Journal of Applied Polymer Science

Functionalization of chitosan via single electron transfer living radical polymerization in an ionic liquid and its antimicrobial activity

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ABSTRACT: In this work, single electron transfer living radical polymerization (SET-LRP) was used to functionalize chitosan in a well-controlled manner. The chitosan-based macroinitiator was first synthesized and then initiated the SET-LRP of methacryloyloxyethyl trimethylammonium chloride (DMC) in ionic liquid system, using Cu^0/*N*,*N*,*N*′,*N*′,*N*″-pentamethyldiethylenetriamine as a catalyst. The grafting of PDMC brushes on chitosan was confirmed and analyzed by Fourier transform infrared spectroscopy and ^1H nuclear magnetic resonance. Transmission electron microscopy reveals that the chitosan copolymer showed self-assembled behavior in acetone. Surface properties of the copolymer have been investigated by environment scanning electron microscopy analysis. The linear relationship between the $\ln([M]_0/[M]_t)$ and time, the linear increase of number-average molecular mass with conversion as well as the low polydispersity index of the polymer confirmed the "living/controlled" features of the polymerization of DMC through SET-LRP. Finally, the chitosan copolymer demonstrates its potential antibacterial application, showing excellent inhibitive capability against *Escherichia coli*. © 2015 Wiley Periodicals, Inc. J. Appl. Polym. Sci. **2015**, *132*, 42754.

KEYWORDS: biomaterials; biopolymers and renewable polymers; copolymers; ionic liquids; self-assembly

Received 9 April 2015; accepted 23 July 2015
DOI: 10.1002/app.42754

INTRODUCTION

Because of growing environmental awareness and the diminishing petroleum-based resources, natural polysaccharides have gained increasing interest owing to their favorable properties, such as renewability, nontoxicity, biodegradability, and cheapness. Moreover, they can be chemically modified to introduce new functional groups, yielding various types of derivatives.[1–5] Chitosan, obtained from partial *N*-deacetylation of chitin, is one of the most abundant biopolymer in nature. It is a linear polysaccharide composed of randomly distributed β (1→4)-linked D-glucosamine and *N*-acetyl-D-glucosamine. As a kind of natural polymer, chitosan has attracted people's attention for its excellent biological characteristics, such as antimicrobial activity, blood-clotting properties, hypotoxicity, and biocompatibility.[6–10] These advantages result in its wide range of biomedical applications, including pharmaceutical and medical applications, environmental protection, textiles, wastewater treatment, biotechnology, cosmetics, food processing, and agriculture.

The antimicrobial properties of chitosan, which is mainly due to its polycationic structure, make chitosan an attractive candidate for antimicrobial agent.[11,12] As an antimicrobial agent, chitosan has a series of virtues, for example, it is nontoxic to the environment, it can minimize the environmental problems by reducing the residual toxicity of the agents (such as forming complexes with various heavy metals), it is nonvolatile, chemically stable, and do not permeate through the skin. Thus, chitosan is widely employed as a cheap and abundant antimicrobial agent. However, the antimicrobial activity of chitosan was prominent only in acidic medium because of its poor solubility in neutral and basic media where chitosan starts to lose its cationic nature, which limited its wide applications to some extent.[13–15] Therefore, chemical modifications have been explored to improve properties of chitosan. Among them, grafting polymerization is an efficient way to increase the solubility in common solvents or improve the antimicrobial activity of chitosan. The free amino group (NH_2) on deacetylated units and the hydroxyl group (OH) at the C3 and C6 on acetylated or deacetylated units will enable the easy occurrence of graft copolymerization on chitosan.[16,17] Single electron transfer-living radical polymerization (SET-LRP), proposed by Percec *et al.*,[18]

is a recently developed "controlled" radical polymerization method. It attracted interests for the construction of well-defined polymers and functional chain ends, which can be followed by polymerization of vinyl monomers for various structures. Thus, it is possible to use this method for the site-specific controlled grafting from functionalized sites on chitosan backbones to produce graft copolymers.

Ionic liquids (ILs), as a good solvent for biological macromolecules, have attracted much attention as greener replacements for traditional volatile organic solvents owing to their unique properties, such as low toxicity, high chemical and thermal stability, nonflammability, and recyclability.[19,20] ILs were first applied as solvents for cellulose,[21] and then led to increasing explosion of interest in the use of ILs for biomass treatment. It has been reported that chitosan could be successfully dissolved in 1-butyl-3-methylimidazolium chloride (BMIMCl),[22] which makes it possible to modify chitosan in a homogeneous and efficient way, providing a brand new platform for the utilization of chitosan. Moreover, the use of IL as a medium could enhance the nucleophilicity of amines and increase the stability of the reagent-activated complexes, thus accelerating a nucleophilic substitution reaction.[23,24] This would shorten the reaction time and benefit the grafting control.

Conventional free-radical polymerization may result in the production of unwanted homopolymer together with the graft copolymer and the undesired chain degradation of the chitosan backbone. To efficiently provide new chitosan-based materials, herein, we report the functionalization of chitosan with methacryloyloxyethyl trimethylammonium chloride (DMC) via SET-LRP to enhance the antimicrobial activity of chitosan molecule. A kind of IL, BMIMCl, was used as a medium to make the reaction under homogeneous conditions, which generally leads to more uniform distribution of substituents (e.g., grafts) along chitosan chains. The structure of the obtained product was characterized, and also the antibacterial activity of chitosan derivative was evaluated.

EXPERIMENTAL

Materials

Chitosan (deacetylation degree of 95%) and IL (BMIMCl, mp.73°C) were obtained from Henan Lihua Pharmaceutical Co. (Henan, China). DMC, triethylamine (TEA), 2-bromoisobutyryl bromide (BiBB), 4-(dimethylamino)-pyridine (DMAP), copper bromide, *N,N,N′,N″,N″*-pentamethyldiethylenetriamine (PMDETA), and other reagents were all of analytical grade and used as received.

Synthesis of Chitosan-Based Macroinitiator (CS-Br)[5]

According to previous work,[25] one gram chitosan, 2 g TEA, and 0.5 g DMAP were dissolved completely in pyridine (30 mL) with stirring, and then kept in an ice bath. Four grams BiBB dissolved in 5 mL pyridine was slowly added in droplets into the solution under argon atmosphere. Then, the flask was sealed and kept for 1 h. After that, the flask was removed from the ice bath, and the reaction was allowed to proceed at room temperature for another 24 h. At the end of the reaction, the products were separated by filtration, and then rinsed with pyridine, acetone, and deionized water, and then dried in a vacuum desiccator at room temperature for 48 h. The yield of CS-Br was 76.3%.

Polymerization of DMC on Chitosan through the SET-LPR Method (CS-Br-PDMC)[6]

Based on the literature,[26] the process of polymer preparation was as follows: 0.5 g CS-Br and 16 cm of copper wire were introduced into flask containing 30 g BMIMCl, and then the monomer DMC was added and dissolved. After that, PMDETA and $CuBr_2$ were added, and the flask was then evacuated five times and back-filled with nitrogen. Reactions were carried out in nitrogen atmosphere at 50°C for 8 h under stirring. For the polymerization, a molar feed ratio of [DMC]/[CS-Br]/[Cu(0)]/[$CuBr_2$]/[PMDETA]of 200 : 1 : 1 : 1 : 0.2 was used. Finally, the mixture was exposed to air and poured into acetone. The precipitate was collected by filtration and washed with acetone and water. The yields of CS-Br-PDMC ranged from 83.3% to 91.6%.

Characterization

Conversion of monomer was determined by gravimetry. The number-average molecular weight [M_n, gel permeation chromatography (GPC)] values and molecular weight distribution (M_w/M_n) values of the polymers were measured on a GPC (equipped with a Waters1515 pump, three columns Styragel HT3, StyragelHT4, and Styragel HT5, and a 2414 differential refractometer detector), with tetrahydrofuran as the eluent and monodisperse polystyrene as standard. The flow rate was 1 mL min^{-1}.

Fourier transform infrared (FTIR) spectroscopy was performed using a GX Infrared spectrophotometer by KBr disk. The ^1H nuclear magnetic resonance (NMR) spectra of the sample were obtained on a Bruker Avance III 500 spectrometer with Chloroform-D ($CDCl_3$) as solvent.

Elemental analysis (EA) of C, N, and H was performed on Vario MICRO Elemental Analyzer. Chitosan is the deacetylated chitin, with a large portion of amine (—NH_2) groups. Therefore, the —$NHCOCH_3$ and —NH_2 groups are both present on the polymeric chain of chitosan. The degree of deacetylation (DDA) value of chitosan samples was calculated from the following equation:[27]

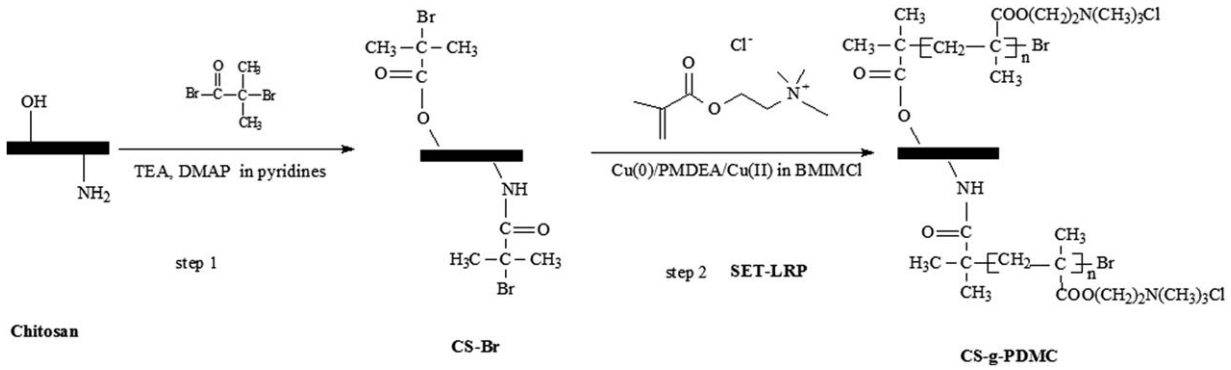
$$\mathrm{DDA}(\%) = \frac{6.875 - \mathrm{C/N}}{1.714} \quad (1)$$

Degree of substitute (DS) of CS-Br was designated as the average number of BiBB groups on each D-glucosamine. It was calculated as C (%)/N (%):[28]

$$\mathrm{DS} = \frac{14}{4 \times 12} \frac{(\mathrm{C/N_{CS\text{-}Br}} - 5.14)}{\mathrm{DDA}(\%)} \quad (2)$$

where the C/N is a percentage ratio of these elements in the chitosan derivative and in the original chitosan. The constant 5.14 is the C/N value of chitosan at a deacetylation degree of 100%, and the constant 4 denotes the number of C atoms of 2-bromoisobutyryl ester groups.

The morphology of the samples was observed by XL30 ESEM-TMP environment scanning electron microscope. The aggregated and self-assembly morphology of chitosan graft copolymer was examined by a Tecnai G2 F20 S-TWIN 200KV transmission electron microscope. To observe the aggregated morphology of graft copolymer in good solvent, samples were prepared according to Meng *et al.*:[29] Five milliliters of chitosan graft copolymer solution in dimethylsulfoxide (w/v = 1/100)



Scheme 1. Synthesis route of chitosan copolymer via SET-LRP.

was slowly dropped into 95 mL acetone under stirring to obtain the solution of copolymer in acetone. Then, one drop of this solution was placed onto a newly cleaved fresh mica surface and was self-dried at room temperature.

Antibacterial Activity

The antibacterial activity of CS-*g*-PDMC was tested on *Escherichia coli* by zone of inhibition experiment. To get the bacterial suspension, a certain amount of bacteria frozen liquid was added into sterile Luria–Bertani liquid culture medium and then cultivated at 37°C in the vibration shaker overnight. Then, the overnight culture was diluted to an optical density at 600 nm (OD_{600}, the value measured by spectrophotometer) of 0.06. Chitosan and CS-*g*-PDMC samples (2.0 mg) were irradiated under UV lamp for 30 min to eliminate the microbial contamination. Each sample was added into 30 mL Luria–Bertani liquid culture medium, which was then inoculated with the bacterial suspension to reach the final bacterial concentration of 10^3 CFU mL^{-1}. For the control test, there was no membrane added. The growth inhibitory effects of the beads were determined by OD_{600} at predetermined time intervals. In the inhibition experiment, the test samples were placed on the *E. coli* agar plates and incubated at 37°C for 24 h before the inhibition zones were measured.

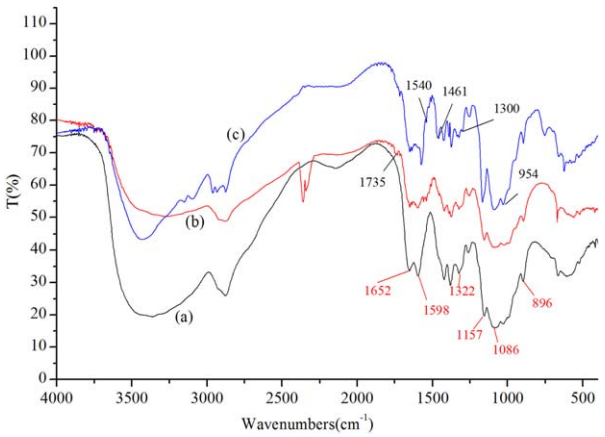


Figure 1. FTIR spectra of CS (a), CS-Br (b), and CS-*g*-PDMC (c). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

RESULTS AND DISCUSSION

Preparation of CS-Br

To prepare graft copolymers using natural polysaccharides as a backbone via SET-LRP, it is essential to introduce alkyl halide onto polysaccharides chains. 2-Bromoisobutyryl ester, known to be an efficient initiator for SET-LRP,[30] was immobilized onto chitosan backbone through the interaction with the —NH_2 or —OH groups in pyridine in the presence of TEA and a catalytic amount of DMAP. The synthesis route is shown in Scheme 1.

The attachment of 2-bromoisobutyrate groups was confirmed by FTIR spectra shown in Figure 1. The FTIR spectrum of CS [Figure 1(a)] shows that the main characteristic peaks of chitosan are at 3446 cm^{-1} (O—H stretch), 2920 cm^{-1} (CH_2 asymmetric stretch), 2875 cm^{-1} (C—H stretch), 1652 cm^{-1} (amide

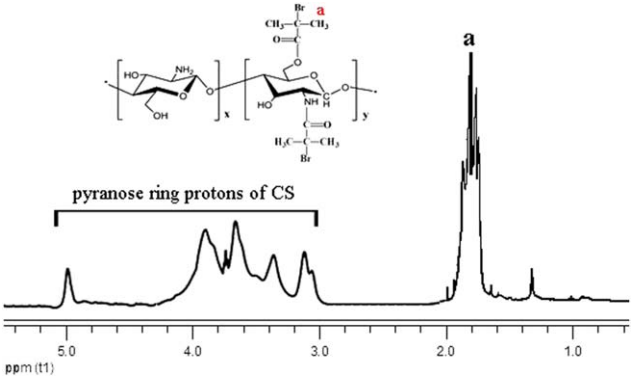


Figure 2. ^{1}H NMR spectrum of CS-Br in $CDCl_3$. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Table I. Element Analysis of CS, CS-Br, and P(CS-Br-DMC)

	N (%)	C (%)	H (%)	C %/ N %	DDA (%)	DS
CS	7.61	39.91	7.36	5.244	95.13	-
CS-Br	6.30	33.33	6.40	5.290	-	0.46
CS-Br-DMC[a]	5.99	38.45	8.28	6.420	-	-

[a] The monomer conversion of CS-Br-DMC calculated from ^{1}H NMR was 10.23%.

Table II. Experimental Conditions and Results of SET-LRP in BMIMCl

No.	[M]/[I]/[Cu^0]/[Cu^{2+}]/[PMDETA][a]	Temp. (°C)	Time (h)	Conversion[b] (%)	M_n[c] (g mol^{-1})	M_w/M_n
1	50 : 0.5 : 0.1 : 0.3 : 0.3	30	8	22.18	3205	1.83
2	100 : 0.5 : 0.1 : 0.3 : 0.3	50	5	40.92	4536	1.56
3	100 : 0.5 : 0.1 : 0.3 : 0.3	80	3	31.43	4275	1.78
4	200 : 0.5 : 0.1 : 0.3 : 0.3	80	4	48.16	5011	1.62
5	200 : 0.5 : 0.1 : 0.3 : 0.3	50	6	58.60	5721	1.49
6	400 : 0.5 : 0.1 : 0.3 : 0.3	50	7	-	-	-

[a] [M]: mole ratio of monomer; [I]: mole ratio of 2-bromoisobutyrate group.
[b] Calculated from gravimetric method.
[c] Measured by GPC.

band), 1598 cm^{-1} (NH_2 band), 1322 cm^{-1} (C—N stretch), 1156 cm^{-1} (bridge O stretch), and 896 cm^{-1} (pyranoid ring stretch).[31,32] Compared with CS, the new absorption peaks of CS-Br spectrum appeared at 1735 cm^{-1} corresponding to the carbonyl group (C═O) characteristic vibration of BiBB [Figure 1(b)], and the reduction of the NH and OH band (around 3400 cm^{-1}) also supported the reaction between BiBB and CS.

The introduction of the BiBB groups to the chitosan chains is further confirmed from NMR measurements. The ^1H NMR spectrum of the CS-Br in $CDCl_3$ is shown in Figure 2. The protons from the methyl groups of the BiBB are detected at around 1.9 ppm, and the peaks from 3.0 to 5.5 ppm are attributed to the pyranose ring of chitosan.

Amount of 2-bromoisobutyrate groups attached onto chitosan was also determined by EA, as can be seen from Table I. CS-Br exhibited a little higher percentage rate of carbon to nitrogen (C %/N %) compared with CS, ascribing to the no nitrogen element of BiBB. The DS of CS-Br were calculated from eq. (2) and listed in Table I.

SET-LRP of DMC from CS-Br

Preliminary research has reported the Cu(0)-mediated SET-LRP of various monomers from polysaccharide-based macroinitiator, using Cu(0) wire or powder with PMDETA or Me_6TREN as catalyst together with low amounts of Cu(II) additive. The polymerization is controlled and results in favorable control over molecular weight and low polydispersity.[33–35]

In this work, the obtained CS-Br efficiently afforded the copper-wire-catalyzed SET-LRP of DMC in BMIMCl (Step 2, Scheme 1). The use of BMIMCl as a reaction medium could result in a homogeneous system. A series of graft copolymers were prepared by varying the ratio between the immobilized initiator and the monomer as shown in Table II. It could be seen that increasing the molar ratio of DMC monomer, raising the temperature, or extending the reaction time may raise the monomer conversion as well as the molecular weight of the polymer. Yet, any further increases in the monomer ratio for similar polymerization times do not cause increases in the monomer conversion. Gels were easily formed, and the reaction was quite difficult to control when the monomer to initiator exceeded 400 : 0.5. Moreover, radical–radical coupling of the propagating chains was prone to occur due to high polymerization temperature.[36] But, the polymerization rate was found to decrease with a decrease in the temperature, and so the polymerization temperature was set at 50°C.

It should be noticed that, in previous studies using chitosan derivatives as the starting materials via "living"/controlled radical

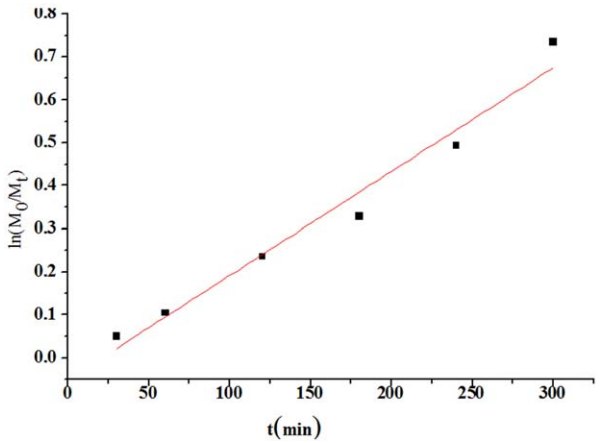


Figure 3. $\ln([M]_0/[M]_t)$ versus time for DMC polymerizing in BMIMCl initiated by CS-Br. [M]/[I]/[Cu^0]/[Cu^{2+}]/[PMDETA] = 200 : 0.5 : 0.1 : 0.3 : 0.3, polymerization temperature is 50°C.[Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

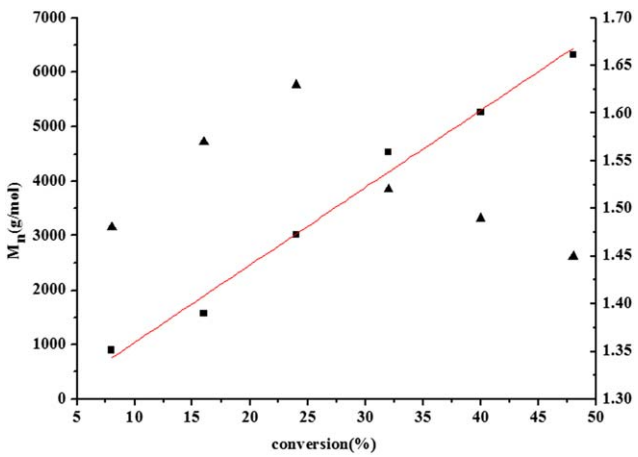


Figure 4. Variation of the M_n and M_w/M_n with the monomer conversion. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

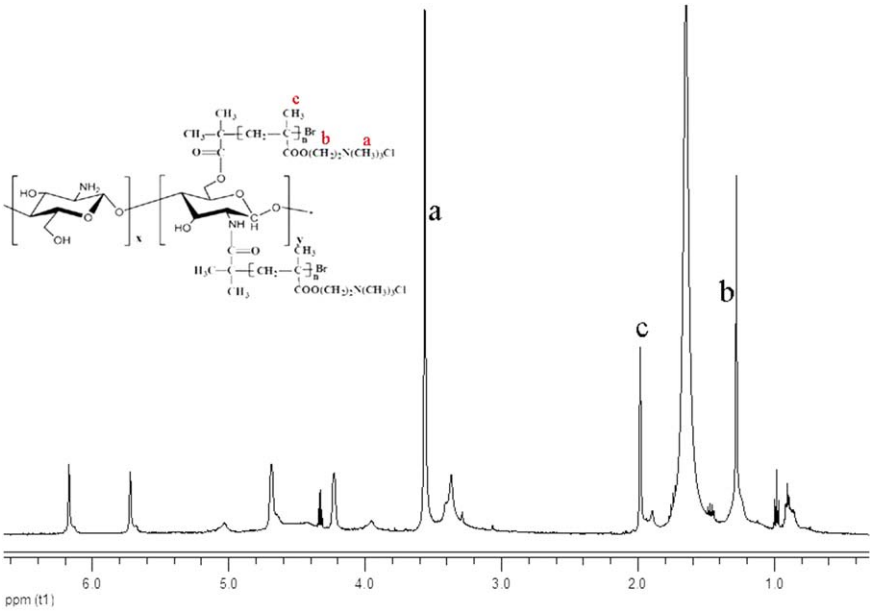


Figure 5. ^{1}H NMR spectrum of CS-*g*-PDMC in $CDCl_3$. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

copolymerization of DMC in mixture of distilled water and methanol,[37] the molecular weight of the chitosan polymer (M_n) was 5272, when the reaction time was longer than 24 h. Obviously, the graft polymerization in IL showed an increase in polymerization rate. This may be due to higher IL polarity, which has also been demonstrated by Harrisson *et al.*[38,39] who investigated the magnitude and effect of solvent on the propagation rate.

Figure 3 shows the kinetic plot of $\ln([M]_0/[M]_t)$ versus time for the SET-LRP of DMC initiated by CS-Br at 50°C, where $[M]_0$ is the initial monomer concentration and $[M]_t$ is the monomer concentration at time *t*. A first-order kinetic plot was observed, indicating that, within this period, the polymerization is of first-order with respect to the monomer. That is, the concentration of the growing radical species in the system is constant during the polymerization. The results of Figure 4 show the relationship between the molecular weight (M_n, GPC) of the polymers and the monomer conversion, and the polydispersity (M_w/M_n, PDI) values of the obtained polymers were low (1.45–1.65) during the polymerization process. The above fact confirmed the controlled/"living" characteristics of SET-LRP of DMC in BMIMCl.

Characterization of CS-*g*-PDMC

The copolymer structures were characterized by FTIR, ^{1}H NMR, and scanning electron microscopy (SEM). Figure 1(c) shows the FTIR spectrum of CS-*g*-PDMC. Characteristic peaks of DMC appeared at 1300 cm^{-1} (—C—N stretching), 1512 cm^{-1} (N—H bending), 1461 cm^{-1} ($—CH_2—N+$, bending), and 954 cm^{-1} ($—N^+(CH_3)_3$, characteristic peaks of

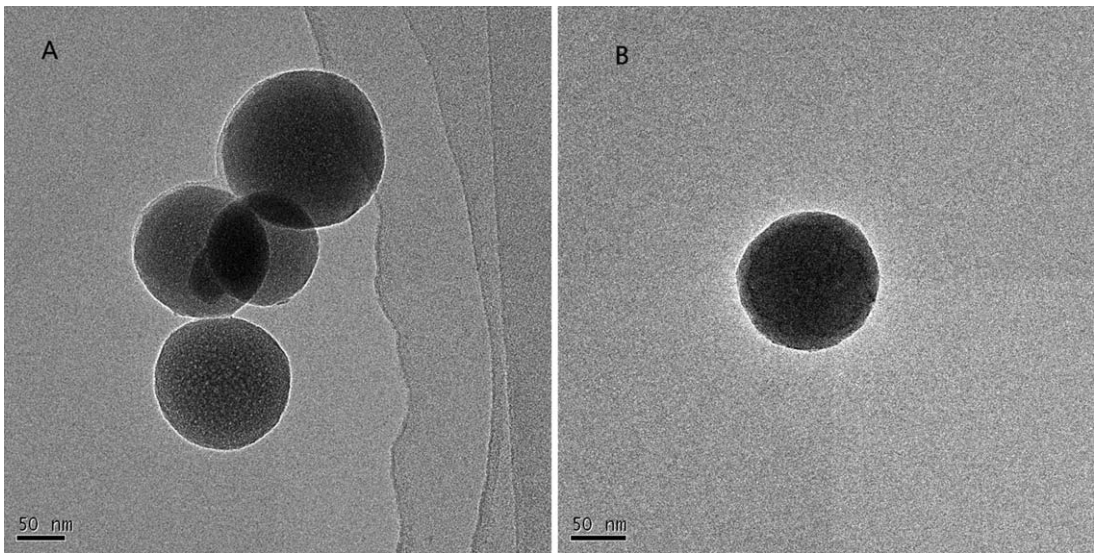


Figure 6. TEM images of chitosan copolymer.

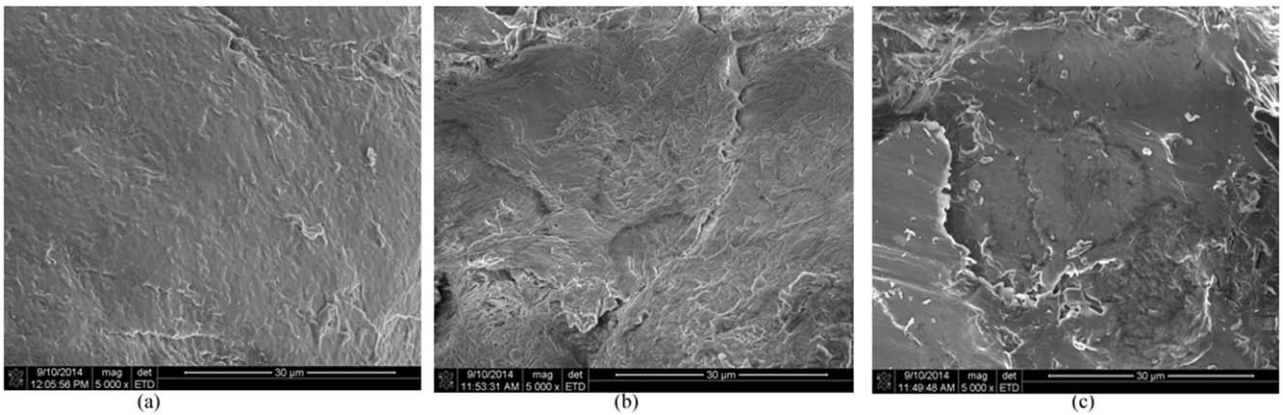


Figure 7. SEM images of chitosan (a), CS-Br (b), and CS-*g*-PDMC (c).

quaternary ammonium group), whose appearance indicates the introduction of DMC onto chitosan [Figure 1(a)].

^{1}H NMR spectrum of CS-*g*-PDMC is shown in Figure 5. It shows that the graft copolymer not only shows the signals of CS-Br, but also has new peaks at 3.56 ppm (a, assigned to H proton of $N^+(CH_3)_3$ group) and 1.278 ppm (b, assigned to H proton of $COO(CH_2)_2$ group), which are due to the presence of the proton in the DMC, further confirming the successful introduction of the DMC group onto CS.

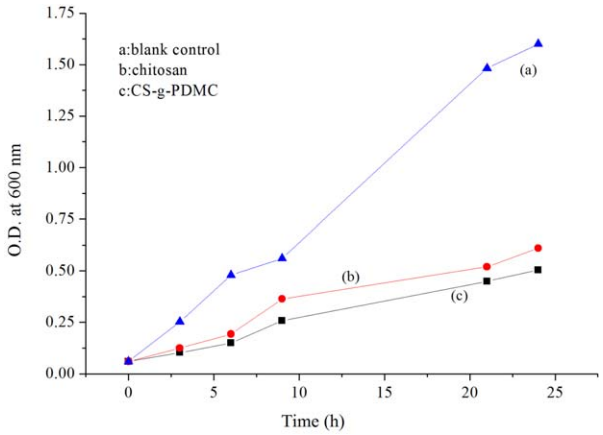


Figure 8. Effect of CS (b) and CS-*g*-PDMC (c) on the growth of *E. coli* at different times. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Morphological property of CS copolymer is investigated by transmission electron microscopy (TEM), as shown in Figure 6. It can be recognized that the copolymer showed self-assembled behavior, have a smooth spherical surface structure, and exhibit well monodisperse spheres in acetone; the typical size of this micelle is about 120–200 nm.

Additional proof for the synthesis of CS-*g*-PDMC was given by their EA data, as shown in Table I. The percentage ratio of carbon to nitrogen (C %/N %) of chitosan graft polymer was changed, which was just higher than that of CS-Br due to the higher C %/N % of DMC.

The surface morphologies of the pristine and functionalized CS were characterized by SEM imaging. As shown in Figure 7(a,b), the surface of the CS microspheres appears relatively smooth [Figure 7(a)]. As compared with the CS microsphere, the condensation reaction of BiBB seems to result in a slightly rougher surface of the CS-Br [Figure 7(b)]. The grafting of DMC polymer brushes causes the surface to be much rougher [Figure

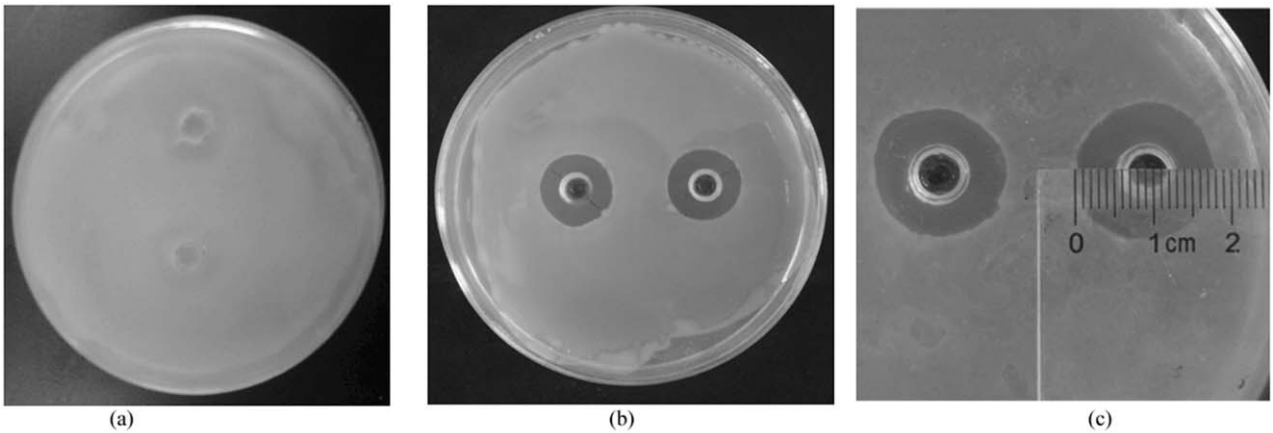


Figure 9. Antimicrobial properties through growth inhibition zone of chitosan (a) and CS-*g*-PDMC (b,c) against *E. coli*.

7(c)]. The introduction of long and dense polymer brushes with a high density of DMC groups onto the CS microspheres is, therefore, expected to change the surface properties and substantially enhance its antibacterial activity.

Antibacterial Properties

The antibacterial activity of chitosan graft copolymer was tested against *E. coli*, and the result is shown in Figure 8. The growth of *E. coli* was affected significantly by Cs-*g*-PDMC compared with unmodified chitosan. The most acceptable mechanism is the electrostatic attraction between positively charged chitosan graft copolymer molecules and negatively charged bacterium cell membrane. The interaction is mediated by the electrostatic forces between the protonated NH_3^+ group of chitosan graft copolymer and the negatively charged bacterium cell surface. When CS had been converted into CS-*g*-PDMC, so that the hydroxyl and amino group of chitosan had been grafted with the quaternary ammonium salt, the positive charge density of the chitosan polymer greatly increased, which led to enhanced adsorption of the polycation onto the negatively charged cell surface. Thus, CS-*g*-PDMC was more easily associated with the cell surface and exhibited better antibacterial activity.

Next, we evaluated the antibacterial activity of the chitosan graft copolymer using the inhibition zone tests. Figure 9 displays the inhibition zones of the CS and CS-*g*-PDMC against *E. coli* after 24 h incubation at 37°C. Clear inhibition zones were observed surrounding CS-*g*-PDMC [Figure 9(b,c)], whereas no obvious inhibition zone was observed around the control CS [Figure 9(a)]. In Figure 9(c), the average diameter of inhibition zone for CS-*g*-PDMC against *E. coli* is 16.0 mm. These results further support the fact that modification of natural chitosan with more antibacterial groups can enhance the antimicrobial activity of the natural chitosan.

CONCLUSIONS

Chitosan was successfully functionalized homogeneously with DMC via SET-LRP method from the bromoacetyl chitosan in BMIMCl. The controlled/"living" characteristics of SET-LRP was confirmed by the linear variation of $\ln([M]_0/[M]_t)$ with time in the first-order kinetics of the copolymerization, linear increase in M_n with conversion, and low PDIs (1.55 in average). The obtained product was characterized by FTIR, ^{1}H NMR, and SEM, and the self-assemble properties of CS-*g*-PDMC was investigated by TEM. The presence of PDMC segments can improve the antimicrobial activity of chitosan, showing much better inhibitive capability against *E. coli*, The results suggested that the obtained chitosan complymer can be applied as antimicrobial material in medical fields.

ACKNOWLEDGMENTS

This research was supported by National Science Foundation of Fujian Province (2015J01049), the Open Research Project of Key Laboratory of Pulp and Paper Science and Technology of Ministry of Education of China (KF201412), Science and Technology Development Fund of Fuzhou University (2012-XY-10, 2014-XQ-11), and National Natural Science Foundation of China (21407026).

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