could be potentially used in some aquaculture antibacterial applications.

Comparative Antibacterial Effectiveness and hemolytic activity. We compared the bioactivity of our star shaped polymers with those of reported polymethacrylate based antibacterial polymers (Table 5). Different from our star polymers, most of the reported antibacterial polymethacrylates were linear polymers. Brayden *et al* reported linear PDMAEMA which showed relatively poor antibacterial activity with high MIC value of 100 μg/mL against *E. coli*.[15] Here our star POSS-g-PDMA showed enhanced antibacterial activity compared with these linear polymers (MIC 10 μg/mL against *E. coli*). Similar with our report, the linear PDMAEMA showed low hemolytic activity with HC_{50} more than 10 000 μg/mL. DeGrado *et al* introduced hydrophobic component into linear PDMAEMA and the antibacterial activity can be tuned by the ratio of DMAEMA to the hydrophobic butyl methycrylate (BMA). After introduction of hydrophobic composition, the antibacterial activity increased (MICs 10-100 μg/mL) but the hemolytic activity increased and the HC_{50} was much lower than its MIC.[10] Instead of introducing hydrophobic component, Yang *et al* reported PEGylated PDAMEMA which showed comparable antibacterial activity while the polymer not so hemolytic because of the hydrophilicity of PEG.[38] Recently, linear long chain PDMAEMA-*co*-Poly(2-hydroxylethylmethacrylate) (PDMA-*co*-PHEMA) also showed good MICs (4-16 μg/mL) but was hemolytic. Notably, although HEMA was somehow hydrophilic, the HC_{50} was still as low as 256-512 μg/mL because the hydrophilicity of HEMA is less than that of PEG. Taking together, the hemolytic activity of

polymers was strongly affected by its hydrophilicity. Because the order of decreasing hydrophilicity: polysulfobetaine≈PEG>PHEMA>>PBMA, PDMAEMA showed reduced hemolytic activity when modified with more hydrophilic polymers. The antibacterial activity of polymers was affected by both polymer structure and polymer compositions. Although the introduction of hydrophobic and hydrophilic component can somehow increase its antibacterial activity of linear PDMAEMA in previous reports, here in our polymers the random and block polymer structure of star polymers also played a key role in the antibacterial activity. That is to say, the random cationic-zwitterionic (or cationic-hydrophilic) star polymers showed poor antibacterial activity while block ones showed good activity.

CONCLUSION

We have synthesized star polymers based on poly[2-(dimethylamino)ethyl methacrylate] (PDMA) and a three dimensional inorganic biocompatible POSS core. For POSS-*g*-PDMA, the antibacterial activity was molecular weight dependent with an "inverted omega" MIC profile. The hemolytic activity of POSS-*g*-PDMA decreased when the molecular weight increased. After quaternization with CH_3I, the antibacterial activity decreased with increase of quaternization degree which was undesirable for antibacterial applications. In addition, cationic-zwitterionic PDMA-poly(sulfobetaine) polymers with both random and block structures were synthesized. The random polymers showed poorer antibacterial activity but lower cytotoxicity and non-hemolytic property. The block polymers, however, showed similar antibacterial and hemolytic activity, and cytotoxicity with pristine POSS-*g*-PDMA. We attribute this difference to the random structure destroying the amphiphilic balance of PDMA and reducing the cationic charge

density while block polymer structures did not change this in partially. The block cationic-zwitterionic polymer enhanced serum stability, and minimized the red blood cell aggregation. Furthermore, these zwitterionic copolymers as well as pristine POSS-*g*-PDMA showed good compatibility with zebrafish embryos and could be potentially used in aquaculture antibacterial applications. Also. the killing kinetics showed that *E. coli* could be killed in 30 min when treated with random and block zwitterionic polymers and pristine POSS-*g*-PDMA.

ASSOCIATED CONTENT

Supporting Information. Figure S1-S6. Table S1-S2 for NMR, DLS, and MIC in 50%MHB and blood.

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Notes

The authors declare no competing financial interest.

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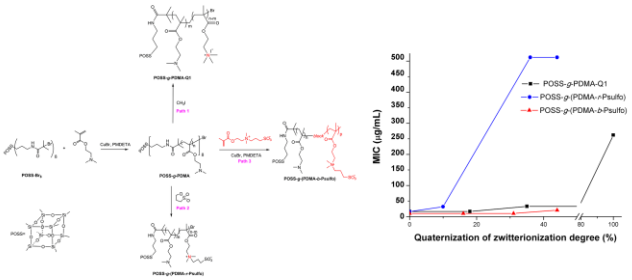
TOC


MIC (μg/mL)
Quaternization of zwitterionization degree (%)
POSS-g-PDMA-Q1
POSS-g-(PDMA-r-Psulfo)
POSS-g-(PDMA-b-Psulfo)