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Quaternized Poly(2-(dimethylamino)ethyl methacrylate)-Grafted Agarose Copolymers for Multipurpose Antibacterial Applications

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Abstract

Polymeric quaternary ammonium salts (QAS) have been widely studied for their antimicrobial acitivities. However, most of them were evaluated and used in solution form, which may limit their other applications, in medical and wound care and surface biofouling prevention. The development of antibacterial agents for use both in solution and in surface biofouling prevention are highly interesting and desirable. In this work, the quaternized poly(2-(dimethylamino)ethyl methacrylate)-grafted agarose (Agr-*g*-QPDMAEMA) copolymers were synthesized via a combination of atom transfer radical polymerization (ATRP) and quaternization of tertiary amine moieties. The resulting Agr-*g*-QPDMAEMA copolymers can be dissolved in aqueous media at low concentrations to evaluate their antibacterial activity in the solution form. They can also be gelated as hydrogels on substrate surfaces to inhibit bacterial adhesion, biofilm formation and bacterial colonization. The Agr-*g*-QPDMAEMA copolymer hydrogels can be further fabricated into antibacteiral patches to inhibit the bacteria growth on the contaminated surfaces.

Keywords: Agarose, quaternary ammonium salts, atom transfer radical polymerization, antibacterial, multipurpose

Introduction

Microbial infections caused by bacteria are a serious health problem to the human population.[1,2] Fortunately, the discovery and development of antibiotics to treat infections caused by bacteria has been an important contribution of modern medicine. However, The misuse and overuse of antibiotics have created antiobiotic resistence in certain bacteria, which require expensive treatments and increase the risk of complications and death for patients with infections.[3,4] A 2013 report from the Centers for Disease Control and Prevention (CDC) has revealed that more than 2 million people in the US become ill annually due to the antibiotic-resistant infections, and 23,000 die from such infections. Thus, the next-generation of antimicrobial agents are needed and much effects have been devoted to discovering of highly efficient compounds, such as antimicrobial peptides (AMPs)[5-7] and cationic compounds.[8-16] These compounds are less sesceptible to the development of resistence by bacteria.[17] The quaternary ammonium salts (QAS) are among the most commonly used cationic antimicrobial agents. It is generally accepted that the mechanism of the bactericidal action of the QAS involves: i) penetration of the coumpound into the cell wall, ii) reaction with the cytoplasmic membrane, followed by membrane disruption, iii) leakage of intercellular matters and degradation of proteins and nucleic acids, and iv) cell wall lysis.[2,17] Among the QAS, the polymeric QAS provide the advantages of non-volatility, long-term chemical stability and activity, enhanced antimicrobial efficacy and reduced residual toxicity.[18] Polymeric QAS based on poly(vinylpyridine), poly(acrylate), poly(acrylamide), poly(diallylammonium), poly(styrene), degradable polyester, conjugated polymers, poly(norbornene), branched poly(ethyleneimine) and modified natural polysaccharides[2,14,17-19] have been reported to eradicate pathogens with impressive results.

The antibacterial activities of most QAS are evaluated in solution forms.[14] For other medical applications, such as wound care and surface biofouling prevention, the antimicrobial moieties need to be physically embedded into or chemically bonded to polymeric matrixes.[1,20] In this case, the actibacterial activities are evaluated mainly at the surfaces in contact with the bacterial suspensions or bacterial aerosols.[21] It would be interesting to develop new antimicrobial agents, which are suitable for multipurpose biomedical applications in both solution and solid form.

Agarose (Agr), a natural polysaccharide extracted from seaweed, can forms a thermoresponsive hydrogel through physical cross-linking.[22] Due to its biocompatibility, non-fouling and non-immunogenic attributes,[23] Agr has been widely explored for applications in the biomedical field.[24,25] Agr is not soluble in cold water, but it can dissolve in hot water and the aqueous Agr solution (minimum concentration of ~ 5 mg/mL) forms a hydrogel as it cools. Agr is soluble/dispersible in aqueous media at a lower concentration. The chemically modified Agr derivative can exhibit a better solubility in water, as the hydrogen bonding within the Agr becomes weaker.[23,24,26,27] Thus, integration of Agr and QAS could lead to the formation of an antibacterial agent, which is soluble in aqueous media at low concentration and solidifies as a hydrogel at high concentration.

In this work, the integration of Agr and QAS was achieved by atom transfer radical polymerization (ATRP) using modified Agr as the macroinitiator and 2-(dimethylamino)ethyl methacrylate (DMAEMA) as the monomer, followed by the quaternization of the tertiary amine group in DMAEMA. The resulting quaternized poly(DMAEMA)-grafted Agr (Agr-*g*-QPDMAEMA) copolymers were characterized for their antibacterial and hemolytic activities in

solution form. The Agr-*g*-QPDMAEMA copolymers were also gelated, and the bacterial adhesion, biofilm formation and bacterial colonization on their surfaces were assayed. The Agr-*g*-QPDMAEMA copolymer hydrogels were then fabricated into antibacterial patches. The inhibition of bacteria growth on the contaminated substrate surfaces by the hydrogel patches was investigated.

Experimental Section

Materials

Agarose (Agr) for biochemistry, 2-bromoisobutyryl bromide (BiBB, 98%), copper(I) bromide (CuBr, 99.99%), 2,2'-bipyridine (bpy, 99%), 2-(dimethylamino)ethyl methacrylate (DMAEMA, 99%) and 1-bromobutane (99%) were purchased from Aladdin Industrial Corporation (Shanghai, China). CuBr was purified by stirring in glacial acetic acid overnight, followed by filtration and rinsing with ethanol. DMAEMA was purified by passing it through a basic alumina column. All other regents and solvents were purchased from Aladdin Industrial Corporation or Kelong Chemical Reagent Plant (Chengdu, China), and were used without further purification.

Synthesis of the alkyl bromide-functionalized Agr (Agr-Br) macroinitiator

The preparation of Agr-based macroinitiator was described as follows: Agr (6 g, ~18.5 mmol of repeat units) was dissolved in 150 mL of *N*,*N*-dimethylacetamide (DMAc), and the mixture was stirred at 120 ^{o}C for 4 h until Agr was completely dissolved. After that, the temperature of reaction mixture was lowered to 0 ^{o}C in an ice bath. Triethylamine (3.87 mL, 27. 7 mmol) in 10 mL of DMAc was added to this solution, followed by the drop-wise addition of BiBB (3.43 mL, 27.7 mmol) in 10 mL of DMAc. The reaction mixture was stirred at room temperature for 24 h, and poured into an excess amount of ethanol. The obtained precipitate was filtered and dried under reduced pressure at 50 ^{o}C for 12 h.

Synthesis of poly(DMAEMA)-grafted Agr (Agr-*g*-PDMAEMA) copolymers

A typical atom transfer radical polymerization (ATRP) of DMAEMA was carried out using CuBr/bpy as the catalyst system and Agr-Br as the macroinitiator. Agr-Br (2 g, ~3.0 mmol of alkyl bromide moieties) was dissolved in 30 mL of *N*,*N*-dimethylforamide (DMF) and stirred at 60 ^{o}C until the complete dissolution. The obtained homogeneous solution was left to cool down to room temperature. Then, CuBr (0.3 g, 2.1 mmol) and DMAEMA (8.89 mL, 52.8 mmol) in 12 mL of DMF were sequentially introduced into the reaction mixture. The solution was degassed with argon for 20 min, and bpy (0.65g, 4.2 mmol) was quickly added into the mixture. The reaction was then sealed, and the mixture was stirred at 60 ^{o}C until the dark brown solution became very viscous. The resulting solution was dialyzed against ethylenediamine tetraacetic acid (EDTA) aqueous solution for three days and against pure deionized water for another four days. The solid Agr-*g*-PDMAEMA1 copolymers were obtained after lyophilization. The resulting copolymer sample prepared from 13.33 mL of DMAEMA (79.1 mmol) is referred to as the Agr-*g*-PDMAEMA2 copolymer.

Quaternization of Agr-*g*-PDMAEMA Copolymers

Agr-*g*-PDMAEMA1 copolymer (3 g) was added to 40 mL of DMF and suspended by stirring at 60 ^{o}C until the complete dissolution. 1-Bromobutane (0.55 mL) in 0.69 mL of DMF was added drop-wise to this solution, and the reaction was maintained at 60 ^{o}C for 5 h under stirring. The reaction mixture was then lowered to room temperature and precipitated into a large excess of ethanol. The precipitate was collected by centrifugation, washed with ethanol 3 times, and dried under reduced pressure. The copolymer obtained is referred to as the quaternized Agr-*g*-

PDMAEMA1 (Agr-*g*-QPDMAEMA1) copolymer. The Agr-*g*-QPDMAEMA2 copolymer was prepared from the Agr-*g*-PDMAEMA2 copolymer using similar procedures.

Determination of antimicrobial property of Agr-*g*-QPDMAEMA copolymers

Minimum inhibitory concentration (MIC) of Agr-*g*-QPDMAEMA1 and Agr-*g*-QPDMAEMA2 copolymers against *Staphylococcus aureus* (*S. aureus* 25923) and *Escherichia coli* (*E. coli* DH5α) was determined by the broth microdilution method.[28] *S. aureus* and *E. coli* were cultured in Mueller Hinton Broth (MHB) by incubation overnight at 37 ^{o}C in the dark. The bacterial suspensions were then dispersed in MHB to give rise to a final concentration of 3×10^5 bacterial cells/mL. Stock solutions of the Agr-*g*-QPDMAEMA1 and Agr-*g*-QPDMAEMA2 copolymers were prepared in MHB, and then serially diluted by 2-fold each time using MHB. Each well of a 96-well plate was filled with 100 μL of medium containing the copolymer, to which 100 μL of bacterial suspension (3×10^5 bacterial cells/mL) was added. The plate was incubated at 37 °C overnight, and MIC was recorded as the lowest concentration of the copolymer that inhibited bacteria growth detected by naked eye.

After the MIC assay, the bacterial suspensions (3×10^5 bacterial cells/mL, 20 mL) in MHB were then introduced into respective 50 mL sterile tubes, each containing 20 mL of the Agr-*g*-QPDMAEMA1 and Agr-*g*-QPDMAEMA2 copolymers at different concentrations (1 × MIC or 2 × MIC) in MHB. The tubes was sealed and placed on an incubator at 37 ^{o}C. The viable cell numbers in the suspensions after 1, 2, 3, 4 and 5 h of incubation were determined using the spread plate method. Bacterial suspensions were spread onto plates in triplicate. Control experiments were carried out in the absence of the Agr-*g*-QPDMAEMA copolymers.

Characterization of hemotoxicity of the Agr-*g*-QPDMAEMA copolymers

Fresh blood collected from a healthy rabbit was immediately mixed with 3.8 wt% sodium citrate solution in a ratio of 9:1 (v/v). After that, the blood was diluted by 25-fold using phosphate buffered saline (PBS, pH = 7.4). The copolymers were dissolved in PBS at concentrations ranging from 0 to 2,500 μg/mL by 2-fold serial dilutions using PBS. The hemolysis assay was carried out in a 24-well plate. One mL of the diluted fresh blood was added into each well, followed by the addition of equal volume of the copolymers at different concentrations. Positive control was treated with 1 mL of 0.1% (v/v) Triton-X, while the negative control was treated with 1 mL of PBS. The plate was incubated at 37 ^{o}C for 1 h to allow interactions between the rabbit red blood cells (RBCs) and the copolymer to taken place,[19,29] before 1 mL of the mixture in each well was transferred into respective 1.5 mL PCR tube. The PCR tube was centrifuged at 1,000 rpm for 5 min, and 100 μL of the supernatant was transferred into a 96-well plate. Four replicates were measured for each copolymer. The optical density (OD), arising from the released hemoglobin, was measured at 576 nm on a microplate reader. The hemolysis (%) was determined with the following equation:

$$\text{Hemolysis} = (OD_{sample} - OD_{negative\ control})/(OD_{positive\ control} - OD_{negative\ control}).$$

Bacterial adhesion and biofilm formation on the Agr-*g*-QPDMAEMA copolymer hydrogel surfaces immersed in bacterial suspensions

Agr-*g*-QPDMAEMA2 copolymer (150 mg) and unmodified Agr (30 mg) was suspended in 10 mL of deionized water and stirred at 90 ^{o}C until the complete dissolution. The addition of unmodified Agr was utilized to enhance the mechanical strength and stability of the copolymer hydrogel. The hot solution (0.25 mL) was added into each well of the 24-well plates. The gelation of the solution was achieved by placing the plate in a 4 ^{o}C fridge overnight. *S. aureus*

and *E. coli* were cultured in tryptic soy broth (TSB) and nutrient broth (NB), respectively, by incubation overnight at 37 °C in the dark. The bacterial suspensions were centrifuged at 2,700 rpm for 10 min. After removal of the supernatant and washing twice with sterile PBS, *E. coli* and *S. aureus* cakes were dispersed in PBS containing 5% (v/v) of TSB and NB, respectively, to give rise to a final concentration of 1×10^5 cells/mL. One mL of the bacterial suspension was added into each well of the hydrogel-deposited 24-well plate, which was then incubated in a 37 °C incubator. After every 24 h, the culture medium in each well was removed, and 1 mL of freshly prepared PBS containing 5% (v/v) of TSB or NB was added into each well. After the predetermined incubation period, the culture medium in each well was discarded and the well was gently washed with PBS for two times to remove any non-adherent or loosely adhered bacteria on the surface. The well was then stained with 50 μL of the combination dye (LVE/DEAD BacLight Bacterial Viability Kit) for 30 min, washed with deionized water, and observed under a Nikon Eclipase Ti microscope equipped with excitation-emission filters of 470/525 and 540/605 nm for green and red fluorescence, respectively.

Bacterial colonization on the Agr-*g*-QPDMAEMA copolymer hydrogel surfaces exposed to bacterial aerosol

Agr-*g*-QPDMAEMA2 copolymer (150 mg) and unmodified Agr (30 mg) was suspended in 10 mL of TSB or NB and autoclaved at 100 ^{o}C for 10 min. When the medium was cool enough to handle, it was poured into the sterile Petri dishes, which was placed at room temperature for 1 h to allow the plates to set. Harvested bacterial cells (*S. aureus* and *E. coli*) were suspended in PBS at a concentration of 1×10^5 cells/mL. The prepared bacterial suspension in PBS was sprayed onto the Agr-*g*-QPDMAEMA copolymer hydrogel surfaces using a commercial parfum atomizer. The Petri dishes were then inverted and incubated at 37 ^{o}C for 18 h to form visible bacterial

colonies. Controlled experiments were carried out using unmodified Agr as the gel precursor.

Prevention of bacterial infection by the Agr-*g*-QPDMAEMA copolymer hydrogel patches

Agr-*g*-QPDMAEMA2 copolymer (150 mg) and unmodified Agr (30 mg) was suspended in 10 mL of TSB or NB and autoclaved at 100 ^{o}C for 10 min. The hydrogel patches were formed by casting the copolymer solution onto the hydrophilic silicon wafer. The hydrogel patches were allowed to stand at room temperature for 1 h, and were subsequently cut into circle shapes with a knife. The resulting hydrogel patches were gently peeled off from the silicon wafer using a ruler.

The contaminated Titanium (Ti) surfaces by *S. aureus* and *E. coli* were built by immersing the pristine Ti substrates in bacterial suspensions (1×10^7 cells/mL for both cells) in PBS for 4 h. The Ti substrates were washed with PBS three times to remove any non-adhered or loosely adhered bacteria. After that, the contaminated Ti substrates were gently placed on the top of the as-formed Agr-*g*-QPDMAEMA2 copolymer hydrogel patches in the sterile Petri dishes. The Petri dishes were then incubated at 37 ^{o}C for 24 h. The Ti substrates after incubation were taken out from the hydrogel patch surfaces, placed in the 24-well plate, and washed with PBS three times. The numbers of viable adherent cells on the Ti surfaces were quantified using the spread plate method, involving ultrasonic cell removal, serial dilution and plating for viable cell counts.[21] Bacterial suspension of each dilution was spread onto plate in triplicate. Controlled experiments were carried out using unmodified Agr hydrogel patches with TSB or NB nutrients.

Characterization

The chemical structures of Agr copolymers were characterized by ^{1}H NMR spectroscopy on a

Varian 600 MHz spectrometer. X-ray photoelectron spectroscopy (XPS) measurements were carried out on a Kratos AXIS Ultra HSA spectrometer equipped with a monochromatized AlKα X-ray source (1468.71 eV photons). FT-IR spectroscopy analysis was carried out on a Bio-Rad FTS-135 spectrophotometer.

Results and Discussion

To prepare the graft copolymers via atom transfer radical polymerization (ATRP) from the agarose (Agr), it is essential to introduce the alkyl bromide initiator onto the Agr. In this work, the bromoisobutyryl-functionalized Agr (Agr-Br) macroinitiator was prepared via the reaction of hydroxyl groups of Agr with 2-bromoisobutyrl bromide (BiBB) (Scheme 1).[26,30] It has been reported that the hydroxyl groups at the *C*-6 positions of polysaccharides are apparently favorable for the substitution reactions.[31] When Agr reacted directly with BiBB, the alkyl bromide initiator was probably introduced at the *C*-6 positions of Agr. The presence of alkyl bromide moieties in the structure of Agr-Br is evidenced by the appearance of methyl protons adjacent to the bromide atom at 1.92 ppm in the ^{1}H NMR spectrum of Agr-Br (Figures 1a and 1b). The degree of alkyl bromide substitution was estimated from ^{1}H NMR spectrum of Agr-Br to be about 37.4%. The chemical structure of Agr-Br was also characterized by X-ray photoelectron spectroscopy (XPS). In comparison to that of Agr (Figure 2a), the XPS C 1s core-level spectrum of Agr-Br (Figure 2c) can be curve-fitted into four peak components with binding energies (BEs) at about 284.6, 286.2, 287.6 and 288.6 eV, attributable to the C-H/C-C, C-O/C-Br, O-C-O and O=C-O species, respectively. The presence of O=C-O peak component is associated with the formation of ester bonds from esterification between BiBB and Agr. The XPS Br 3d core-level spectrum of Agr-Br (Figure 2d) at the BE of approximately 70.5 eV, attributable to the covalently C-Br species,[32] indicates that the bromoisobutyrate has been successfully coupled to the hydroxyl groups of the Agr. The coupling of BiBB to Agr is further confirmed by the presence of C=O stretch in the ester group at about 1730 cm^{-1} in the FT-IR spectrum (Figure S1, Electronic Supplementary Information, ESI). Thus, the alkyl bromide initiators for ATRP have been successfully introduced on the Agr.

ATRP of 2-(dimethylamino)ethyl methacrylate (DMAEMA) from Agr-Br mactoinitiator was conducted in *N*,*N*-dimethylforamide (DMF) using CuBr/2,2'-bipyridine (bpy) as the catalyst system. The resulting poly(DMAEMA)-grafted Agr (Agr-*g*-PDMAEMA) copolymers with different PDMAEMA grafting contents were achieved by varying the macroinitiator to monomer ratios. Table 1 summarizes the characteristics of the Agr-*g*-PDMAEMA1 and Agr-*g*-PDMAEMA2 copolymers. With the increase in macroinitiator to monomer ratio from 1:17.4 to 1:26.1, the PDMAEMA graft content in the Agr-*g*-PDMAEMA copolymers increase from 64.9% to 68.9%. ^{1}H NMR spectra of the Agr-*g*-PDMAEMA1 and Agr-*g*-PDMAEMA2 copolymers (Figures 1c and 1d) show new chemical shifts at about 3.99, 2.20, 1.76 and 0.83 ppm, which are characteristic proton signals of methylene protons adjacent to ester group, methyl protons adjacent to tertiary amine, methylene protons in the main backbone, and methyl protons adjacent to the vinyl bond in DMAEMA repeat units, respectively. The XPS C 1s core-level spectrum of the Agr-*g*-PDMAEMA2 copolymer (Figure 2e) can be curve-fitted into five peak components with BE s of about 284.6, 295.5, 286.1, 287.6 and 288.5 eV, attributable to the C-H/C-C, C-N, C-O, O-C-O and O=C-O species, respectively. The presence of C-N species and the decrease in C-O and O-C-O species are consistent with the grafting of PDMAEMA side chains.

In the final step of synthesis, the tertiary amine moieties in the grafted PDMAEMA chains were converted to polymeric quaternary ammonium salts (QAS) through quaternization with 1-bromobutane.[33,34] The ammonium ions can be easily verified by XPS N 1s core-level spectrum, as the positively-charged nitrogen is expected at a higher N 1s BE.[35,36] In comparison to the XPS N 1s core-level spectrum of Agr-*g*-PDMAEMA2 copolymer (Figure 2f), the XPS N 1s core-level spectrum of Agr-*g*-QPDMAEMA2 copolymer (Figure 2h) can be curve-fitted into two peak

components with BEs at about 399.1 and 402.0 eV,[37] attributable to tertiary amine and positively-charged nitrogen, respectively. The degrees of quaternization in the grafted PDMAEMA chains are determined from the ratios of postively-charged nitrogen peak component and total nitrogen spectral area in the XPS N 1s core-level spectra of Agr-*g*-QPDMAEMA1 and Agr-*g*-QPDMAEMA2 copolymers to be about 71% and 76%, respectively.

Having successfully characterized the chemical structures and compositions of the Agr copolymers, the antimicrobial activities against Gram-negative (*Staphylococcus aureus* (*S. aureus*)) and Gram-positive (*Escherichia coli* (*E. coli*)) bacteria of the Agr-*g*-QPDMAEMA copolymers were investigated using the broth mirodilution method.[28] The Agr-*g*-PDMAEMA copolymers were not studied becuase they are not soluble in aqueous media. The minimum inhibitory concentration (MIC) values for the Agr-*g*-QPDMAEMA1 and Agr-*g*-QPDMAEMA2 copolymers are presented in Table 1. The respective MIC values of the Agr-*g*-QPDMAEMA1 and Agr-*g*-QPDMAEMA2 copolymers are 64 and 64 μg/mL against *E. coli*, and 128 and 64 μg/mL against *S. aureus*. The results suggest that these copolymers are bacteriostatic (or growth inhibiting) against both *E. coli* and *S. aureus*, and the MIC values of these copolymers against *E. coli* and *S. aureus* seem to approach a comparable level with other quaternized PDMAEMA copolymers.[33,38] To further ascertain the bactericidal activities of these copolymers, their time-dependent killing efficiencies were assayed by co-culturing them at 1 × MIC and 2 × MIC concentrations with *E. coli* and *S. aureus*. As shown in Figures 3a and 3b, significant loss of viable *E. coli* and *S. aureus* are observed. The killing efficiencies of the Agr-*g*-QPDMAEMA1 and Agr-*g*-QPDMAEMA2 copolymers reach more than 98.5% for both bacteria at 1 × MIC and 2 × MIC concentrations, after 5 h of contact. However, the Agr-*g*-QPDMAEMA copolymers

exhibit a high antibacterial efficiency against *E. coli*, reaching more than 99.4% at both concentrations, even only after 3 h of contact. It is generally recognized that the double cellular membrane of *E. coli* may complicate the polymer permeation and the inner membrane disruption, resulting in a slower cell death than that of *S. aureus*.[33,39] Interestingly, the higher antibacterial capability of cationic polymers against *E. coli* than against *S. aureus* has also been reported.[40] This phenomenon presumably is due to the fact that *S. aureus* has a very thick (20-80 nm) and highly crosslinked peptidoglycan layer (polysaccharide with amino acid side chains) in the outer cell wall,[41] which may interact with Agr polysaccharide via hydrogen bonding to retard the permeation of the Agr-based copolymers as well as the disruption of cellular membrane. The peptidoglycan layer between the outer and inner membrane of *E. coli* has a thickness of 6-8 nm,[40] and may not be sufficient to hinder the polymer permeation.

As a measure of biocompatibility, the hemolysis of rabbit red blood cells (RBCs) induced by the copolymers were investigated. Hemolytic activity is generally defined by the HC_{50}, which represents the concentration required to lyse 50% of the RBCs. Due to the limited solubility of these copolymers in phosphate buffered saline (PBS, pH = 7.4), the maximum concentration used was 2,500 μg/mL for the Agr-*g*-QPDMAEMA1 and Agr-*g*-QPDMAEMA2 copolymers. The hemolysis assay is shown in Figure 4 and the HC_{50} values for these copolymers are present in Table 1. As shown in the hemolysis assay, the maximum hemolysis is still under 20% at all concentrations tested for both copolymers. This results indicate that these copolymers at the concentration up to 2,500 μg/mL have negligible toxicity for rabbit erythrocyte. The selectivity, defined as HC_{50}/MIC, can be used to describe the cell specificity towards bacteria versus RBCs. The selectivity values of the Agr-*g*-QPDMAEMA1 and Agr-*g*-QPDMAEMA2 copolymers are

larger than 39 towards *E. coli*, and larger than 19 and 39, respectively, towards *S. aureus*. These values are comparable to other cationic polymer systems,[42-44] indicating that these copolymers are potentially suitable for healthcare applications.

Agr can form a thermoresponsive hydrogel through physical crosslinking.[22] The intriguing gelation property of Agr can be used for solidifying the Agr-*g*-QPDMAEMA copolymers in aqueous media, resulting in an antibacterial hydrogel. Antimicrobial hydrogels are extremely attractive biomaterials for use as wound dressings and fillers.[45] The antibacteral Agr-*g*-QPDMAEMA copolymer hydrogels were prepared by dissolving the copolymers in tryptic soy broth (TSB) and nutrient broth (NB), followed by pouring the hot mixture into Petri dishes to stand. Bacterial colonization on the surfaces of antibacterial hydrogels after exposure to bacterial aerosol was first examined to simulate bacterial contamination from air and droplets. Bacterial suspensions (containing 1×10^5 cells/mL of *E. coli* or *S. aureus*) in PBS were sprayed onto the surfaces of unmodified Agr and Agr-*g*-QPDMAEMA2 copolymer hydrogels (Figure 5a). The contaminated hydrogel surfaces in Petri dishes were then incubated at 37 ^{o}C for 18 h. Optical images of Petri dishes after the incubation period are shown in Figure 5b-5e. Numerous colonies of *E. coli* and *S. aureus* can be observed by naked eye on the unmodified Agr hydrogel surfaces in Figures 5b and 5d, respectively. On the other hand, the respective colonies of *E. coli* and *S. aureus* on the Agr-*g*-QPDMAEMA2 copolymer hydrogel surfaces in Figures 5c and 5e are barely discernible to the naked eye. These results indicate that the Agr-*g*-QPDMAEMA2 copolymer hydrogels exhibit bactericidal activity against aerosolized bacteria.

To investigate the effectiveness of the Agr-*g*-QPDMAEMA copolymer hydrogel surfaces against bacterial adhesion and biofilm formation, the Agr-*g*-QPDMAEMA2 copolymer was dissloved in PBS and the hot solution was added into the wells of 24-well plates. After gelation, bacterial suspension containing 1×10^5 cells/mL of *E. coli* (or *S. aureus*) in NB (or TSB) and PBS mixed solution (5/95, v/v) was added into each well of the 24-well plates. Controlled experments were carried out using blank well of the 24-well plates. Regrescentative merged fluorescence microscopy images of the blank and hydrogel-deposited wells after incubation in *E. coli* (or *S. aureus*) suspension for 1 day are shown, respectively, in Figures 6a and 6d (or Figures 6g and 6j). There are many single live *E. coli* and *S. aureus* cells (stained green) adherent to the surface of blank wells, while only a few single live *E. coli* and *S. aureus* cells adhere to the hydrogel surfaces. Many researches have reported that the dead cells can accumulate on the cationic bactericidal surfaces, causing loss of antibacterial activity.[14,21,46] However, the accumulation of dead bacteria (stained red) on the Agr-*g*-QPDMAEMA2 copolymer hydrogel surfaces is rare. This phenomenon may be due to the fact that highly hydrophilic Agr-based coatings can form a hydration layer at the water-hydrogel interface,[24] serving as a physical and energy barrier to discourage the accumulation of dead bacteria on the surface.[21,47] After incubation for 4 days in the NB (or TSB) and PBS mixed solutions, more uniformly distributed bacteria with small numbers of cell clusters are evident on the surfaces of blank wells (Figures 6b and 6h). Much more bacteria clusters and thicker films are present on the surfaces of blank wells (Figure 6c and 6i) after incubation in the NB (or TSB) and PBS mixed solutions for 7 days. In contrast, the wells deposited with the Agr-*g*-QPDMAEMA2 copolymer hydrogels still have few cells and are free of bacteria clusters on Day 4 (Figures 6e and 6k), and even up to Day 7 (Figures 6f and 6l). The results suggest that the Agr-*g*-QPDMAEMA2 copolymer hydrogel

exhibits good performance in inhibiting bacterial adhesion and biofilm formation in aqueous media. The copolymer hydrogel also exhibits durable antibacterial activity.

The above results have shown that the copolymer hydrogels can inhibit bacterial contamination from air and aerosol, as well as bacterial adhesion and biofilm formation in aqueous media. The inhibition of bacteria growth on the contaminated substrate surfaces by the copolymer hydrogel patches was also studied. Titanium (Ti) and its alloys have been widely used for biomedical implants due to their excellent mechanical properties, corrosion resistance and biocompatibility.[48] However, the pristine Ti substrate surfaces are susceptible to bacterial infection which may lead to failure of implants.[49] Thus, contaminated Ti substrates were chosen as the model surfaces to investigate the inhibiting efficiency of the copolymer hydrogel patches. Ti substrates were first immersed into the bacterial suspensions (containing 1×10^7 cells/mL of *E. coli* or *S. aureus*) in PBS for 4 h. The Ti substrates were washed with PBS for three times to remove any non-adhered or loosely adhered bacteria. After that, the contaminated Ti substrates were gently placed on the top of the as-formed unmodified Agr and Agr-*g*-QPDMAEMA2 copolymer hydrogel patches. Quantitative determination of the viable cells on the respective Ti substrates was conducted using the spread plate method and the results are shown in Figure 7. The numbers of viable *E. coli* and *S. aureus* cells on the Ti substrates after incubation with bacterial suspensions for 4 h are 5.5×10^5 and 4.8×10^5 cells/cm^2, respectively. After contact with the unmodified Agr hydrogel patches with nutrients at 37 ^{o}C for 24 h, the contaminated Ti substrates reach cell densities of 1.2×10^7 and 4.4×10^6 cells/cm^2, respectively, for *E. coli* and *S. aureus*. However, the numbers of viable *E. coli* and *S. aureus* cells on the contaminated Ti substrates after contact with the copolymer hydrogel patches with nutrients are only 5.4×10^3

and 2.3×10^4 cells/cm^2, which correspond to 99% and 95% decrease from that of the original contaminated Ti substrates. The results indicate that the Agr-*g*-QPDMAEMA2 copolymer hydrogel patches can effectively inhibit the bacteria growth on the contaminated Ti surfaces.

Conclusions

The quaternized poly(2-(dimethylamino)ethyl methacrylate)-grafted agarose (Agr-*g*-QPDMAEMA) copolymers were successfully synthesized via a combination of atom transfer radical polymerization (ATRP) and quaternization of tertiary amine moieties. The Agr-*g*-QPDMAEMA copolymers exhibit comparable minimum inhibitory concentration (MIC) values for *Staphylococcus aureus* (*S. aureus*) and *Escherichia coli* (*E. coli*) and hemotoxicity to rabbit red blood cells (RBCs). The Agr-*g*-QPDMAEMA copolymers can form hydrogels at a high concentration. The hydrogel surfaces can effectively inhibit the bacterial adhesion and biofilm formation in bacterial suspensions. The hydrogel surfaces can also prevent the bacterial colonization upon contact with the aerosolized bacteria. After fabrication the copolymer hydrogels into antibacterial patches, the bacteria growth on the contaminated substrate surfaces can be significantly reduced. This study provides a general approach for the preparation of Agr-based antibacterial agents with multipurpose biomedical applications. It can be readily extended to the modification of Agr with other quaternary ammonium salts (QAS). This study also broadens the antibacterial applications of polymeric QAS from solutions to substrate surfaces, without the usage of reactive chemistry or complicated treatment.

Electronic Supplementary Information

Electronic supplementary information (ESI) available: Figure S1, FT-IR spectra of the Agr, Agr-Br, Agr-*g*-PDMAEMA2 and Agr-*g*-QPDMAEMA2 copolymers. See DOI: **.****/**********

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Legends for Schemes and Figures

Scheme 1. Synthesis routes of the Agr-Br macroinitiator, Agr-*g*-PDMAEMA and quaternized Agr-*g*-QPDMAEMA copolymers.

Figure 1. ^{1}H NMR spectra of the (a) Agr (b) Agr-Br (c) Agr-*g*-PDMAEMA1 and (d) Agr-*g*-PDMAEMA2 copolymers in DMSO-d_6 at 50 ^{o}C.

Figure 2. XPS C 1s core-level spectra of the (a) Agr (c) Agr-Br (e) Agr-*g*-PDMAEMA2 and (g) Agr-*g*-QPDMAEMA2 copolymers, Br 3d core-level spectra of the (b) Agr and (d) Agr-Br, and N 1s core-level spectra of the (f) Agr-*g*-PDMAEMA2 and (h) Agr-*g*-QPDMAEMA2 copolymers.

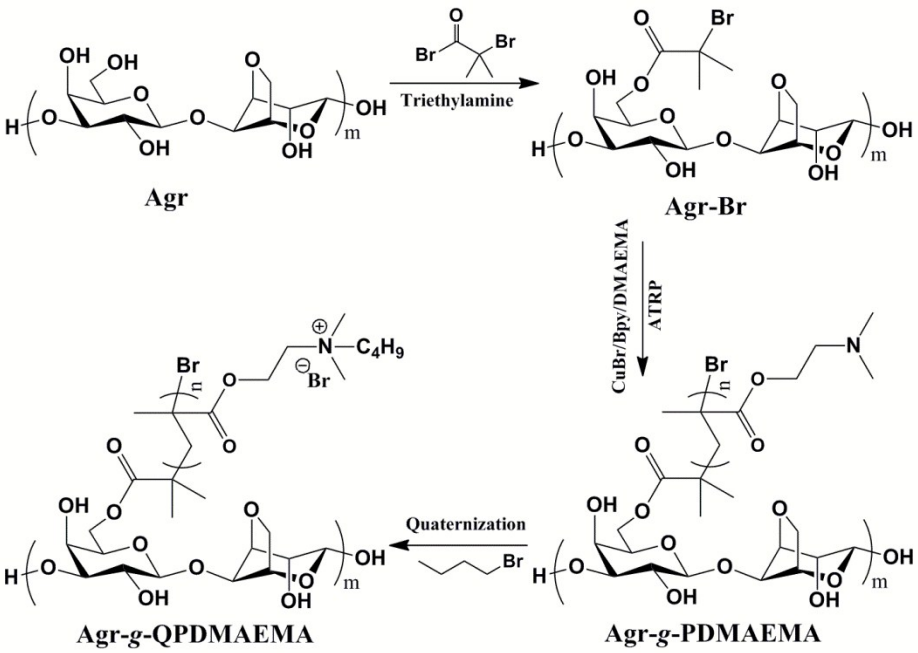
Figure 3. Time-dependent killing efficiencies of the Agr-*g*-QPDMAEMA1 and Agr-*g*-QPDMAEMA2 copolymers at concentrations of 1 × and 2 × MIC against (a) *E. coli* and (b) *S. aureus*.

Figure 4. Relative hemolytic activity of the Agr-*g*-QPDMAEMA1 and Agr-*g*-QPDMAEMA2 copolymers after 1 h of cultuing with rabbit RBCs.

Figure 5. (a) Schematic diagram illustrating the assay for the inhibition of aerosolized bacterial colonization on the hydrogel surface; optical images of the (b,d) unmodified Agr and (c,e) Agr-*g*-QPDMAEMA2 copolymer hydrogel surfaces after exposure to bacterial aerosol of *E. coli* and *S. aureus*, respectively.

Figure 6. Bacterial adhesion and biofilm formation on the blank and copolymer hydrogel-deposited wells of the 24-well plates after exposure to bacterial suspensions of *E. coli* and *S. aureus* for 1, 4 and 7 days. (Scale bar = 40 μm)

Figure 7. The numbers of viable cells on the contaminated Ti substrate surfaces before and after contact with unmodified Agr and Agr-*g*-QPDMAEMA2 copolymer hydrogel patches. Inset shows the schematic diagram for the preparation of the hydrogel patch.



Scheme 1

Table 1: Characteristics of the Agr copolymers

	Degree of alkyl bromide substitution[a]	Polymerization condition[b]	[Agr]: [PDMAEMA] weight ratios[c]	Degree of quaternization in the grafted PDMAEMA[d]	HC_{50}[e]	MIC[f] (Selectivity[g])	
						E. coli	*S. aureus*
Agr-Br	37.4%						
Agr-*g*-PDMAEMA1		1:17.4:0.7:1.4	35.1:64.9				
Agr-*g*-PDMAEMA2		1:26.1:0.7:1.4	31.1:68.9				
Agr-*g*-QPDMAEMA1				71.4%	>2,500	64 (>39)	128 (>19)
Agr-*g*-QPDMAEMA2				75.8%	>2,500	64 (>39)	64 (>39)

[a.] determined by ^{1}H NMR spectrum of Agr-Br: using the intergrated area ratios of methyl protons adjacent to the bromide atom and *C*1'-H and *C*2-OH in Agr

[b.] the molar ratio of macroinitiator:DMAEMA:CuBr:bpy

[c.] determined by ^{1}H NMR spectra of Agr-*g*-PDMAEMA1 and Agr-*g*-PDMAEMA2 copolymers: using the intergrated area ratios of methyl protons adjacent to the vinyl bond in DMAEMA and *C*1'-H and *C*2-OH in Agr

[d.] determined from the corresponding postively-charged nitrogen peak component and total nitrogen spectral area ratios in the XPS N 1s core-level spectra of Agr-*g*-PDMAEMA1 and Agr-*g*-PDMAEMA2 copolymers

[e.] the capability of these copolymers to produce 50% of hemolysis of rabbit RBCs at a given concentration (μg/mL)

[f.] the lowest concentration (μg/mL) of these copolymers that inhibited bacteria growth

[g.] the selecity of these copolymers against bacteria versus rabbit RBCs (HC_{50}/MIC)

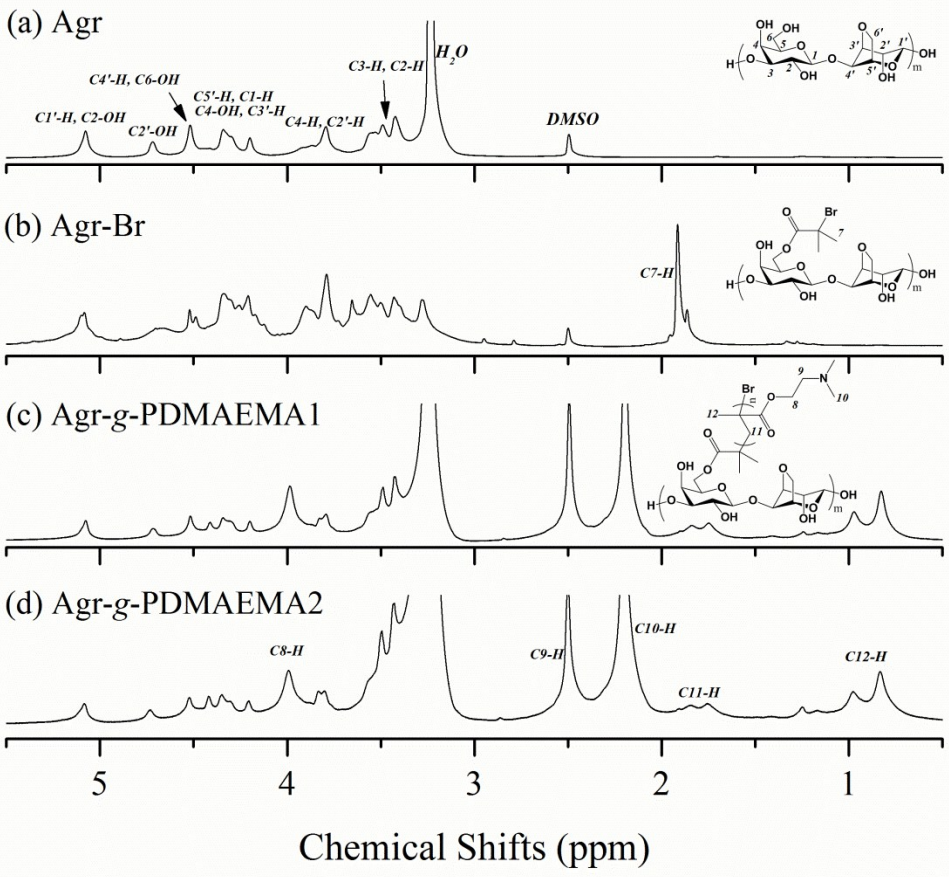


Figure 1

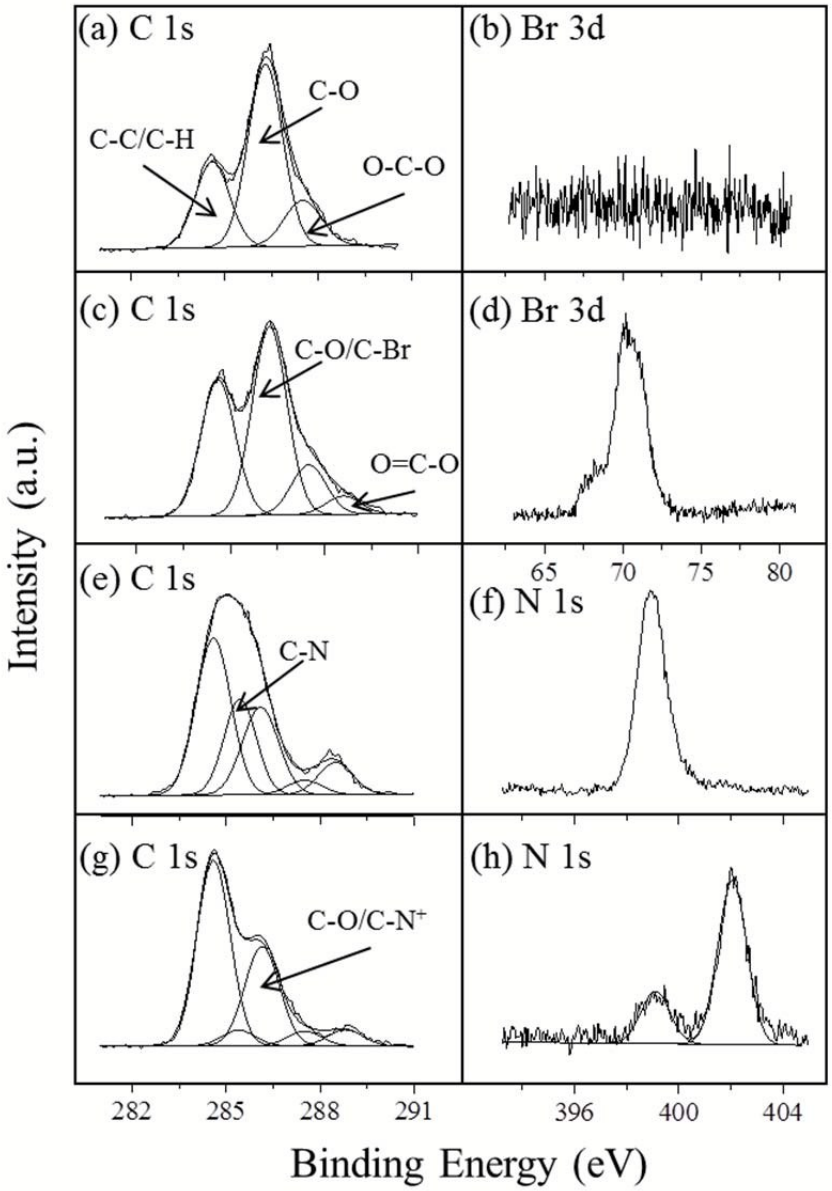


Figure 2

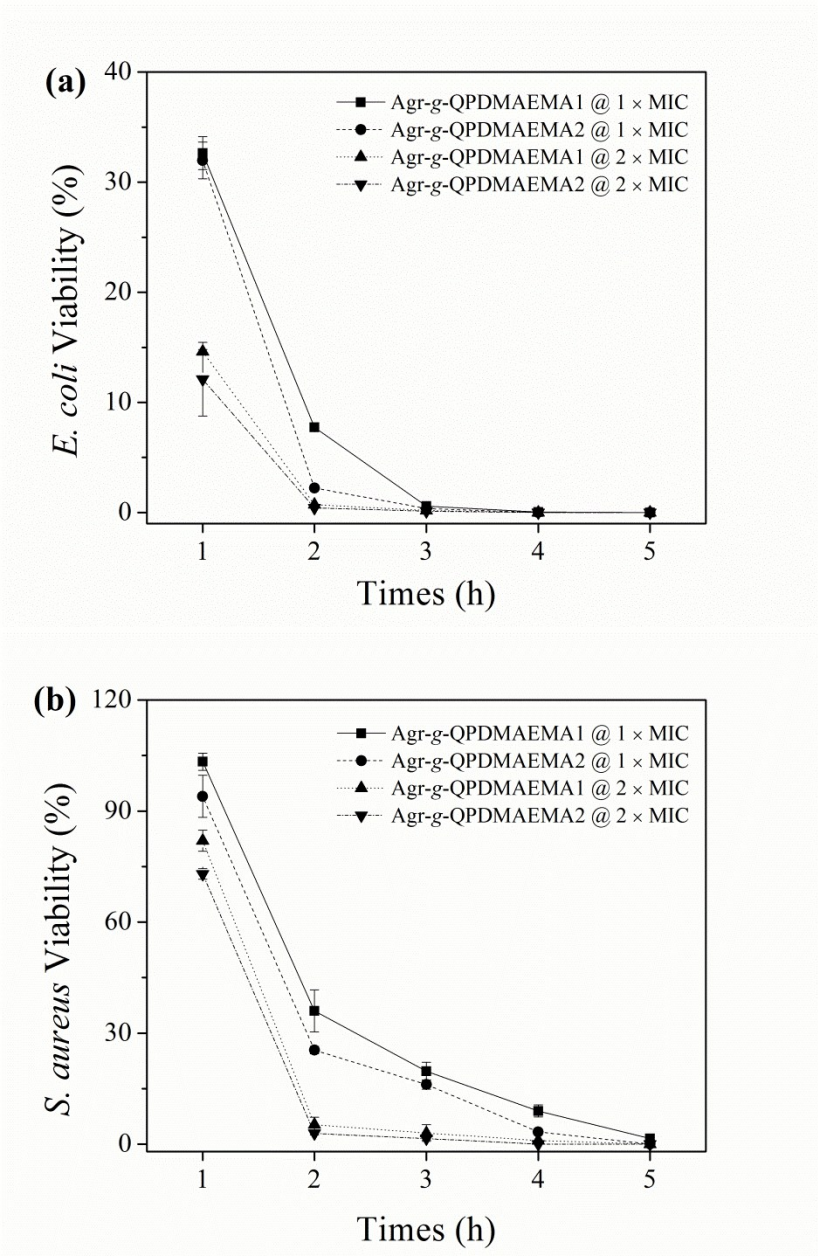


Figure 3

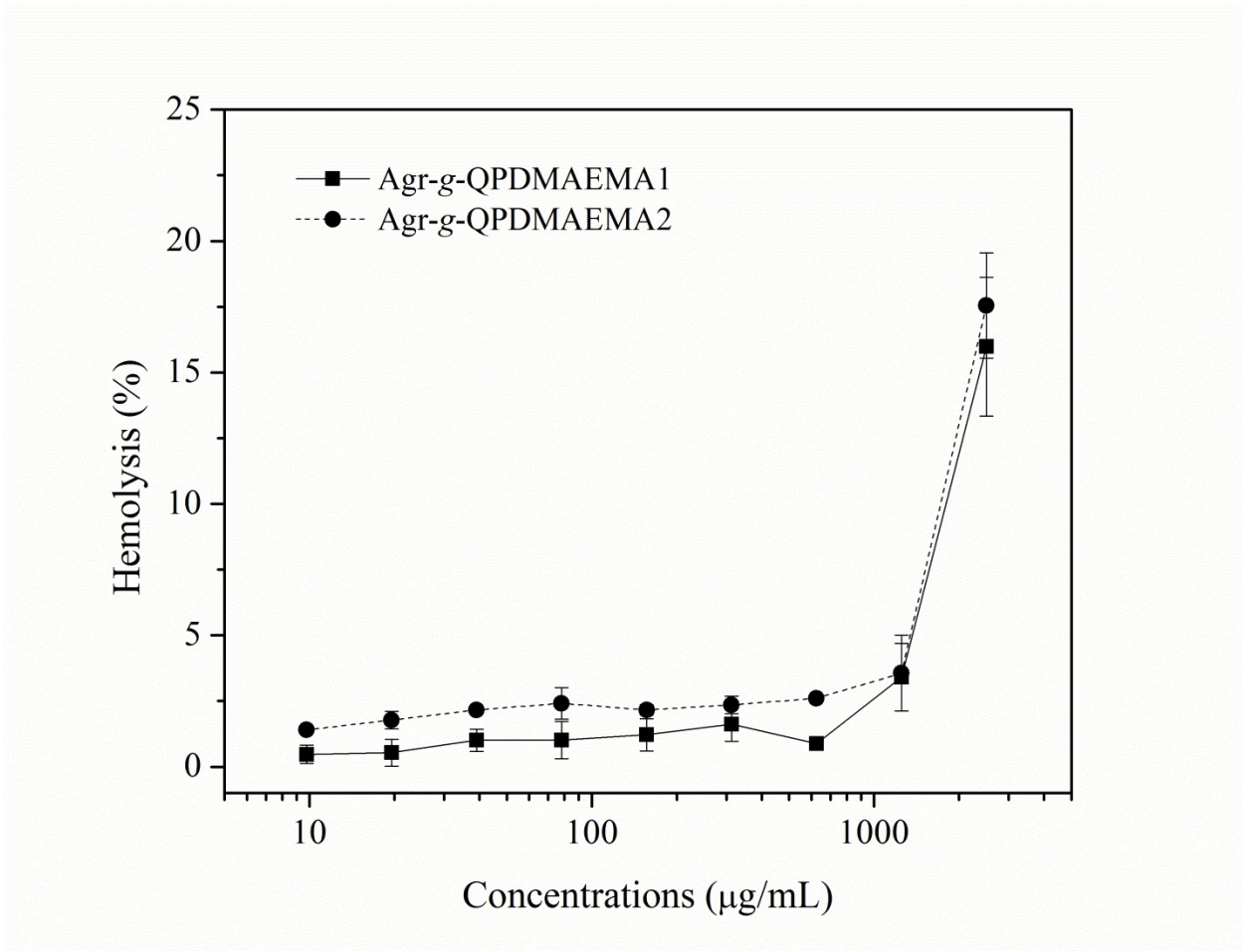


Figure 4

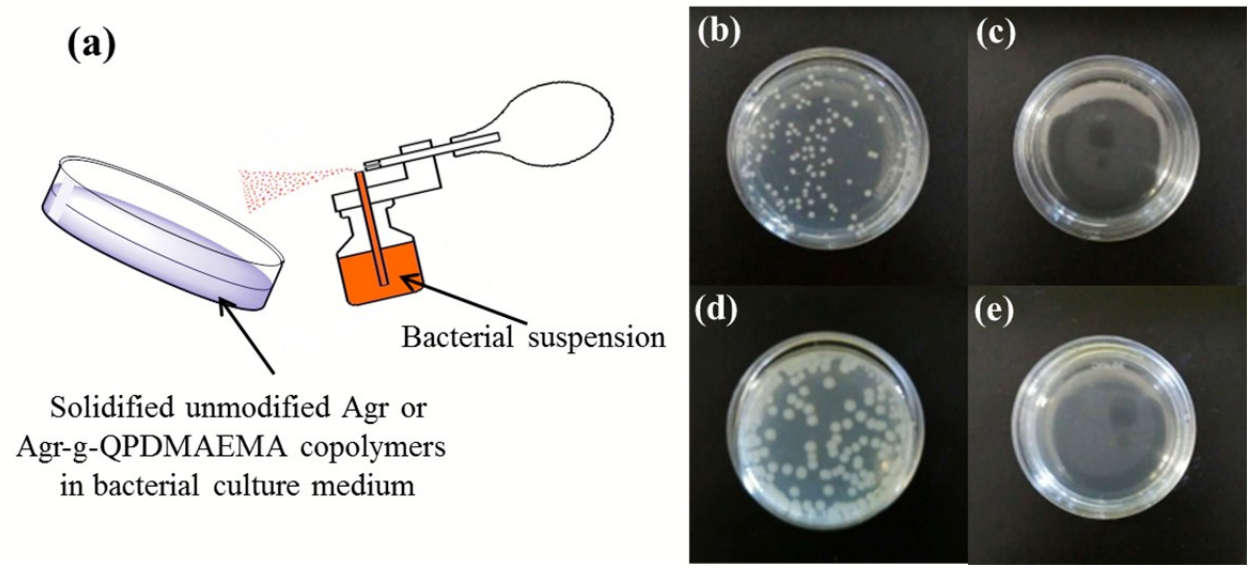


Figure 5

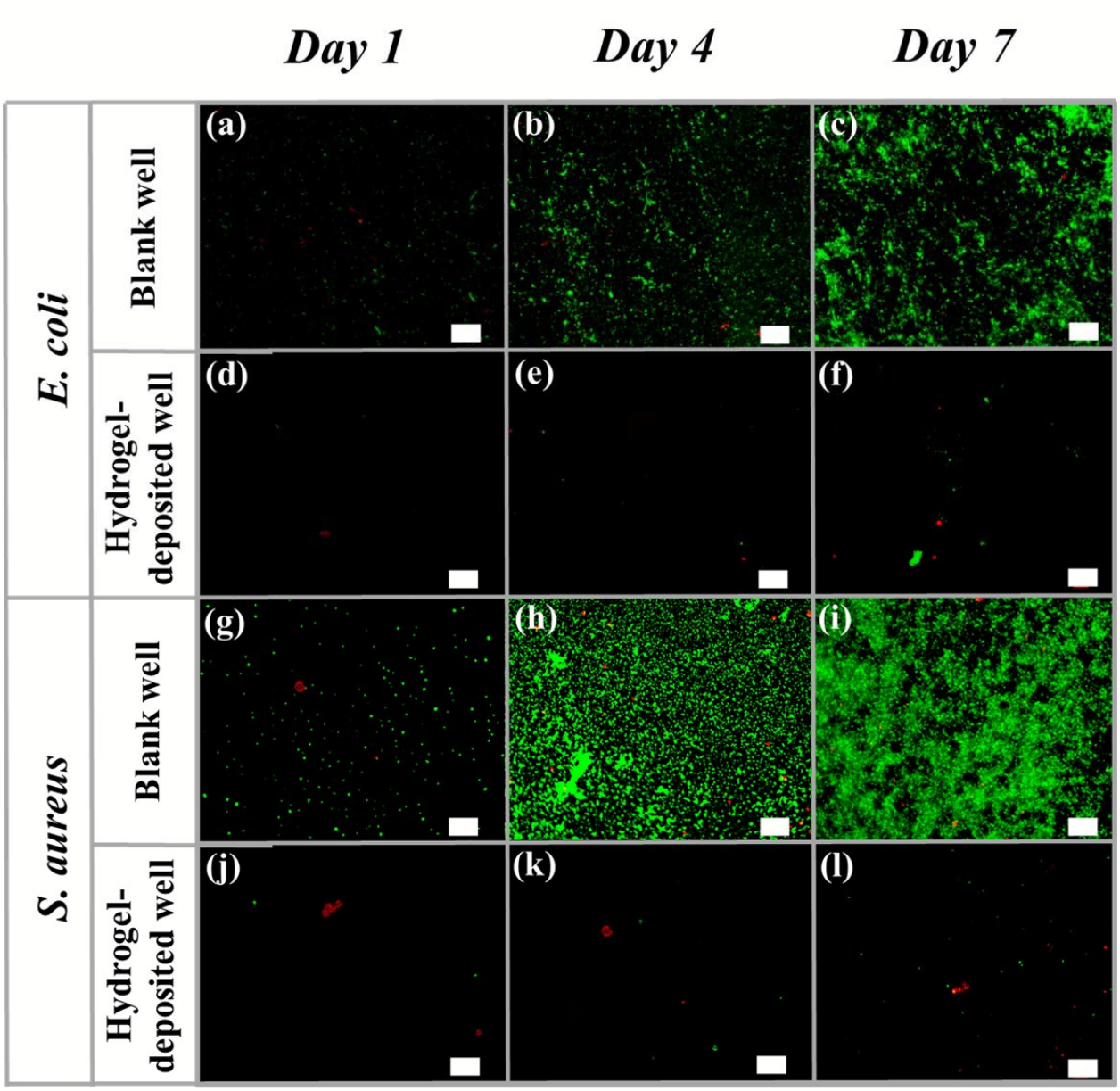


Figure 6

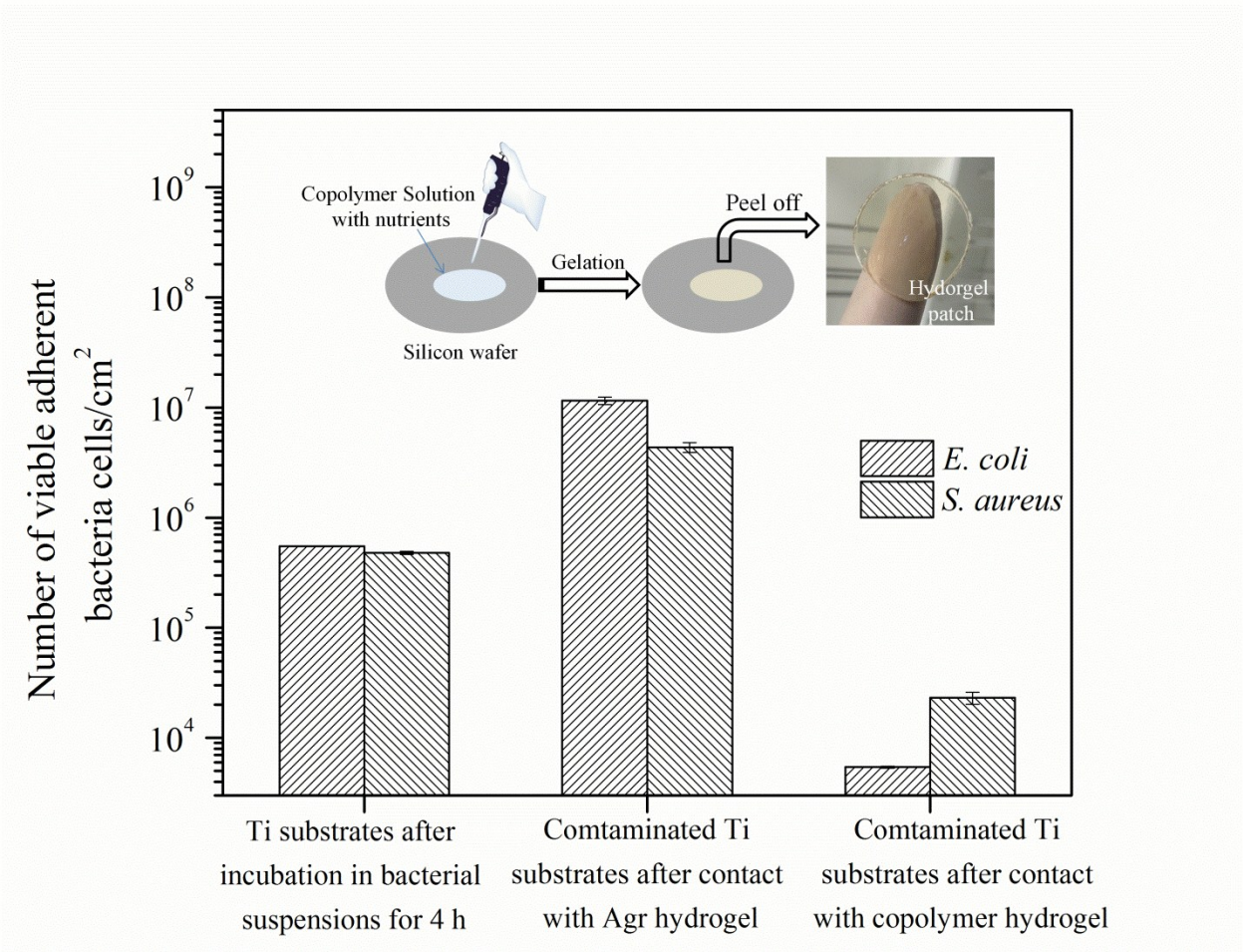
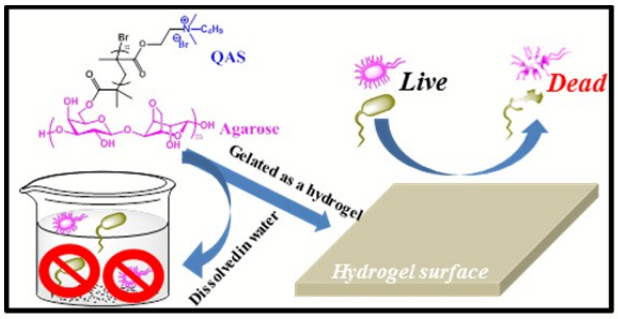


Figure 7

TOC

Title: Quaternized poly(2-(dimethylamino)ethyl methacrylate)-grafted agarose copolymers for multipurpose antibacterial applications

Authors: L. Q. Xu*, N. N. Li, J. C. Chen, G. D. Fu, E. T. Kang



Polymeric quaternary ammonium salts-functionalized agarose not only exhibit good antibacterial activity in solution form, but also can be solidified to construct antibacteiral surfaces.