Carbohydrate Polymers 98 (2013) 1547–1552

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Antibacterial hydrogel coating by electrophoretic co-deposition of chitosan/alkynyl chitosan

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ARTICLE INFO

Article history:
Received 17 May 2013
Received in revised form 18 July 2013
Accepted 19 July 2013
Available online 29 July 2013

Keywords:
Alkynyl chitosan
Antibacterial activity
Electrophoretic co-deposition
Chitosan
Composite hydrogel

ABSTRACT

Despite much effort has been paid to develop aseptic implant devices, the infection associated with medical implant still remains a significant problem. Here, we report a potential coating material derived from a natural biopolymer chitosan. Firstly, chitosan functionalized with alkynyl moiety (ACS) was prepared by reaction between chitosan and 3-bromopropyne. The structure of the alkynyl chitosan was characterized by FT-IR, ^{1}H NMR, XRD, TGA and element analysis. The minimum inhibitory concentration (MIC) of ACS with a degree of substitution (DS) of 0.40 was 0.03% against *Escherichia coli* (*E. coli*) and *Staphylococcus aureus* (*S. aureus*). Subsequently, the alkynyl chitosan was co-deposited with chitosan on stainless steel wire to fabricate a composite hydrogel. The composite hydrogel exhibited better antibacterial activities than pure chitosan hydrogel.

1. Introduction

Polymer coating adhered on the surface of medical implants and devices with antibacterial activities have attracted great interests in the last decades for its ability to reduce implant-associated infections (Li et al., 2011; Zhou et al., 2011). Antimicrobial polymers such as chitosan and hydroxypropyltrimethyl ammonium chloride chitosan have been applied as antibacterial coatings (Muzzarelli et al., 1990; Xu, Wang, Guo, Lei, & Tang, 2011). Nevertheless, developing new chitosan derivatives or composites with superior antibacterial activity remain a continuing challenge. Herein, we functionalized chitosan with alkynyl groups and prepared a composite coating with antibacterial activities by electrophoretic co-deposition of chitosan and alkynyl chitosan.

Chitosan is an amino polysaccharide obtained by the deacetylation of chitin (Muzzarelli et al., 2012a; Muzzarelli, Greco, Busilacchi, Sollazzo, & Gigante, 2012; Pillai, Paul, & Sharma, 2009). Chitosan and its derivatives has been widely used as implant coatings for its intrinsic properties such as non-toxic, osteoconductive, pH responsive, anti-microbial, biocompatible and cell adhesive (Jayakumar, Prabaharan, Nair, & Tamura, 2010; Jayakumar, Prabaharan, Sudheesh Kumar, Nair, & Tamura, 2011; Muzzarelli, 2009; Muzzarelli et al., 2012b; Park & Kim, 2010). Solution casting (López-Lacomba et al., 2006), silane reactions and layer-by-layer assembly (Cai, Rechtenbach, Hao, Bossert, & Jandt, 2005) were used to coat the implanted surface with chitosan. Recently, it was reported that chitosan can undergo sol–gel transition at the cathode surface (Liu, Gaskell, Cheng, Yu, & Payne, 2008; Liu et al., 2013; Payne & Raghavan, 2007). It is the high pH at the cathode surface that induces the sol–gel transition of chitosan (chitosan is insoluble at pH above its p*K*a of 6.3). Our previous study showed that chitosan can be electrodeposited on titanium surface as implant coating (Shi, Wu, Li, Wei, & Du, 2013). Other study also showed that silk fibroin (Zhang et al., 2011) and gelatin (Jiang et al., 2010) can be electrochemically co-deposited with chitosan to enhance the shear bond strength and cellular affinity of pure chitosan coating.

In the present study, alkynyl chitosan with superior antibacterial activity was synthesized and characterized by Fourier transform infrared spectra (FT-IR), ^{1}H nuclear magnetic resonance (^{1}H NMR), X-ray diffraction (XRD), thermogravimetric analysis (TGA) and element analysis. The antibacterial property of alkynyl chitosan was evaluated by methods of disk diffusion and MIC. For a proof of concept demonstration, the alkynyl chitosan was co-deposited with chitosan on a stainless steel wire to test the potential usage of this chitosan derivative as antibacterial coating. The antibacterial activity of the composite coating was further evaluated by disk diffusion method. The pH responsive and antibacterial alkynyl chitosan may have important applications in the biomedical fields.

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0144-8617/$ – see front matter © 2013 Published by Elsevier Ltd.
http://dx.doi.org/10.1016/j.carbpol.2013.07.042

2. Experiment

2.1. Materials

Chitosan with a molecular weight of 2.0×10^5 Da and degree of deacetylation 0.85 was purchased from Sigma–Aldrich. 3-bromopropyne was purchased from Shanghai Darui fine chemical Co., Ltd. Agar, peptone and beef extract were purchased from Shanghai Reagent Co., Ltd (China). *E. coli* and *S. aureus* were supplied by the microbiology laboratory of School of Resource and Environment Science, Wuhan University. All other reagents were of analytical grade and were used without further purification.

2.2. Preparation of alkynyl chitosan

Alkynyl chitosan was synthesized by reacting chitosan with 3-bromopropyne. In a typical process, 1 g chitosan was added into 40 mL isopropanol and stirred at 25 °C for 30 min. Then, 10 mL NaOH (4 M) was added and stirred vigorously for 30 min. 3-bromopropyne (0.486 mL) was mixed with the above solution and the mixture was stirred at 70 °C for 4 h. Then, it was neutralized using 1% HCl. The resulting solution was precipitated by acetone and washed with 70% ethanol and ethanol three times respectively. Pale yellow alkynyl chitosan powder was obtained after vacuum dried. Alkynyl chitosan coded as ACS1, ACS2, ACS3 and ACS4 were prepared by changing the molar ratio of chitosan unit to 3-bromopropyne monomer as 1:0.5, 1:1, 1:1.5 and 1:2.

2.3. Characterization of alkynyl chitosan

Fourier transform infrared spectra (FT-IR) of alkynyl chitosan were recorded by using the KBr pellets method on a Nicolet 5700 Fourier transform infrared spectrometer.

The ^{1}H nuclear magnetic resonance (^{1}H NMR) was recorded on a Varian INOVA-600 spectrometer with 2% DCl as solvent. The concentration of alkynyl chitosan was 3 wt%. Chemical shifts were given in ppm using tetramethylsilane (TMS) as an internal reference.

X-ray diffraction (XRD) test was conducted on an X-ray diffract meter (D8-Advance, Bruker). The XRD patterns with CuK_α radiation ($\lambda = 0.154$ nm) at 40 kV and 50 mA were recorded in the region of 2θ from 7° to 45°.

Thermo gravimetric analysis (TGA) was carried out in a Pyris TGA linked to a Pyris diamond TA Lab System (Perkin-Elmer Co., USA). The samples were scanned at a heating rate of 10 °C min^{-1} under flow of nitrogen.

Scanning electron micrograph (SEM) measurements were conducted on a Hitachi X-650 microscope (Japan). The electrodeposited hydrogel were frozen in liquid nitrogen, and then freeze-dried. The hydrogel was sputtered with gold, observed and photographed.

The degree of substitution (DS) of alkynyl chitosan was determined by elemental analysis (Vario III, Elementar, Germany) and DS values were calculated as Eq. (1).

$$\frac{C\ (\%)}{N\ (\%)} = \frac{6 \times 12 \times (1 - DS) + 9 \times 12 \times DS}{1 \times 14} \quad (1)$$

2.4. Electrodeposition of the alkynyl chitosan hydrogel

Alkynyl chitosan hydrogel was co-deposited with chitosan. In a typical run, chitosan and alkynyl chitosan were dissolved in 1% HCl to prepare 2 wt% solutions respectively. The deposited solutions were prepared by mixing the two solutions according to volume ratio of chitosan to alkynyl chitosan as 1:0, 1:0.5, 1:1, 1:1.5 and 1:2. NaCl (0.25 wt%) was added to facilitate the deposition. The pH of the solution was adjusted to 5.0 by adding sodium hydroxide solution (1 M). A platinum foil was used as anode and stainless steel wire with 10 cm long and a diameter of 0.5 mm was used as cathode for the deposition of alkynyl chitosan. The platinum foil and stainless steel wire were cleaned by acetone, alcohol and water consecutively under sonication before electrodeposition. The distance between the electrodes was kept at 2 cm. The electrodes were connected to a power supply and a constant voltage (2 V) was applied. A hydrogel was formed on the wire after 180 s' deposition and rinsed briefly with ultrapure water.

2.5. Antibacterial assay

The antibacterial activities of the alkynyl chitosan were measured by disk diffusion method. *E. coli* and *S. aureus* were chosen as the bacteria to evaluate the antibacterial activities. 500 mL of nutrient medium (pH 7.0) containing beef extract 2.5 g, peptone 5 g, agar 10 g and NaCl 2.5 g was autoclaved at 126 °C for 30 min. 50 μL bacteria suspension (10^6 cfu mL^{-1}) was added onto the agar plates containing 15 mL nutrient medium. The bacteria suspensions were homogeneously dispersed by using coated rods and paper disks containing 1 wt% chitosan, ACS1, ACS2, ACS3 and ACS4 solution respectively were pasted on the agar plates. The inhibition zones were measured on a microscope after incubation at 37 °C for 24 h.

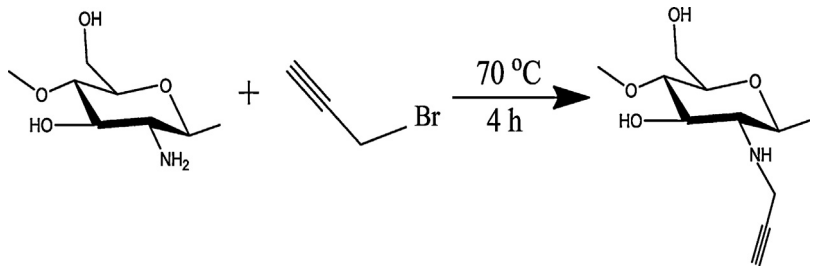
The minimum inhibitory concentration (MIC) of the alkynyl chitosan was also evaluated. In this test, 18 mL nutrient medium, 1 mL microorganism and 1 mL test solution (sterile water, chitosan, ACS1, ACS2, ACS3 and ACS4) were added into the petri dish. The final concentrations of the test samples were 0.1 wt%, 0.08 wt%, 0.05 wt%, 0.03 wt%, 0.01 wt% and 0 wt% respectively. The lowest concentrations of the alkynyl chitosan and chitosan that will inhibit the visible growth of *E. coli* and *S. aureus* after incubation at 37 °C for 48 h were defined as the minimum inhibitory concentrations (MICs).

In the case of antibacterial activities of the composite hydrogels, the method used was similar to the disk diffusion method except that the paper disks were replaced by the hydrogels.

3. Results and discussion

The alkynyl chitosan was synthesized through reaction between 3-bromopropyne and amine on the chitosan backbone. The process for the preparation of alkynyl chitosan is shown in Scheme 1. Alkynyl chitosans with various DS were obtained by changing the molar ratio of chitosan unit to 3-bromopropyne monomer, as illustrated in Table 1. The DS value of the alkynyl chitosan enhanced from 0.283 to 0.508 with increased amount of 3-bromopropyne.

The structure of alkynyl chitosan was initially characterized by using Fourier transform infrared spectra (FT-IR). In the FT-IR spectra of chitosan (Fig. 1), there are three characteristic absorption bands at 1653 cm^{-1} (amide I, C=O stretching), 1597 cm^{-1} (amide II, —NH_2 bending) and 1381 cm^{-1} (—CH_2 bending). The strong band at around 3400 cm^{-1} was assigned to the stretching vibration for O—H, the extension vibration of N—H, and inter-molecular hydrogen bonds of the polysaccharide moieties (Li, Hu, Ren, Worley, & Huang, 2013; Sajomsang, Gonil, & Tantayanon, 2009). As compared with the chitosan spectrum, a new peak appeared at 2117 cm^{-1} in the spectrum of alkynyl chitosan. The peak at 2117 cm^{-1} was the characteristic absorbance peak of alkynyl group, suggesting the successful introduction of alkynyl group on chitosan backbone (Bao et al., 2010; Kulbokaite, Ciuta, Netopilik, & Makuska, 2009). Other newly appeared or strengthened absorbance peaks around 3250, 1423, and 630 cm^{-1} represent the alkyne C—H stretching, alkane C—H bending and alkyne C—H bending, respectively (Bao et al., 2010).



Scheme 1. Process for the synthesis of alkynyl chitosan.

Table 1
Conditions and results of the alkynyl chitosan prepared by using 3-bromopropyne as the modifier.

Sample ID	Molar ratio[a]	Time (h)	Temperature (°C)	C%	N%	H%	DS
ACS1	0.5	4	70	38.00	6.473	6.593	28.30%
ACS2	1	4	70	38.65	6.521	6.740	30.49%
ACS3	1.5	4	70	40.27	6.532	6.792	39.75%
ACS4	2	4	70	41.30	6.403	6.690	50.84%

[a] Molar ratio of 3-bromopropyne monomer to chitosan unit.

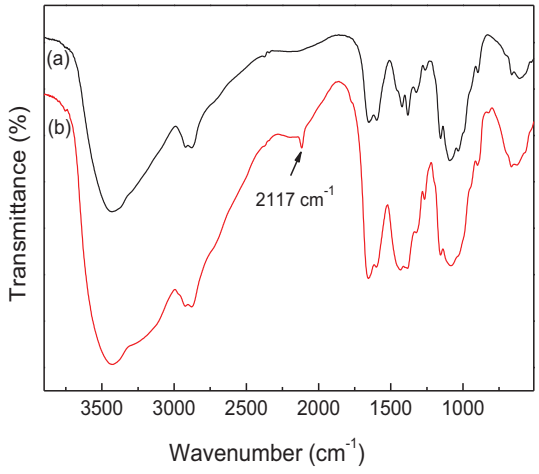


Fig. 1. FT-IR spectra of original chitosan (a) and alkynyl chitosan with DS 39.75% (b).

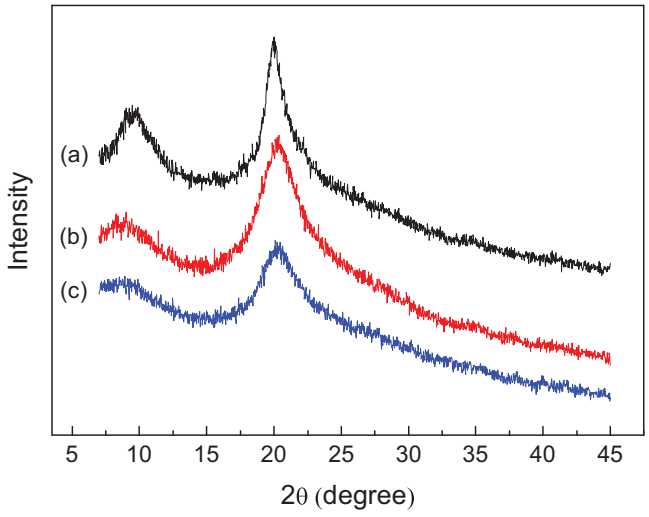


Fig. 3. X-ray diffraction patterns of original chitosan (a), ACS1 (b) and ACS4 (c).

The structure of alkynyl chitosan was further studied by ^{1}H NMR. Fig. 2 shows the ^{1}H NMR of ACS3. As shown in the spectrum, the basic ^{1}H NMR of chitosan is δ = 1.95 ppm (—$COCH_3$), 3.08 ppm (H2), 3.31–3.85 ppm (H3–H6), 4.81 ppm (H1) (Domard, Gey, Rinaudo, & Terrassin, 1987). The signals appearing at δ = 2.81–2.95 ppm (b) can be ascribed to methylene protons (2H, —CH_2—), and the alkynyl proton (a) signal at δ = 1.95 ppm is overlapped with that of CS acetyl groups (Bao et al., 2010).

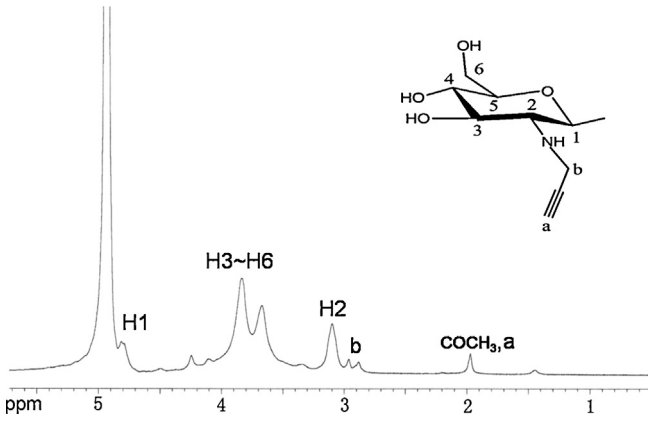


Fig. 2. The ^{1}H NMR spectrum of alkynyl chitosan (ACS3) using 2% DCl as solvent.

The alkynyl chitosan was further studied by X-ray diffraction (XRD). As shown in Fig. 3(a), the peak at 9.4°and 20.3° were the characteristic peaks of chitosan (Clark & Smith, 1935). The characteristic peaks in the spectra of alkynyl chitosan (ACS1, ACS4) dramatically declined which indicated that the introduction of alkynyl groups into chitosan disrupted the crystalline structure of chitosan to some extent (Ma et al., 2008; Zhang, Ping, Zhang, & Shen, 2003).

Fig. 4 shows the TGA and DTG of the chitosan and alkynyl chitosan (ACS1, ACS2, ACS3). The TGA of chitosan showed two different stages of weight loss as other literatures reported (Li et al., 2013). The first stage weight loss extended from 45 to 131 °C, and T_{max} was 73 °C. This may be ascribed to the loss of adsorbed and bound water. The second stage of weight loss was in the range of 234–418 °C and the T_{max} was 302 °C. This was due to the degradation of chitosan biopolymer (Kumar, Koh, Kim, Gupta, & Dutta, 2012). The thermal behavior of alkynyl chitosan was similar to that of chitosan, which indicates that incorporation of alkynyl groups in chitosan does not significantly affect its thermal performance.

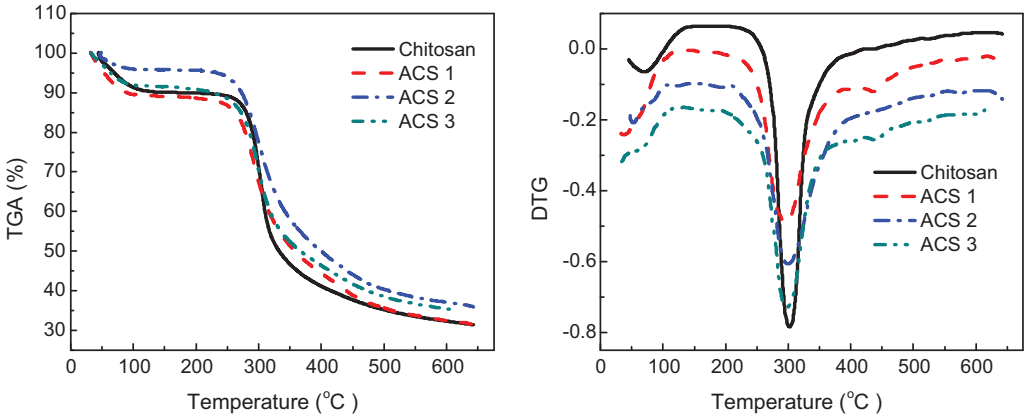


Fig. 4. TGA and DTG curves of chitosan and alkynyl chitosan (ACS1, ACS2, ACS3).

The antibacterial activity of alkynyl chitosan was firstly determined by disk diffusion method. As shown in Fig. 5, both chitosan and alkynyl chitosan show antibacterial activities against both *E. coli* and *S. aureus*. It can be found that the inhibition zone for the filter paper containing 1 wt% alkynyl chitosan was larger than the filter paper containing 1 wt% chitosan. The exact mechanisms for microbial inhibition by chitosan and its derivative are still not known (Lim & Hudson, 2003). The most acceptable mechanism is the amino groups interact with the predominantly anionic components on the bacterial surface and change the membrane permeability (Ding et al., 2012; Dutta, Tripathi, & Dutta, 2011; Dutta, Tripathi, Mehrotra, & Dutta, 2009; Rabea, Badawy, Stevens, Smagghe, & Steurbaut, 2003). In the case of alkynyl chitosan, though the amine groups in the alkynyl chitosan backbone decreased, more alkynyl groups grafted in the chitosan chains make the alkynyl chitosan more hydrophobic. It was reported that hydrophobic chitosan derivatives have higher hydrophobic affinity to the bacteria cell composed of mainly phospholipids (hydrophobic) and membrane protein, and as a result, show higher antimicrobial activity (Kim, Choi, Chun & Choi, 1997; Lim & Hudson, 2003).

Minimum inhibitory concentration (MIC) method was further used to evaluate the antibacterial activities of alkynyl chitosan. The results of this study are presented in Table 2. The MICs of chitosan against both *E. coli* and *S. aureus* was 0.08%, whereas the MICs of alkynyl chitosan against both *E. coli* and *S. aureus* were lower than chitosan. This was consistent with the antimicrobial results determined by disk diffusion method. The antimicrobial activity of ACS3 against *E. coli* was lower than other samples. This may attribute to the synergy effect of amino groups and hydrophobicity of the alkynyl chitosan.

Table 2
MIC results of chitosan and alkynyl chitosan against *E. coli* and *S. aureus*.

Microorganism	Sample ID				
	ACS1	ACS2	ACS3	ACS4	CS
Escherichia coli	0.05%	0.05%	0.03%	0.05%	0.08%
Staphylococcus aureus	0.03%	0.03%	0.03%	0.03%	0.08%

In subsequent experiment, we prepared alkynyl chitosan hydrogels by using electrodeposition method to test the potential usage of alkynyl chitosan in biomedical field. A stainless steel wire (diameter 0.5 mm) with 10 cm long served as working electrode was partially immersed in 2 wt% alkynyl chitosan solution. After applying a cathode voltage (−2 V) for 180 s, we observed a hydrogel formed on the wire. However, the mechanical strength was too weak to obtain an intact hydrogel. It was the loss of crystallinity and decrease of amine groups in the deposited hydrogel that may cause the poor mechanical strength.

Chitosan can be electrodeposited on the cathode electrode and form a hydrogel with good mechanical strength. In order to enhance the mechanical strength of the alkynyl chitosan hydrogel, chitosan was chosen to co-deposit with alkynyl chitosan so as to obtain an integrated hydrogel. The deposited solutions were prepared by mixing the chitosan (2 wt%) and alkynyl chitosan (2 wt%) according to volume ratio as 1:0, 1:0.5, 1:1, 1:1.5 and 1:2. Fig. 6(a) shows the scheme of the co-deposition of alkynyl chitosan and chitosan. By applying a cathode voltage (−2 V) for 180 s, we can obtain a well shaped hydrogel (average diameter 2 mm) on the wire as shown in Fig. 6(b). The morphology of the composited hydrogel was shown in Fig. 6(c). Obviously, the hydrogel exhibited a porous structure

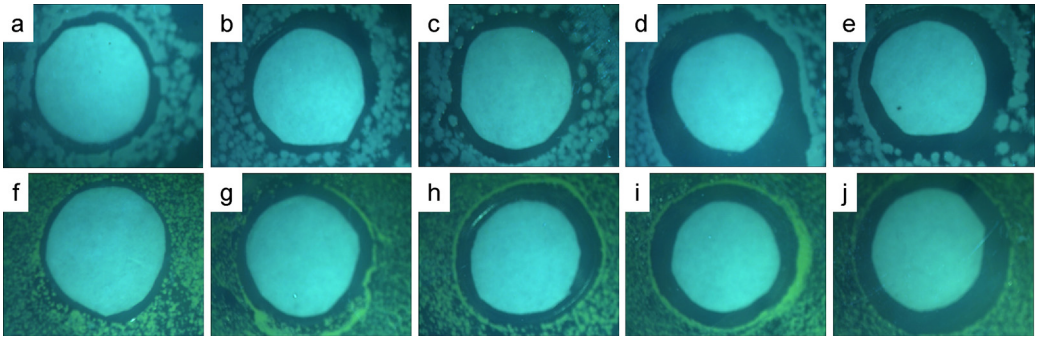


Fig. 5. Antimicrobial results of chitosan and alkynyl chitosan against *E. coli* and *S. aureus* by using disk diffusion method. Images (a–e) are paper disks containing 1 wt% chitosan, 1 wt% alkynyl chitosan (ACS1, ACS2, ACS3 and ACS4), respectively against *E. coli*; Images (f–j) correspond to paper disks containing 1 wt% chitosan, 1 wt% alkynyl chitosan (ACS1, ACS2, ACS3 and ACS4), respectively against *S. aureus*.

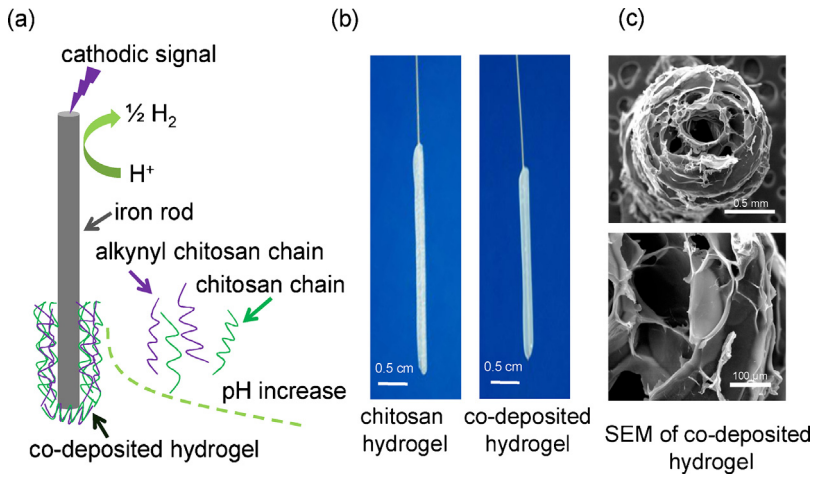


Fig. 6. Scheme of electrophoretic co-deposition of chitosan/alkynyl chitosan (a), photographs of the chitosan hydrogel and hybrid hydrogel deposited on stainless steel wire (b) and SEM images of the co-deposited hydrogel with a volume ratio of chitosan (2 wt%) to alkynyl chitosan (2 wt%) as 1:1 (c).

and the diameter of the pores was in the range of 20–100 μm. The gelation of chitosan and alkynyl chitosan on cathode was due to the electrochemically generated concentration gradient of reactant OH^- ions, and their subsequent neutralization of the NH_3^+ groups on chitosan or alkynyl chitosan in solution near the cathode (Liu et al., 2010; Meyer, Liu, Shi, Yang, Bentley & Payne, 2009; Shi et al., 2008).

The antimicrobial activities of the co-deposited hydrogels were determined by method similar to the disk diffusion method except that the paper disks were replaced by the hydrogels. As shown in Fig. 7, small inhibition zones (about 0.5 mm) were observed in the test of pure chitosan hydrogel against either *E. coli* or *S. aureus*. The neutralization of NH_3^+ groups in the chitosan chains during the electrodeposition may cause the weaker antibacterial activities. The inhibition zones of the hybrid hydrogels with different amount of alkynyl chitosan (ACS3) were larger than that of pure chitosan hydrogel. The hydrophobicity of chitosan derivative may facilitate the integration between bacteria cell and chitosan derivative chains, leading to the high antimicrobial activity (Lim & Hudson, 2003).

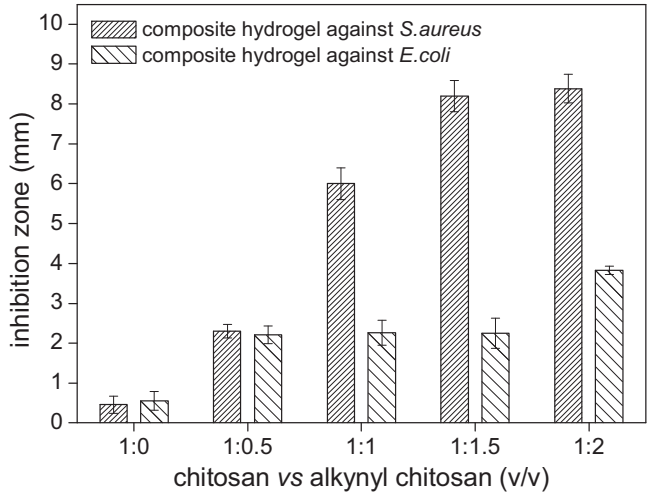


Fig. 7. Antimicrobial assay of composite hydrogel prepared by mixing the chitosan (2 wt%) and alkynyl chitosan (ACS3, 2 wt%) according to volume ratio of 1:0, 1:0.5, 1:1, 1:1.5 and 1:2, respectively against *E. coli* and *S. aureus* by using a similar disk diffusion method. The inhibition zone was calculated by diameter of area that no bacteria were grown.

4. Conclusions

In this study, we have demonstrated that the alkynyl chitosan was successfully synthesized and confirmed by FT-IR, ^{1}H NMR, XRD, TGA and element analysis. The alkynyl chitosan show better antibacterial activity against *E. coli* and *S. aureus* than pure chitosan assessed by MIC and disk diffusion method. At the same time, we fabricated a composite hydrogel by electrochemical co-deposition of chitosan and alkynyl chitosan. The antibacterial activities of the composite hydrogels were higher than the pure chitosan hydrogel. We believe the composite hydrogel of chitosan and alkynyl chitosan can be applied on other implant substrate and used as implant coatings.

Acknowledgments

This work was financially supported by National Natural Science Foundation of China (grant no. 21007049), Program for New Century Excellent Talents in University (NECT-10-0618), and Natural Science Foundation of Hubei Province (2011CDB268).

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