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Graphical Abstract

Fully Biodegradable Antibacterial Hydrogels via Thiol-ene "Click" Chemistry†

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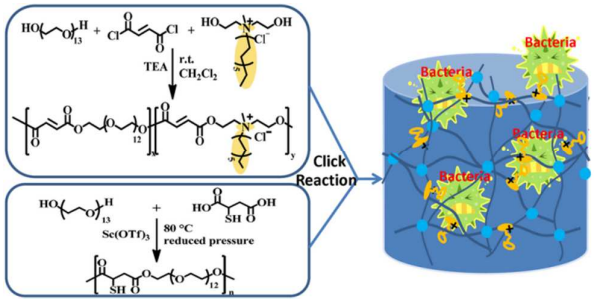
Novel biodegradable antimicrobial hydrogels were prepared facilely via thiol-ene "click" reaction under human physiological conditions using multifunctional poly(ethylene glycol) (PEG) derivatives as precursors, which is promising to be used as biomaterials.



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Fully Biodegradable Antibacterial Hydrogels via Thiol-ene "Click" Chemistry†

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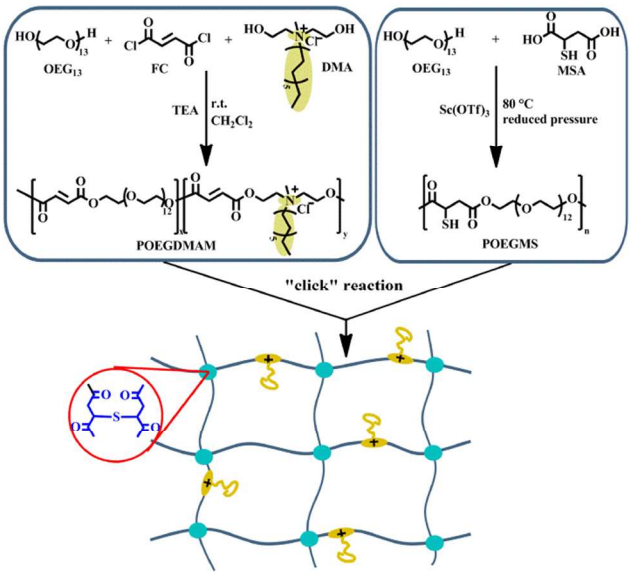
In this work, fully biodegradable antimicrobial hydrogels were prepared facilely via thiol-ene "click" reaction under human physiological conditions using multifunctional poly(ethylene glycol) (PEG) derivatives as precursors. Water soluble and degradable PEG derivatives with multi-enes and multi-thiols were synthesized by polycondensation of oligo(ethylene glycol) (OEG) with "clickable" monomers, respectively. Ammonium groups with long alkyl chain were incorporated into one of the precursors covalently, using dodecyl bis(2-hydroxyethyl) methylammonium chloride as comonomer. Proton nuclear magnetic resonance (^{1}H-NMR), gel permeation chromatography (GPC) and Fourier transform infrared spectroscopy (FT-IR) were used to characterize the precursors and hydrogels. This kind of cationic PEG-type hydrogels showed strong antibacterial abilities against both gram-negative and gram-positive bacteria due to the ammonium moieties. Moreover, the hydrogel with fewer ammonium moieties still possessed significant antibacterial ability, but low toxicity, which has potentials as medical materials.

Introduction

Bacterial infection is a serious problem in many areas, especially biomaterials. According to World Health Organization (WHO) statistics, at any time, over 1.4 million people worldwide suffer from infectious complications acquired in hospital, which have much to do with the use of medical devices.[1-5] Hydrogels are three-dimensional polymer networks that are able to retain a large fraction of aqueous solvent within their structure. Due to their high water contents and soft consistency, which is similar to natural tissue, hydrogels resemble natural living tissue more than any other class of synthetic biomaterials.[6-8] Therefore, hydrogels have received extraordinary attention as biomaterials used for biomedical applications,[9] such as tissue engineering,[10-12] wound dressings materials,[13-15] immunoisolation[16] and drug delivery.[17-19] Thus, fabricating hydrogels with antibacterial property is meaningful for the biomedical field.

In the past few decades, hydrogels based on poly (ethylene glycol) (PEG) have been widely studied and used as biomaterials,[20-26] due to the unique properties of PEG, such as good biocompatibility, non-immunogenicity, and resistance to protein adsorption.[27] On the other hand, it has been reported that polymers with pendant quaternary ammonium salts (QAS) groups attached by covalent bonds exhibit strong antibacterial activity.[28-30] So it is promising to incorporate ammonium groups into the backbone of PEG hydrogel to prepare PEG hydrogels with good stability and antibacterial property. In order to fabricate such kind of antibacterial PEG hydrogel, it is necessary to design and synthesize PEG derivatives with multiple QAS and cross-linkable groups as precursors. Recently, our research group has developed a low-cost and large-scale synthesis of biodegradable PEG derivatives containing multiple functional groups by polycondensation of diol oligo(ethylene glycol) (OEG) with functional diacids under mild conditions in the presence of suitable catalyst.[31] PEG derivatives with multi-enes or multi-thiols were thus synthesized, which were used as precursors to prepare biodegradable PEG-type hydrogels via thiol-ene "click" reaction by simply mixing the PBS solution of them under human physiological environment.[32]

In this study, based on the concept of our previous work, we designed and synthesized a novel PEG derivative containing multiple QAS and "clickable" double bonds (POEGDMAM) by the direct polycondensation of OEG, fumaryl chloride (FC) and dodecyl bis(2-hydroxyethyl) methylammonium chloride (DMA) in the presence of triethylamine. Herein, inexpensive and commercial available DMA was used as comonomer, which could realize "one pot" synthesis of "clickable" PEG derivative with QAS directly without further quaternization. Additionally, another corresponding "clickable" PEG derivative containing multiple thiol groups (POEGMS) was synthesized by the polycondensation of OEG and mercaptosuccinic acid (MSA) according to our previous report.[32] Finally, the cationic hydrogels could be obtained through thiol-ene "click" reaction between POEGDMAM and POEGMS in aqueous media under human physiological conditions as shown in Scheme 1. The gelation time, swelling behaviour and degradation behaviour of the hydrogels were investigated in detail. This kind of cationic hydrogels exhibits strong antibacterial ability, while low toxicity, which is promising to implement their biomedical applications.



Scheme 1 Synthesis of cationic hydrogel

Experimental

Materials

Diol oligo(ethylene glycol) (ethylene glycol) (OEG13, Mn = 600, Sinopharm Chemical Reagent Co., China) was dried by azeotropic distillation with toluene to remove water. Triethylamine (TEA) and dichloromethane was dried over CaH_2 and distilled before use. Fumaryl chloride (TCI, Japan), mercaptosuccinic acid (MSA, Aladdin, China) and dodecyl bis(2-hydroxyethyl) methylammonium chloride (Xiamen Pioneer Technology Co., China) and other reagents were used as received.

Synthesis of POEGDMAM

A typical reaction was described as follows: The dried OEG_{13} (6.06 g, 10.1 mmol) , dodecyl bis(2-hydroxyethyl) methylammonium chloride (DMA, 0.408 g, 1.26 mmol) and triethylamine (2.3 g, 22.72 mmol) were dissolved in 50 mL of anhydrous methylene chloride and cooled by an ice bath. Fumaryl chloride (1.74 g, 11.36 mmol) was added to the solution dropwise via a funnel over 2 h under vigorous stirring. The reaction was continued under an argon atmosphere for 24 h at room temperature. Afterward, the solvent was removed through rotary evaporation, and the residue was dissolved in ethyl acetate. After removing triethylamine hydrochloride salt by filtration, the crude product was precipitated into cold diethyl ether, washed three times by diethyl ether and dried in vacuum to constant weight (4.97 g, yield: 67.4 %).

Preparation of cationic hydrogels

In a typical experiment, POEGDMAM (0.1 g, 0.15 mmol of double bonds) and POEGMS (0.11 g, 0.15 mmol of thiol groups) were separately dissolved in phosphate buffered saline (PBS, 0.02 mol/L, pH = 7.0-8.0) at a concentration of 20 wt-%. These two solutions were mixed well to form the cationic hydrogels.

Characterization

^{1}H NMR was measured on Bruker Avance DMX500 NMR spectrometer (500 MHz) at room temperature with $CDCl_3$ as the solvent. Molecular weights and molecular weight distributions were determined by gel permeation chromatography (GPC) equipped with Waters 208 apparatus equipped with Waters 2410 RI detector (set at 60 °C). The eluent was DMF at a flow rate of 1.0 mL/min. The number-average (Mn) was calculated using a calibration curve which was obtained from polymethylmethacrylate standards with low polydispersity indices. Fourier transform infrared spectroscopy (FT-IR) spectra were recorded on a VECTOR 22 spectrometer. Gelation time was determined by tube-inversion method. In detail, POEGDMAM and POEGMS were separately dissolved in PBS solution. Then these two solutions were mixed well with equal mole of "clickable" groups and the gelation time was defined as the time at which the sample showed no ability to flow.

Swelling tests

Hydrogels for swelling tests were prepared in a PBS solution (0.02 mol/L, pH = 7.4) with a precursor concentration of 20 wt-%. Swelling tests were performed by immersing the weighed dry hydrogels (by lyophilization) in PBS (0.02 mol/L, pH=7.4) (refreshed everyday) at 37 °C in triplicate. At regular time intervals, the swollen hydrogels were weighed after removal of the buffer on the surface. The swelling ratio (SR) of the hydrogels was calculated from the equation:
SR (%) = (Ws-Wd)/Wd × 100 %,
where Ws is the weight of swollen hydrogel and Wd is the weight of dry hydrogel.

Degradation experiments

Hydrogels for degradation experiments were prepared in a PBS solution (0.02 mol/L, pH = 7.4) with a precursor concentration of 20 wt-%. Each hydrogel (1 mL) was placed into a capped bottles containing 20 mL phosphate buffer solution (PBS) (0.02 mol/L, pH = 7.4). Then the bottle was incubated at 37 °C and the buffer solution was changed every day. At predetermined time intervals, the samples were withdrawn, rinsed with distilled water for five times and then lyophilized. Weight loss was determined by the following formula:
Weight Loss (%) = [(Weight of initial dry hydrogel-Weight of residual dry hydrogel)/Weight of initial dry hydrogel] × 100 %.

Minimum inhibitory concentrations (MIC)

The minimum inhibitory concentrations (MICs) of polymers were measured using a broth microdilution method.33 Briefly, bacteria cells were grown overnight at 37 °C in broth to a midlog phase and diluted to 106 colony forming units (CFU) mL-1. A dilution series for the cationic polymers was made with concentrations ranging from 500 to 1 μg/mL. 100 μL of diluted polymer solution was introduced into each well of a 96-well plate, followed by the addition of 100 μL of the bacterial solution, to give a final inoculum of 5 × 105 CFU/mL. The plates were incubated at 37 °C for 24 h. The MIC was taken at the concentration that no growth of bacteria was detected. Broths containing bacterial cells only and containing cationic polymers only were used as positive control and negative control, respectively. The MIC test was repeated three times for each sample.

Inhibition zone tests

Hydrogels for inhibition zone tests were prepared in a PBS solution (pH = 7.4) with a precursor concentration of 20 wt-%, which were covered on the LB agar containing bacteria. The plates were cultured at 37 °C overnight. Hydrogels without quaternary ammonium salt (QAS) were taken as controls. Antibacterial activity was judged by the ability of the material to inhibit bacterial growth on the agar surface in contact with it. After incubation for 24 h, the growth zone of inhibition was recorded by a digital camera.

Haemolysis assay

Hydrogels for haemolysis assay were prepared in a PBS solution (pH = 7.4) with a precursor concentration of 20 wt-%. The toxicity of the hydrogels and their precursors against mammalian cells was tested using fresh human red blood cells (hRBCs). Briefly, hRBCs were washed 3 times with PBS. For polymers, 100 μL of red blood cell suspension in PBS (4 % in volume) was placed in each well of a 96-well plate and 100 μL of polymer solution was then added to the well. For hydrogels, 100 μL of red blood cell suspension in PBS (4 % in volume) was placed on the hydrogel (0.1 mL) in each well of a 48-well plate and 100 μL of PBS was then added to the well. The plates were incubated for 1 h at 37 °C. The cell suspensions were taken out and centrifuged at 3000 g for 5 min. Aliquots (100 μL) of supernatant were transferred to 96-well plates, and hemoglobin release was monitored at 576 nm using a Microplate Spectrophotometer (Sunrise-Basic TECAN, Austria). Two control groups were provided for this assay: untreated hRBC suspension as the negative control and hRBC suspension treated with 0.1 % Triton X-100 as the positive control. Each assay was performed in 3 replicates. The percentage of hemolysis was defined as follows:
Hemolysis (%) = [(ODsample − ODnegative control) / (ODpositive control − ODnegative control)] × 100 %.

Results and discussion

Synthesis and characterizations of POEGDMAMs

Cationic PEG derivatives with multiple QAS and double bonds in backbone were synthesized by polycondensation of OEG, DMA and FC with various molar ratios of OEG to DMA. The 1H NMR spectra of the polycations, as well as their neutral analogue were presented in Fig. 1. The characteristic peaks of each building block were clearly detected and assigned. Meanwhile, compared with the spectrum of POEGDMAM-0, the signals of DMA units (Ha, Hb, Hc) were observed in the spectra of POEGDMAM-1, POEGDMAM-2 and POEGDMAM-3. According to the integral ratio of peak Hg (OEG unit) to peak Hb (DMA unit), the molar ratios of in copolymers could be calculated, which are basically consistent with the feed ratios. The cationic polymers, as well as their analogue without ammonium groups, were also characterized by GPC as summarized in Table 1. The relative molecular weights decreased with the increase of QAS ratio, which may be due to the shrinkage of polymer chains with more QAS moieties in DMF, leading to smaller unimolecular coil in DMF. All these results indicate that PEG derivatives containing multiple QAS and double bonds in backbone with controlled chemical compositions were successfully synthesized.

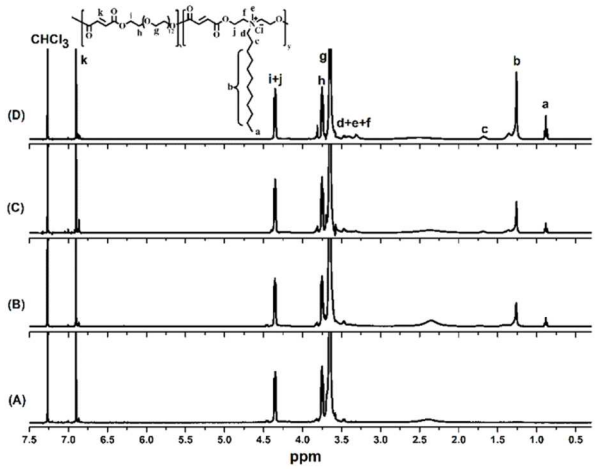


Fig. 1 1H NMR spectra of (A) POEGDMAM-0, (B) POEGDMAM-1, (C) POEGDMAM-2 and (D) POEGDMAM-3.

Table 1 Syntheses of "clickable" cationic PEG derivatives with various chemical compositions

Product	OEG: DMA (in feed)	OEG: DMA (in polymer)	Mna	PDI a
POEGDMAM-0	-	-	13,300	2.97
POEGDMAM-1	1: 0.125	1: 0.132	12,700	2.64
POEGDMAM-2	1: 0.250	1: 0.239	8,800	2.03
POEGDMAM-3	1: 0.500	1: 0.439	5,600	1.47

a Measured by GPC using DMF as eluent at 60 °C and calibrated with PMMA standards

Preparation and characterizations of hydrogels

Thiol-ene "click" reaction was employed to prepare the cationic hydrogels. POEGDMAM and POEGMS were separately dissolved in PBS at the same concentration. The cationic hydrogels could be prepared by simply mixing these two solutions based on the equal mole of "clickable" groups. The hydrogels prepared from the cationic polymers POEGDMAM-0, POEGDMAM-1, POEGDMAM-2, POEGDMAM-3 were named as Hydrogel-0, Hydrogel-1, Hydrogel-2, Hydrogel-3, respectively. FT-IR spectra of lyophilized Hydrogel-2 (20 wt-% solid content, in 0.02 mol/L pH 7.4 PBS) and its precursors were recorded as displayed in Fig. 2. The peak at 1645 cm−1 in the spectrum of POEGDMAM-2 was assigned to the stretching mode of double bonds from fumaryl units. Moreover, the peak at 2547 cm-1 in the spectrum of POEGMS is the absorption of thiol group. Obviously, absorption peaks ascribed to double bonds and thiol groups disappeared in the spectrum of Hydrogel-2, indicating that most of the double bonds and thiols have reacted, and the cationic hydrogels were thus prepared successfully.

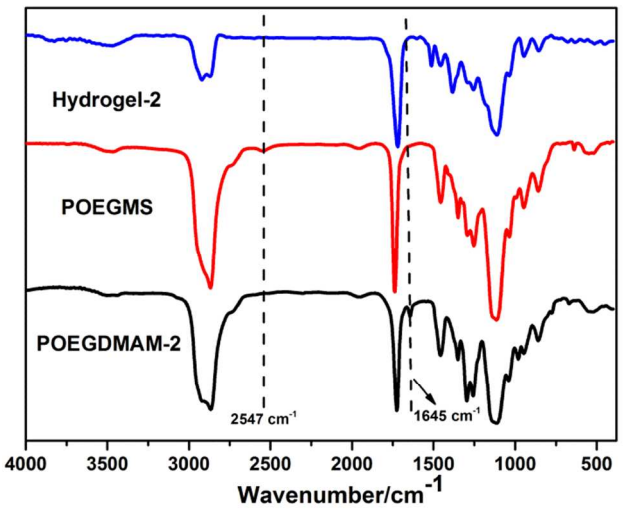


Fig. 2 FT-IR spectra of Hydrogel-2 and its precursors.

The influences of pH value, OEG/DMA molar ratio, and concentration of precursors on "click" gelation time were systematically investigated by inverting the vials method. Fig. 3A shows that gelation time prolonged with the decrease of OEG/DMA molar ratio, probably due to the lower solubility of POEGDMAM with higher DMA content. Moreover, for the same sample, gelation time shortened with the increasing pH value of solution, which is attributed to the fact that the Micheal type "thiol-ene" click reaction followed base catalysis mechanism. Fig. 3B shows that at a constant pH value of 7.4, gelation time shortened considerably with increasing of precursor concentration from 5 to 20 wt-%, because of the increased functional groups' density in the solutions. In brief, by tuning the parameters, such as pH value, OEG/DMA molar ratio and polymer concentration, gelation time of the hydrogels could be adjusted from 18 s to 2 min. The rapid gelation and controlled gelation time make this kind of hydrogels to be suitable candidates as chemically crosslinked injectable hydrogels, which should have better mechanical property than physically crosslinked hydrogels.

Swelling ratio is an important parameter for evaluating the performance of hydrogels, especially for bio-applications. In this study, the water absorption capability of the lyophilized hydrogels was determined by a gravimetric method. Curves of swelling ratios as a function of time for the lyophilized hydrogels are presented in Fig. 4. All the hydrogels with different QAS proportions basically reached equilibrium swelling within 9 h. The swelling rates, as well as the equilibrium swelling ratios, of the hydrogels slightly decreased with increase of QAS fractions, which may be ascribed to the hydrophobicity of the dodecyl in QAS moieties.

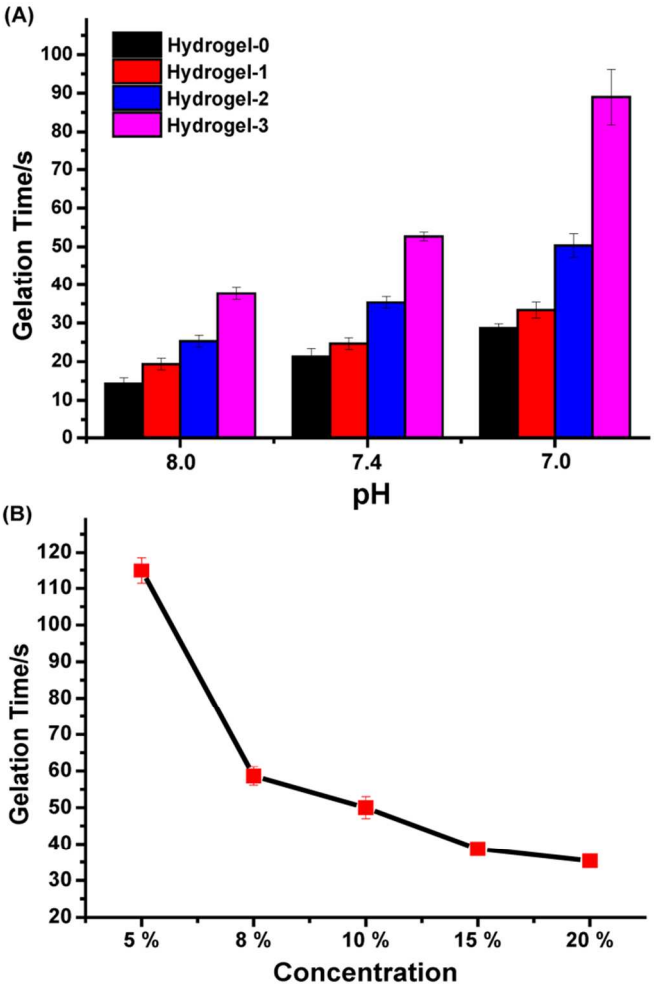


Fig. 3 Gelation time of hydrogels with a solid content of 20 wt-% under various pH values (A), and gelation time of Hydrogel-2 with various concentrations of precursors in pH 7.4 PBS (B).

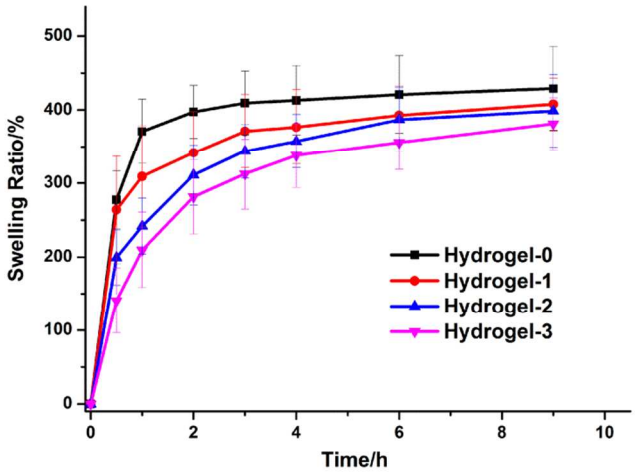


Fig. 4 Swelling curves of hydrogels.

Hydrolytic degradation

Each building block of the hydrogel precursors was connected by ester linkages, which leads to the biodegradability of the hydrogels due to the hydrolysis of ester bonds. Fig. 5 shows the

weight loss profiles versus hydrolytic degradation time for the antibacterial hydrogels by using buffer solution method. All of hydrogels completely degraded about 30 days. The degradation behaviour could be divided into two stages. During the first three weeks, the ester linkages started to break due to hydrolysis, leading to some defects in the hydrogel. However, the hydrogels still kept their 3D-network. The slight weight loss was apparently caused by extraction of a soluble fraction of the hydrogels due to not exhausted conversion of "click" reaction. In the fourth week, the weight of the hydrogels started to decrease continuously and the hydrogels were fully degraded. During this stage, the 3D-network architecture of the hydrogels began to decompose and water soluble fraction was formed, leading to accelerated degradation rate. The degradation experiments indicated that these antibacterial hydrogels could be decomposed to small molecular chains, which would be passed out of body through metabolism. Thus they would not be harmful to the body or the environment.

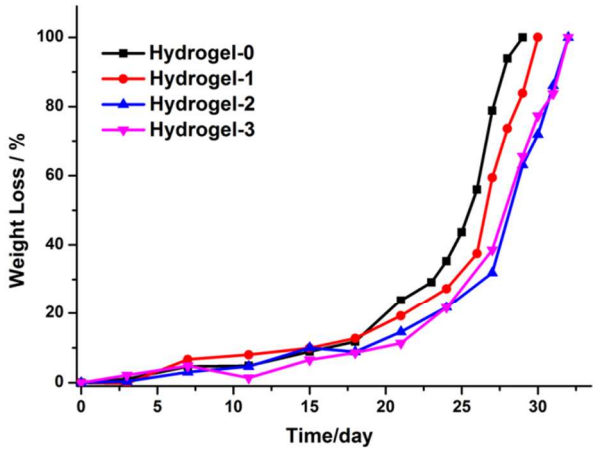


Fig. 5 Weight losses of hydrogels as a function of hydrolytic degradation time.

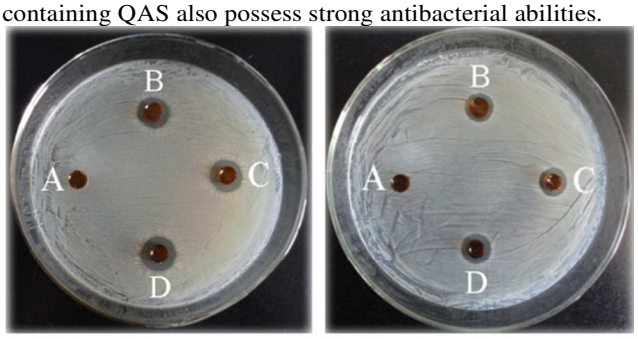
Antibacterial Properties

According to our previous report,34, 35 the polymers with longer alkyl group in quaternary ammonium moiety show higher antibacterial activity. Thus, QAS group with dodecyl was used to provide the hydrogels with antibacterial ability. The antibacterial activities of these cationic copolymers and hydrogels were probed against two representative clinically relevant bacterial strains, namely S. aureus (Gram-positive) and E. coli (Gram-negative). The antibacterial activity of hydrogels' precursors was evaluated by measuring their minimum inhibition concentrations (MIC) using a broth microdilution assay. MIC values for all precursors tested are shown in Table 2. All the polymers with QAS groups showed good antibacterial property. Moreover, the MIC values of cationic POEGDMAMs decreased with the increasing of QAS content, indicating the polymer POEGDMAM-3 with more QAS groups possessed higher antibacterial activity. It is well known that the antibacterial activity of QAS is affected mainly by positive charge density and the hydrophobic/hydrophobic interactions between the alkyl substituent and the hydrophobic inner part of the bacterial cell wall and membrane, which could also be concluded from our results. All of these facts demonstrated that cationic POEGDMAMs exhibited good antibacterial activity against both gram-positive and gram-negative bacteria.

Table 2 MIC and HC50 of POEGDMAMs

Polymer	MIC (μg/mL)		HC50
	S.aureus	E.coli	(μg/mL)
POEGDMAM-0	-	-	>2560
POEGDMAM-1	80	80	1280
POEGDMAM-2	40	40	640
POEGDMAM-3	20	20	320
POEGMS	-	-	>2560

Inhibition zone method was employed to study the antibacterial properties of hydrogels prepared from cationic PEG derivatives. As shown in Fig. 6, inhibition zones could be clearly observed around the cationic hydrogels for both of these two bacteria, while no inhibition zones around Hydrogel-0 were observed. Therefore, the hydrogels prepared by precursors containing QAS also possess strong antibacterial abilities.



S.aureus *E.coli*

Fig. 6 Inhibition zone tests of hydrogels against S. aureus and E.coli: (A) Hydrogel-0; (B) Hydrogel-1; (C) Hydrogel-2; (D) Hydrogel-3.

Hemolytic Activity

The biocompatibility of antibacterial hydrogels and precursors was evaluated via haemolysis assays using human red blood cells. One metric for defining haemolytic activity is HC50, the concentration of antibacterial that kills 50 % of mammalian cells.36 The HC50 values of precursors were summarized in Table 2. The cationic polymer with higher MIC value also possessed a higher HC50 value. The selectivity (HC50/MIC) of all these cationic polymers is 16, indicating the polymers had a modest extent of selectivity toward the bacterial cells over mammalian cells. There is no difference of selectivity among these cationic polymers, which might be due to their similar ratio of hydrophilicity and hydrophobicity. Moreover, the crosslinker POEGMS showed no hemolytic activity, namely good biocompatibility, which was consistent with our previous result.32

The hemolytic activity of hydrogels was shown in Fig. 7. Hydrogel-1 exhibited low haemolysis, while the haemolysis of Hydrogel-2 and Hydrogel-3 with high QAS contents reached about 50 %. However, according to the inhibition zone results in Fig. 6, Hydrogel-1 with low QAS density still exhibits enough antibacterial activity. Thus, such cationic hydrogels with fewer QAS moieties possess both strong antibacterial activity and low toxicity, which might be suitable candidate as biomaterials.

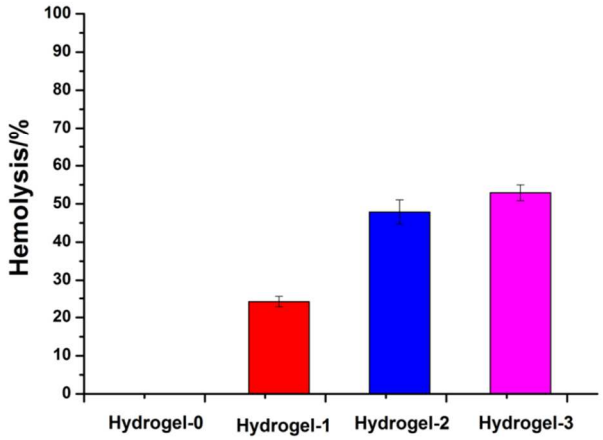


Fig. 7 Hemolysis of hydrogels with QAS.

Conclusions

Fully biodegradable cationic hydrogels were prepared facilely under human physiological conditions through thiol-ene "click" crosslinking of "clickable" PEG derivatives containing QAS groups, which could be synthesized by polycondensation in a large scale. The gelation time of the hydrogels could be controlled by adjusting some parameters, such as pH value, OEG/DMA molar ratio and polymer concentration, making it possible to prepare injectable hydrogels with chemical crosslinkages. The PEG derivatives with QAS moieties in backbone and their corresponding cationic hydrogels exhibit excellent antibacterial activity against both gram-negative and gram-positive bacteria. The biocompatibility tests indicate that the toxicity of the hydrogels reduced with the decrease of QAS density. The hydrogel with fewer QAS moieties (Hydrogel-1) possesses both strong antibacterial ability and low toxicity, which holds potentials as antibacterial materials for biomedical applications, such as wound dressings, coatings for medical devices, injectable tissue engineering scaffold materials and so on.

Acknowledgements

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Notes and references

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