Preparation, Characterization, and Antibacterial Activities of Para-Biguanidinyl Benzoyl Chitosan Hydrochloride

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ABSTRACT: *Para*-biguanidinyl benzoyl chitosan hydrochloride (*p*-BGBC) is prepared with chitosan (CTS) and *para*-biguanidinyl benzoyl chloride, which is synthesized by acidchloride reaction of *para*-biguanidinyl benzonic acid hydrochloride (*p*-BGBA), as starting material in the medium consisted of $MeSO_3H$ and dimethyl sulfoxide (DMSO). Structure of *p*-BGBC is characterized by FT-IR, ^1H NMR and gel permeation chromatography (GPC), and its antimicrobial activities are evaluated against a Gram-negative bacterium *Escherichia coli* (*E. coli*) and a Gram-positive bacterium *Staphylococcus aureus* (*S. aureus*). Compared with CTS hydrochloride, *p*-BGBC has much stronger antimicrobial activities, which increase with the increase of its degree of substitution (DS) of guanidinylation. When the DS of *p*-BGBC achieves or exceeds 36.8%, its antibacterial activities against the tested bacteria are higher than that of Bromo-Geramium. © 2012 Wiley Periodicals, Inc. J Appl Polym Sci 125: 1146–1151, 2012

Key words: *para*-biguanidinyl benzoyl chitosan hydrochloride; antibacterial activity; synthesis

INTRODUCTION

Chitosan, which is a totally or partially deacetylated derivative of chitin, is a natural biopolymer consisting of β-(1–4)-2-amino-2-deoxy-D-glucopyranose (GlcN) repeating unit and includes a small amount of *N*-acetyl-D-glucosamine (GlcNAc) residues.[1–3] Owing to its biocompatibility, biodegradability and biological activities, chitosan, as a functional biopolymer, has won acceptance in many diverse fields, such as food,[4–6] biomedicine,[1,7,8] biomaterials[9–11] and cosmetics,[12,13] etc. However, the poor solubility of chitosan at pH higher than 6.0 is a main obstacle for its application in more extensive fields, especially as antibacterial agent.[14] To overcome this obstacle, many attempts have been taken up to improve the solubility of chitosan, such as carboxyalkylation,[15,16] quaternization,[17,18] sulfatation,[19] betainatation,[20] guanidinylation,[21] etc. Among these methods, the guanidinylation of chitosan has attracted increasing attention recently.[21–23]

Guanidine is a ubiquitous element in many natural products.[24] The guanidine-containing compounds have been isolated from algae, sponges, and microorganisms, etc.[24–26] Guanidinium group being highly basic (pKa = 11.5 ~ 12.5) is fully protonated at physiological pH and exists in form as guanidinium ion,[27–30] which functions as an efficient recognition moiety of anionic substrates such as carboxylate, phosphate, and nitronate-containing biomolecules.[31,32] Guanidine group is also responsible for the biological activity of many pharmaceutically active compounds.[24] Guanidine derivatives could exhibit a variety of coordination modes and a range of donor properties, therefore, they could be regarded as the efficient ligands for a wide range metal ions.[33,34] As the guanidinium ion is able to adopt a transition state assembly with the substrates to reduce the activation energy or to stabilize anionic intermediates, and is able to orient specific substrates based on their electronic properties, it also participates in numerous enzymatic transformations.[35]

Recently, a number of guanidinylating modifications for different polymers to improve their solubility and antibacterial activities have been reported,[29,30,36,37] especially for the chitosan.[21,22] But no report has been published through using acylation to introduce the guanidinium group into the chitosan by now. The purpose of our study is to synthesize the derivative of chitosan, in which the biguanidinyl existed in the biopolymer chain with

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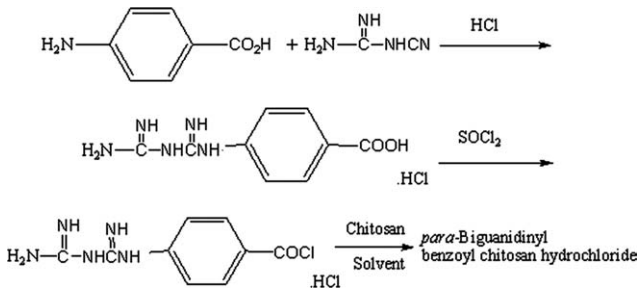

Scheme 1 Synthesis of *para*-biguanidinyl benzoyl chitosan hydrochloride

high substituting degree, through the acylating reaction between chitosan and active guanidinylating agent. Meanwhile, the antibacterial activities of chitosan and its derivative bearing guanidine groups are also investigated.

The preparation of active guanidinylating agent and the derivative of chitosan containing biguanidinium group are presented as Scheme 1.

MATERIALS AND METHODS

Materials

Chitosan was purchased from Sinopharm Chemical Reagent Co., Ltd (Shanghai, People's Republic of China), its viscosity-average molecular weight (M_v) was 3.13×10^5 and degree of deacetylation was determined to be 92.3% by potentiometric titration. 4-Aminobenzoic acid (PABA) and dicyandiamide(DCDA) were purchased from Shanghai Linfeng Chemical Reagent Co., Ltd (Shanghai, People's Republic of China). Sodium hydroxide was purchased from the third factory of chemical reagent of Tianjin (Tianjin, People's Republic of China). Bromo-Geramium was purchased from Sinopharm Chemical Reagent Co. Ltd. A Gram-positive bacterium *Staphylococcus aureus* (*S. aureus*) and a Gram-negative bacterium *Escherichia coli* (*E. coli*) were provided by the Microbiology Laboratory of Nanjing University of Technology (Nanjing, People's Republic of China), and they were inoculated on a gel containing 1% peptone, 2% agar, 3% meat extract, and 0.5% NaCl. All other chemicals were of reagent grade and used without further purification as received.

Preparation of *para*-biguanidinyl benzonic acid hydrochloride (*p*-BGBA)

Totally, 13.7 g of PABA (ca. 0.10 mol) was dissoved in 120.0 mL of hydrochloric acid aqueous solution (mass concentration was ca. 6.0%), and the temperature of mixture was kept at 25°C for about 0.5 h under stirring. Then 21 g of DCDA (ca. 0.25 mol) was added into the above mixture by 8 ~ 10 batches. The mixture was heated to 55°C using water-bath and reacted at this temperature with continuous stirring. After 4.0 h, the temperature of reaction mixture was cooled to lower than 10°C using ice-water bath and filtered. The reside was washed with ethanol and dried under vacuum at 60°C. The product was stored in vacuum prior to use and its yield was 86.2 %.

Preparation of *para*-biguanidinyl benzoyl chitosan hydrochloride (*p*-BGBC)

Totally, 2.58 ~ 10.32 g of *p*-BGBA was dissolved in 10 ~ 30 mL of dimethyl sulfoxide (DMSO) at room temperature. Then, 10 ~ 20 mL of $SOCl_2$ was added dropwise to the mixture with stirring and allowed to react at 60°C for ca. 2 h. Then, DMSO solution containing *para*-biguanidinyl benzoyl chloride was obtained by remove the $SOCl_2$ under vacuum at 60°C.

Totally, 1.64 g of chitosan was dissolved in 20 mL of $MeSO_3H$-DMSO (V/V = 1.0/1.0) at room temperature under continous stirring for 1 h, then the DMSO solution containing *para*-biguanidinyl benzoyl chloride prepared above was added into the mixture. The reaction was stirred at 50°C for 5 h, then ca. 100 mL of cooled ethanol was added into the reactant to stop the reaction and to precipitate the derivative of chitosan. The precipitate was collected by filtration, and washed with cooled ethanol. After the ethanol was removed, the precipitate was dissolved in ca. 50 mL of H_2O and dialyzed in distilled water for 1 day to remove low-molecular compounds. The solution was concentrated and poured into acetone to precipitate the derivative of chitosan. The precipitate was collected by filtration, washed with acetone five times, and lyophilized under vacuum. The products were stored in vacuum prior to use and their yields were 78.2 ~ 85.1%.

FT-IR and ^{1}H NMR spectra analysis

Fourier transform infrared spectra of PABA, DCDA, *p*-BGBA, CTS hydrochloride, and *p*-BGBC were recorded in the frequency range of 400–4000 cm^{-1} using FT-IR spectrophotometer (Nicolet Nexux FT-IR 670 spectrometer). The samples were formed into pellets with KBr.

^{1}H NMR spectra were obtained with Bruker DRX-500 spectrometer that equipped with a trinuclei inverse probe with a Z gradient at 500.13 MHz and 30°C ± 0.5°C. The ^{1}H NMR spectra of *p*-BGBA and *p*-BGBC were measured in DMSO-d_6 solution while that of CTS hydrochloride was measured in D_2O solution, and the samples were dissolved in a 5 mm diameter tube at a concentration of ca. 20 mg mL^{-1}.

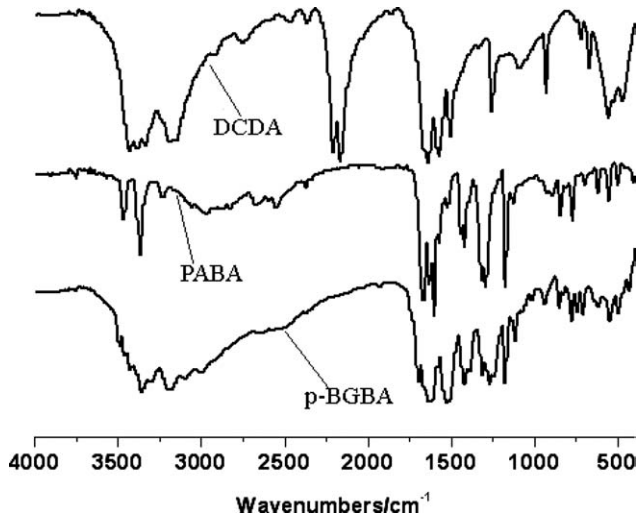

Figure 1 FT-IR spectra of DCDA, PABA, and *p*-BGBA.

Mensuration of degree of substitution (DS) and weight average molecular weights (M_w)

The DS, defined as the number of *para*-biguanidinyl benzoyl groups per 100 sugar residues of chitosan, was calculated by *C/N* ratio according to eq. (1).

$$\mathrm{DS} = \frac{7 \times \mathrm{C/N} + 12\mathrm{DD} - 48}{10\mathrm{DD} - 31} \times 100 \qquad (1)$$

where *C/N* was the mass ratio of carbon versus nitrogen of samples, DD was the degree of deacetylation of chitosan.

The M_w of samples was measured by gel permeation chromatography (GPC) with poly(ethyl oxide) as standard on a Water-208 apparatus (column: Ultrahydrogel 500 and 1000; eluent: water; flow rate: 1.0 mL min^{-1}; column temperature: 32°C; concentration of samples: 0.8 mg mL^{-1}).

Evaluation of antimicrobial activity

The agar plate method was used to determine the minimum inhibitory concentration (MIC) of PABA, CTS hydrochloride, Bromo-Geramium and *p*-BGBCs. The samples were prepared at a concentration of 0.1024% (w/v), and were autoclaved at 121°C for 30 min. Duplicate two-fold serial dilutions of each sample were added into nutrient broth (beef extract 5 g, peptone 10 g to 1000 mL distilled water, pH 7.0) for final concentration of 0.0512, 0.0256, 0.0128, 0.0064, 0.0032, 0.0016, 0.0008, and 0.0004%. Some samples were prepared by decuple diluting method. The culture of each bacterium was diluted by sterile distilled water to ca. 100 CFU/mL. A loop of each suspension of bacterium was inoculated on nutrient medium with sample or control added. After inoculation, the plates were incubated at 37°C for 48 h, and the colonies were counted and the MIC values were obtained.

RESULTS AND DISCUSSION

Identification of resonances in the spectra

The FT-IR spectra of PABA, DCDA and *p*-BGBA were shown in Figure 1, and that of CTS hydrochloride and *p*-BGBC were shown in Figure 2.

In the FT-IR spectrum of DCDA, 2160, and 2210 cm^{-1} were assigned to C≡N stretching vibration, 1570 and 1510 cm^{-1} were assigned to *N*—H bend vibration, 1640 cm^{-1}, 3430, and 3190 cm^{-1} were assigned to C=N and *N*—H stretching vibration, respectively. In the FT-IR spectrum of PABA, 1601, 1572 and 1442 cm^{-1} were assigned to C=C stretching vibration of benzene ring, 1629 cm^{-1} was assigned to *N*—H bend vibration, 1666 cm^{-1}, 3364 and 3463 cm^{-1} were assigned to C=O and *N*—H stretching vibration, 843 cm^{-1} was assigned to 1,4-substitution of benzene ring. Compared with PABA, the C=O stretching vibration and peak of 1,4-substitution of benzene ring were shifted to 1696 cm^{-1} and 851 cm^{-1} in the FT-IR spectrum of *p*-BGBA, respectively. The absorption bands at 1628 cm^{-1}, 1530 cm^{-1}, and 3187 to 3455 cm^{-1} in the spectrum of *p*-BGBA were assigned to C=N stretching vibration, *N*—H bend and stretching vibration of guanidinium group, respectively.

In the spectrum of CTS, 1655, 1593, 1317, 1380 cm^{-1} were assigned to primary, secondary, and tertiary amides, and —CH_3 bend vibration, 3447 and 1084 cm^{-1} could be attributed to the O—H and C—O vibration, respectively. In the spectrum of *p*-BGBC, 1633 cm^{-1} was assigned to *N*—H bend vibration of amide, two strong peaks at 1716 and 1523 cm^{-1} were ascribed to the C=O stretching vibration of —COOH group and *N*—H bend vibration of guanidinium group, respectively.

The ^{1}H NMR spectrum of *p*-BGBA and its ascription was shown in Figure 3, and the ^{1}H NMR spectra of CTS hydrochloride and *p*-BGBC were shown in Figures 4 and 5, respectively.

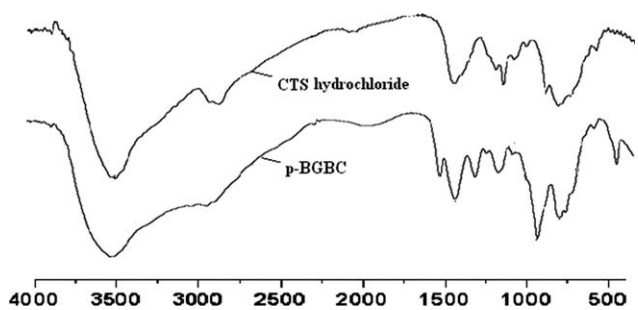

Figure 2 FT-IR spectra of CTS hydrochloride and *p*-BGBC.

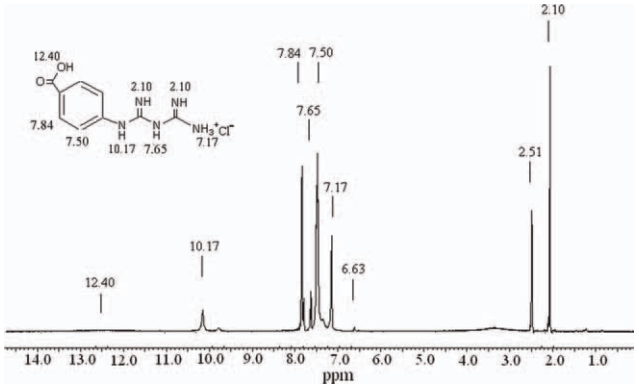

Figure 3 ^{1}H NMR spectrum of *p*-BGBA and its ascription.

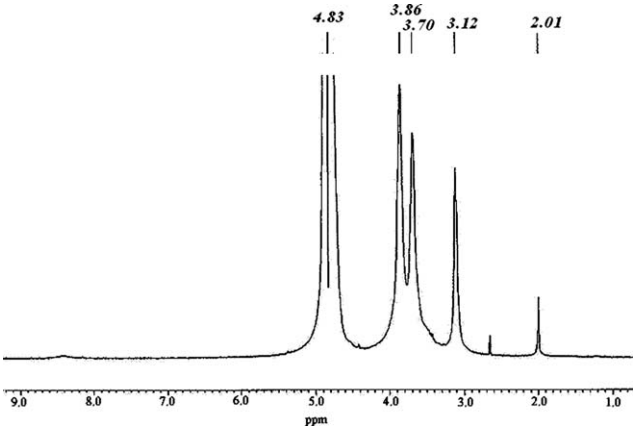

Figure 4 ^{1}H NMR spectrum of CTS hydrochloride.

The peaks at 3.12 and 2.01 ppm in ^{1}H NMR spectrum of CTS hydrochloride were assigned to the proton of C-2 of GlcN and acetyl of GlcNAc unit, and the peaks at 4.83 and 3.4 $\sim$ 4.3 ppm were assigned to the hydrogen of C-1 and C-3 to C-6 of GlcN and GlcNAc, respectively. In the ^{1}H NMR spectrum of *p*-BGBC, the peak at 2.38 and 5.37 ppm were assigned to the proton of —C—NH and —C=NH of guanidinium group, respectively. The peaks at 2.89–4.83 ppm were assigned to proton of sugar unit of chitosan, and the peak at 2.49 ppm was ascribed to the remnant proton of DMSO-d_6.

Antimicrobial activity of p-BGBA, CTS hydrochloride, Bromo-Geramium, and p-BGBCs

The antimicrobial activities of p-BGBA, CTS hydrochloride, Bromo-Geramium, and p-BGBCs against *S. aureus* and *E. coli* were shown in Table I.

The results of antimicrobial experiments showed that the MICs of CTS hydrochloride against the tested bacteria were higher than that of Bromo-Geramium, p-BGBA, and p-BGBC under the experimental condition, which should be owed to its low protonated degree in the neutral condition. The antibacterial activities of p-BGBA, CTS hydrochloride, Bromo-Geramium and p-BGBCs against *S. aureus* are higher than that of them against *E. coli*. The reason resulted in this difference may be concerned with the different structure of cell walls between *S. aureus* and *E. coli*[38]. *S. aureus* is a typical Gram-positive bacterium, its cell wall is fully composed of peptide polyglycogen. The peptidoglycan layer is composed of networks with plenty of pores, which allows foreign molecules to enter into the cell of *S. aureus* without difficulty. On the contrary, the *E. coli* is a typical Gram-negative bacterium, its cell wall is made up of a thin membrane of peptide

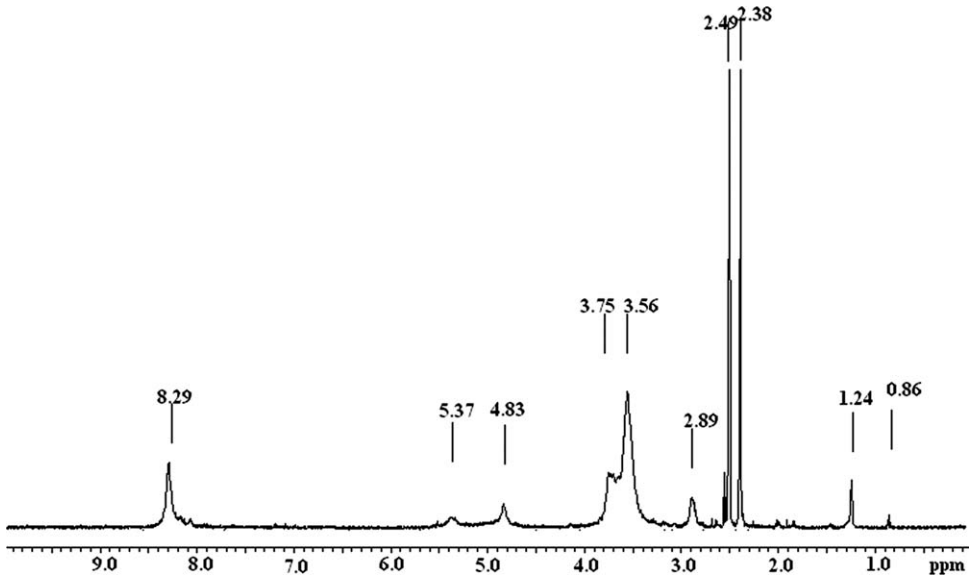

Figure 5 ^{1}H NMR spectrum of *p*-BGBC.

TABLE I
The MIC Value of *p*-BGBA,CTS Hydrochloride, Bromo-Geramium, and *p*-BGBCs Against *S. aureus* and *E. coli*

Sample	DS, %	M ($\times 10^5$)	MIC for *S. aureus*, μg mL^{-1}	MIC for *E. coli*, μg mL^{-1}
CTS hydrochloride	0	3.13	512	>512
p-BGBA[a]			16	32
p-BGBC1	16.2	3.49	32	64
p-BGBC2	27.1	3.58	16	64
p-BGBC3	36.8	3.81	16	32
p-BGBC4	44.7	4.24	8	16
Bromo-Geramium[b]			16	64

p-BGBC1, *p*-BGBC2, *p*-BGBC3 and *p*-BGBC4 were the product of chitosan modified by 10, 20, 30, and 40 mmol *p*-BGBA per 2.32g CTS, respectively. *M* is weight-average molecular weights (M_w) of samples except CTS is viscosity molecular weight (M_v).
[a] The molecular weight of *p*-BGBA is 257.68.
[b] The molecular weight of Bromo-Geramium is 384.44.

polyglycogen and an outer membrane constitute of lipopolysaccharide, lipoprotein, and phospholipids, this bilayer structure results in the foreign molecules to enter into the *E. coli* cell wall with more difficulties.

Because guanidinium group, which is a strongly basic group, could be fully protonated at physiological pH, the introduction of guanidinium group for chitosan chain could result in the increase of its cationic property. Meanwhile, the trace metal cations, which are necessary for the microorganism's growth, could be selectively chelated by the guanidinium group. All these could promote the derivative of chitosan to associate with anions on the bacteria cell wall easily, suppress their biosynthesis, disrupt the mass transport across the cell wall, and accelerate the death of the bacteria[39], so the antimicrobial activities of p-BGBCs against the tested bacteria are better than that of CTS, and this activities increased with the increase of DS of the guanidinium group. The existence of guanidinium group could endow the p-BGBA with excellent chelating ability to metal cations, and promote it to protonation under experimental condition, so the p-BGBA also showed good antibacterial activity against the tested bacteria. Since the antibacterial activities of Bromo-Geramium mainly depended on its cationic property, the antimicrobial activities of p-BGBCs and p-BGBA are stronger than that of Bromo-Geramium some extent.

CONCLUSION

Para-biguanidinyl benzoyl chitosan hydrochloride (*p*-BGBC) was prepared with chitosan and *para*-biguanidinyl benzonic acid hydrochloride as starting material through acidchloride reaction and grafting reaction. The antimicrobial activities of *p*-BGBCs against *S. aureus* and *E. coli* are higher than that of CTS hydrochloride. The synergistic effect between the protonation of guanidinium group at physiological pH and its ability to chelate the trace metal cations could endow the *p*-BGBC with better antibacterial activities than Bromo-Geramium some extent. The increase of degree of substitution(DS) of the guanidinium group for *p*-BGBC could result in the increase of its antibacterial activities against *S. aureus* and *E. coli*. These characters could ensure the *p*-BGBCs to find more extensive applications in the field of antibacterial material.

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