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Carbohydrate Polymers 61 (2005) 472–479
Carbohydrate Polymers
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Preparation and characterization of poly(ethylene glycol)-*g*-chitosan with water- and organosolubility

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Received 11 November 2004; revised 24 June 2005; accepted 28 June 2005
Available online 8 August 2005

Abstract

A new scheme was proposed for synthesizing poly(ethylene glycol)-*g*-chitosan (PEG-*g*-CS), where methoxy poly(ethylene glycol) iodide (MPEG-I) (M_n 2000) was used for *N*-substitution of triphenylmethyl chitosan (TPM-CS) in organic medium. The graft copolymers were obtained by subsequent removal of protecting groups with dichloroacetic acid. By varying PEG-I/TPM-CS feed ratio, the grafting levels (GL) of PEG can be adjusted. The chitosan derivatives were characterized by FTIR, ^{1}H NMR, ^{13}C NMR and DSC. All the copolymers were soluble in water over wide pH range. Furthermore, organosolubility of the hybrids in DMF and DMSO was also achieved when the DS value more than 24%. The lysozyme degradation rate of the copolymers in aqueous neutral medium decreased with the increase of GL value.

Keywords: Chitosan; Poly(ethylene glycol); Graft copolymer; PEG-*g*-Chitosan; Solubility

1. Introduction

Chitosan (CS) is composed of β-(1→4)-2-amino-2-deoxy-D-glucopyranose residues with little or no β-(1→4)-2-acetamido-2-deoxy-D-glucopyranose units. As the *N*-deacetylated derivative of chitin, chitosan is not only naturally abundant but also has many distinctive properties such as biocompatibility, biodegradability, antimicrobial activity, nontoxicity and remarkable affinity to proteins. Therefore, chitosan and its derivatives have been receiving increasing attention in the fields of biotechnology, pharmaceutics, textile, food, cosmetics, and other industries (Li, Dunn, Grandmaison, & Goosen, 1997; Ravi-Kumar, 2000).

In recent years, novel applications of chitosan as peptides, vaccines and genes delivery systems as well as scaffolding material have been carried out (Bernkop-Schnürch, 2000; Illum et al., 2001; Liu & Yao, 2002; Sundararajan & Howard, 1999). However, the poor solubility of chitosan in both water and organic solvents due to its rigid crystalline structure limits its effective utilization in this field.

To overcome this drawback, it is necessary to convert chitosan to water- or organic solvents-soluble derivatives for further biomedical applications. Chemical modification through graft copolymerization is anticipated to be quite promising because it can provide a wide variety of molecular designs by controlling the characteristics of the side chains, including molecular structure, length, and number, and thus obtain novel hybrid materials with desired properties of both natural polysaccharides and synthetic polymers. Compared with chitin and cellulose, the reactivity of chitosan is greatly enhanced by the presence of primary amine groups in the repeat unit and thus it could be specifically modified by controlled chemical reactions (Kurita, 2001). Thereby, a variety of graft copolymers of chitosan were synthesized and evaluated as metal chelating agents (Yazdani-Pedram & Retuert, 1997), surfactant (Ngimhuang et al., 2004), hydrogel (Lee et al., 2004; Yao et al., 2003), drug delivery systems (Gupta, & Ravi-Kumar, 2001; Shim, & Nho, 2003) and other biomedical materials (Kweon, 1998; Yalpani, Marchessault, Morin, & Monasterios, 1991).

Poly(ethylene glycol) (PEG) has been employed extensively in pharmaceutical and biomedical fields because of

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doi:10.1016/j.carbpol.2005.06.022

its outstanding physico-chemical and biological properties, including hydrophilicity, solubility in water and in organic solvents, lack of toxicity, ease of chemical modification and absence of antigenicity and immunogenicity, which allow PEG to be used for many biomedical and biotechnological applications (Harris & Zalipsky, 1997). Recently, several researchers have extensively investigated PEGylation of chitosan and presented some methods to improve the affinity to water or organic solvents (Aiba, 1993; Bentley, Roberts, & Harris, 1998; Harris et al., 1984; Saito, Wu, Harris, & Hoffman, 1997; Sugimoto et al., 1998).

Reductive amination using PEG-aldehyde in aqueous organic acid was the most typical method for grafting PEG onto chitosan. However, the preparation of PEG-aldehyde was generally inconvenient and the degree of conversion was also unsatisfactory (Sugimoto et al., 1998). In addition, Bentley et al. (1998) found that air oxidation of PEG-aldehyde could occur readily and aldol condensation might emerge during the reaction resulting in polymerization of PEG-aldehyde. Recently, Gorochovceva and Makuška (2004) synthesized a novel water-soluble *O*-PEGylated chitosan by etherification between *N*-phthaloyl chitosan and PEG monomethyl ether iodide using Ag_2O as catalyst. Chitosan-*O*-MPEG graft copolymers with different degree of substitution (DS) (5–197%) were obtained. However, we found it difficult to remove the trace amount of Ag_2O dispersed in the final product and to achieve desired solubility in water or common organic solvents unless the copolymer possesses a high DS. Since the copolymer was designed for biomedical applications, it is desirable to avoid any toxic catalyst during the reaction. In addition, the protecting groups should also be thoroughly removed. For this purpose, a simple and convenient procedure for *N*-PEGylation of chitosan was developed, by which chitosan-PEG hybrids with different degree of substitution could be synthesized without need of any catalyst and the solubility was significantly improved in both water and common organic solvents. The methods for removing the protecting groups were also carefully investigated. The copolymers were characterized by ^{1}H NMR, ^{13}C NMR and DSC. The enzymatic degradation of PEG-*g*-chitosan was also investigated.

2. Experimental

2.1. Materials

Chitosan (Sigma, St Louis, MO) was first dissolved in 2% aqueous acetic acid solution, filtered, and then precipitated out by adding concentrated NaOH solution. The precipitate was lyophilized after being washed with distilled water until a neutral pH was reached. The degree of deacetylation (DD) of chitosan was found to be 90% by ^{1}H-NMR analysis and the viscosity-average molecular weight (M_v) was determined to be 2.1×10^5 using Mark-Houwink equation by the method of Wang, Bo, Li, and Qin. (1991).

Poly(ethylene glycol) monomethyl ether (MPEG, M_n 2000) was purchased from Sigma Chemical Co. Triphenylchloromethane was obtained from Acros. Pyridine was dried by calcium hydride for several days before use. Lysozyme from hen egg white was supplied by Biodee Biotechnology Co. (Beijing, China). All the other reagents were of analytical grade and used as received without further purification unless otherwise stated.

2.2. N-Phthaloylation of chitosan

Chitosan precursors were prepared as reported by Nishimura, Kohgo, Kurita, and Kuzuhara (1991). A mixture of purified chitosan (5.00 g, 31 mmol of glucosamine units) and phthalic anhydride (13.8 g, 91 mmol) in *N*,*N*-dimethylformamide (DMF) (100 ml) was heated at 130 °C with magnetic stirring under a nitrogen atmosphere. After 7 h, the resulting clear solution was precipitated into 1000 ml of ethanol after cooling to room temperature and collected by filtration. The resulting product was dried in vacuum at room temperature to give *N*-phthaloylchitosan (yield: 96%). IR (KBr): ν 3450 (O–H), 2930 (C–H, pyranose), 1773 (C=O, imide), 1716 (C=O, imide), 1394 (C=C, phth), 1150–1010 (C–O, pyranose), 721 cm^{-1} (arom, phthaloyl).

2.3. 6-O-Triphenylmethylation of N-phthaloylchitosan

To solution of *N*-phthaloylchitosan (5.00 g, 17.2 mmol) in pyridine (75 ml) was added chlorotriphenylmethane (14.4 g, 51.6 mmol), the mixture was heated at 90 °C with magnetic stirring for 24 h under a nitrogen atmosphere. The mixture was cooled to room temperature and precipitated into 750 ml of ethanol. The precipitate was collected by filtration, washed with 150 ml of ethanol and dried in vacuum at room temperature to give *N*-phthaloyl-6-*O*-triphenylmethylchitosan (yield: 98%). IR (KBr): ν 3450 (O–H), 3080–3031 (C–H, trityl), 2970–2870 (C–H, pyranose), 1651 (C=O, imide), 1596 (C=O, imide), 1490 (C=C, trityl), 1448 (C=C, trityl), 1384 (C=C, phth), 1156–900 (C–O, pyranose), 763 (arom, trityl), 747 (arom, trityl), 703 cm^{-1} (arom, trityl).

2.4. Hydrazinolysis of N-phthaloyl-6-O-triphenylmethylchitosan

N-phthaloyl-6-*O*-triphenylmethylchitosan (5.4 g, 10.1 mmol), hydrazine monohydrate (30 ml) and distilled water (60 ml) were mixed and heated at 90 °C for 16 h. After the mixture was cooled to room temperature, the solid suspended in the mixture was filtered and washed with distilled water, ethanol and ether. The solid was dried in vacuum at room temperature to give 6-*O*-triphenylmethylchitosan (yield: 92%). IR (KBr): ν 3450 (O–H), 3080–3027 (C–H, trityl), 2981–2870 (C–H, pyranose), 1652 (amide I,

N-acetyl), 1598 (amide II, N-acetyl), 1490 (C=C, trityl), 1448 (C=C, trityl), 1156–900 (C–O, pyranose), 763 (arom, trityl), 747 (arom, trityl), 703 cm^{-1} (arom, trityl).

2.5. Iodization of MPEG

MPEG iodide was synthesized based on the reports of Gorochovceva and Makuška (2004). MPEG-2000 (50 g, 25 mmol) was dissolved in toluene (100 ml) and the solvent was distilled to azeotropically remove water. After drying, triphenyl phosphite (19.7 ml, 75 mmol) and methyl iodide (4.67 ml, 75 mmol) were added and the mixture was stirred in a closed ?ask at 120 °C for 6 h in darkness. The reaction mixture was cooled, dissolved in 100 ml toluene, precipitated to 2000 ml ether, filtered, washed several times with ether and dried in a vacuum oven at room temperature to give pale yellow MPEG iodide. The yield of the product was 89%. IR (KBr): ν 2870, 1642, 1460, 1352, 1296, 1250, 1150–1050, 949, 847 cm^{-1}.

2.6. N-PEGylation of 6-O-triphenylmethylchitosan

6-*O*-Triphenylmethylchitosan (1 g, 2.5 mmol) was dissolved in 50 ml of dried (DMF). The amount of MPEG iodide was selected as 0.6 equiv (3 g, 1.5 mmol), 0.8 equiv (4 g, 2 mmol), 1.2 equiv (6 g, 3 mmol) and 1.6 equiv (8 g, 4 mmol), respectively, corresponding to the free amino groups in 6-*O*-triphenylmethylchitosan. To the solution, MPEG iodide was added and the mixture was heated at 60 °C with stirring for 10 h under nitrogen atmosphere, then precipitated into 1000 ml of ether. The precipitate was collected by filtration and redried in vacuum at room temperature. Then, the mixture was washed with acetone and dried in vacuum to give PEG-*g*−6-*O*-triphenylmethylchitosan. Relative yields cannot be determined because the exact degrees of substitution of the graft copolymers were not determined. IR (KBr): ν 3440 (O–H), 3080–3027 (C–H, trityl), 2884 (C–H, pyranose), 1654 (amide I, N-PEG), 1494 (C=C, trityl), 1448 (C=C, trityl), 1110 (C–O), 767 (arom, trityl), 750 (arom, trityl), 703 cm^{-1} (arom, trityl).

2.7. Deprotection of N-PEGylated 6-O-triphenylmethylchitosan (two methods)

Method A: 4.5 g of PEG-*g*-triphenylmethyl-chitosan was dissolved in 100 ml of 50% acetic acid. The mixture was stirred at room temperature for 2–5 h. After that, 50 ml of triethylamine was added to the above solution dropwisely with vigorous stirring. The precipitate generated during neutralization was filtered and washed with water until the pH value of the washing solution reached about 7. The precipitate (PEG-*g*-chitosan) was freeze dried and stored in refrigerator.

Method B: Each group of the above PEG-*g*-6-*O*-triphenylmethylchitosan was added to 30 ml of dichloroacetic acid. The mixture was stirred at room temperature. After 40–60 min, clear viscous solution was obtained. The solution was poured into 500 ml cold ethanol-ether (1:4, v/v). The precipitate generated was filtered, washed with acetone for several times and dried by lyophilization to give PEG-*g*-chitosan. The yields of the products were 86% (0.6 equiv), 83% (0.8 equiv), 78% (1.2 equiv), and 76% (1.6 equiv), respectively, for two consecutive steps. IR (KBr): ν 3430 (O–H), 2920 (C–H, pyranose), 1645 (amide I, N-PEG), 1095–951 cm^{-1} (C–O, pyranose).

2.8. Characterization

^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker Avance DMX500 spectrometer. Chitosan and its derivatives were dissolved in 1% (v/v) CD_3COOD/D_2O or D_2O according to their solubility. As for MPEG iodide, $CDCl_3$ was used. FTIR spectra were recorded in KBr pellets on a Bruker Vector 22 FTIR instrument. The DSC measurements were performed with a Perkin Elmer Pyris 1 instrument. Samples of 8–10 mg were heated from ambient temperature to 180 °C with a heating rate of 10 °C/min, then cooling naturally to 0 °C and subjected to a second run at the same rate under nitrogen atmosphere.

2.9. Solubility tests

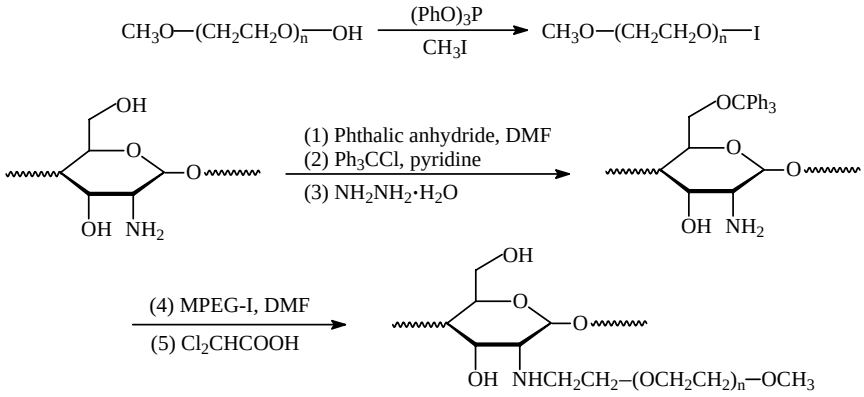
The solubility of the graft copolymers was evaluated in 0.1 M phosphate buffer solution (PBS, pH 7.4), 0.1 M $Na_2CO_3/NaHCO_3$ buffer (pH 10.0), CH_3Cl, DMF and DMSO. The samples were soaked in each solvent at the concentration of 5 mg/ml and the solubility after 24 h was observed.

2.10. Enzymatic degradation of PEG-g-chitosan

PEG-*g*-chitoan solutions (2.5 g/ml) were prepared by dissolving the copolymer in 0.1 M PBS at pH 7.4. Lysozyme (7.5 mg/ml) was dissolved in distilled water, which was added to the above solution to initiate the degradation. Lysozyme/PEG-*g*-chitosan feed ratio in weight was 0.065. The mixture was incubated in 37 °C shaker. The enzymatic activity of lysozyme was monitored by the turbidimetric assay procedure using *Micrococcus lysodeikticus* cells as substrates (Ivanov, Galaev, Kazakov, & Mattiasson, 2001). The specific viscosity, η_{sp}, of the copolymer samples was measured using an Ubbelohde capillary viscometer at 30 °C and calculated by the following formula:

$$\eta_{sp} = \frac{t - t_0}{t_0}$$

where t_0 and t were the eluent time of solvent and PEG-*g*-chitosan solution, respectively. The degradation of PEG-*g*-chitosan was expressed by relative specific viscosity between the degraded and original sample (Zhang & Neau, 2001).



Scheme 1. Preparation of PEG-*g*-chitosan.

3. Results and discussion

3.1. Synthesis of PEG-g-chitosan

Chemical modifications of chitosan under heterogeneous conditions are usually accompanied by various problems including poor extent of reaction, difficulty in regioselective substitution, structural non uniformity of the products. In order to conduct the modification reactions in a well-controlled manner, it is necessary to convert chitosan to organosoluble precursors. The regioselective protection strategy developed by Nishimura et al. (1991) was applied in this work (Scheme 1). It was reported that treatment of chitosan with phthalic anhydride generally results in partial *O*-phthaloylation in addition to the *N*-substitution (Rout, Pulapura, & Gross, 1993). As a consequence, after 6-*O*-triphenylmethylation and removal of the *N*-phthalimido moiety, the DS of the 6-*O*-triphenylmethylchitosan should be less than 1.0. However, there was no undesirable influence on the subsequent reactions appeared in this condition.

PEG monomethyl ether (M_n 2000) was used for the functionalization of PEG in order to avoid the crosslinking reaction by bifunctional PEG derivatives. Iodization of MPEG was carried out though the intermediate, quaternary phosphonium salt, which was made by mixing methyl iodide with triphenyl phosphite at 120 °C. ^{1}H NMR spectra was useful in structural and purity determination (Fig. 1). The degree of the conversion (DC) from -OH to -I groups, estimated by the relative intensities between –OCH_3 ($\delta = 3.38$ ppm) and –CH_2I ($\delta = 3.26$ ppm), was 1.08, implying the existence of PEG activated on both ends. There was no evidence indicating the molecular weight of PEG decreased. The product stored in desiccator was very stable and ^{1}H NMR analysis showed that no decomposition occurred even after one month. Anyway, iodization has the advantages of simple procedure and high DC over other approaches to the functionalization of PEG. In addition, MPEG-I also shows high storage stability.

The use of organosoluble 6-*O*-triphenylmethylchitosan as a precursor enabled the nucleophilic substitution reactions to conduct in homogeneous reaction in polar organic medium, resulting in high DS and good control of the modification reaction. Alkyl iodides are the most reactive alkyl halides, and hence the *N*-substitution reactions could proceed smoothly. After 5–7 h, the reaction mixtures showed no precipitation when dropped into acetone or water, indicating the attainment of high grafting ratio. The extent of these reactions independent of the reaction time after 10 h. As discussed above, there were still some primary hydroxyls existing in 6-*O*-triphenylmethyl-chitosan, which could also react with PEG iodide, especially in alkaline systems. In view of this point, no catalyst was employed in our study.

To deprotect the triphenylmethyl groups of PEG-*g*−6-*O*-triphenylmethylchitosan, Ouchi, Nishizawa, and Ohya (1998) reported the method using 50% (v/v) acetic acid for 2 h, but it was found that by IR completely deprotected products were not available even after 5 h in this study. Fully removal of the triphenylmethyl moiety was attained by using dichloroacetic acid within 60 min (Fig. 2).

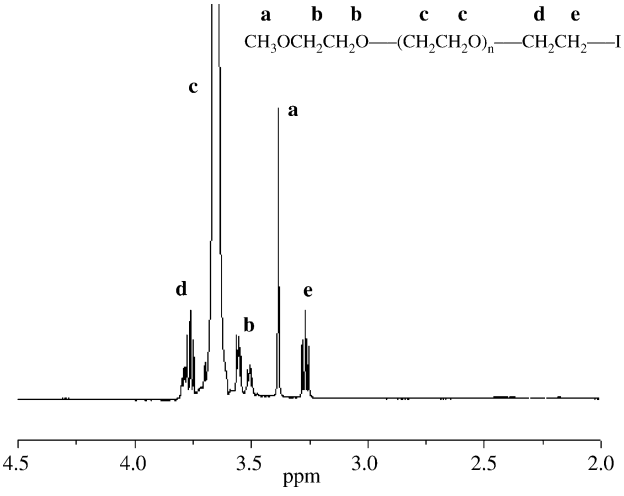


Fig. 1. ^{1}H NMR spectrum of PEG iodide using $CDCl_3$ as solvent.

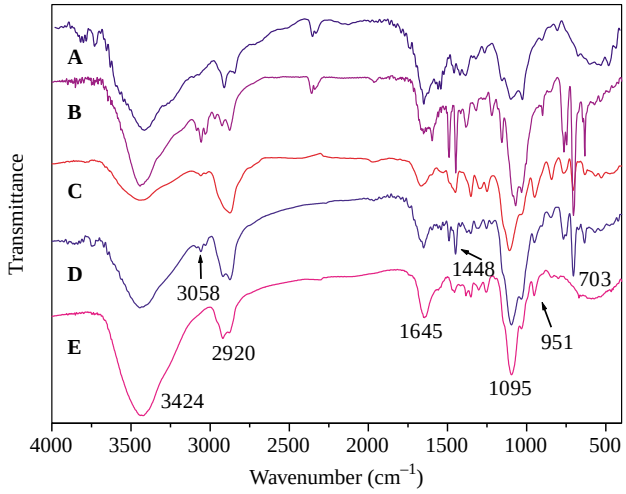
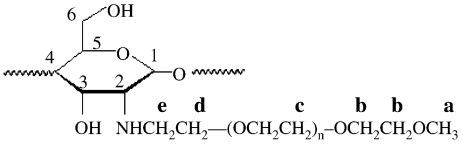


Fig. 2. IR spectra of chitosan (A), 6-*O*-triphenylmethylchitosan (B), PEG-*g*-6-*O*-triphenylmethylchitosan (C), PEG-*g*-chitosan (deprotected by acetic acid) (D) and PEG-*g*-chitosan (deprotected by dichloroacetic acid) (E).

3.2. Characterization of the copolymer

Structure changes of chitosan and its derivatives were confirmed by FTIR spectra (Fig. 2). The 6-*O*-triphenylmethylchitosan shows new absorptions or intensified ones at 2883, 1112, 948 and 842 cm^{-1} attributed to the introduction of PEG. The peaks at 3080–3027 (C–H, trityl), 763, 747 and 703 (arom, trityl) cm^{-1} disappeared after the treatment by dichloroacetic acid suggesting that complete removal of the 6-*O*-triphenylmethyl moieties was achieved.



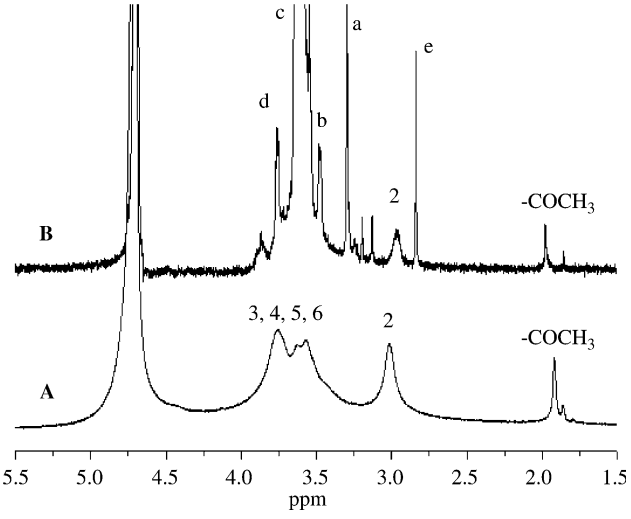


Fig. 3. ^{1}H-NMR spectra of chitosan in 1% (v/v) CD_3COOD/D_2O (A), and PEG-*g*-chitosan with DS 24% in D_2O (B) at 30 °C.

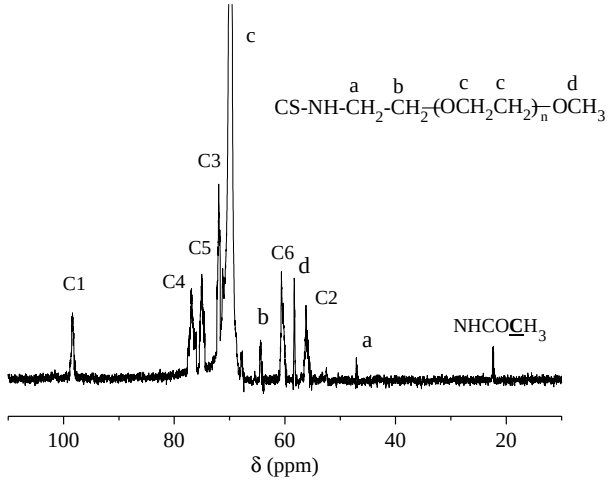


Fig. 4. ^{13}C NMR spectrum of PEG-*g*-chitosan with DS 24% in D_2O at 30 °C.

More information about the graft copolymers was obtained by NMR analysis. In comparison with chitosan, peaks correspond to -NH-CH_2CH_2O- appeared at 2.8 and 47.1 ppm on the ^{1}H NMR (Fig. 3) and ^{13}C NMR (Fig. 4) spectra of PEG-*g*-chitosan, respectively. The sharp single

Table 1
Preparation of PEG-*g*-chitosan

Sample No.	Feed ratio (PEG/CS, mol/mol)	DS[a] (%)	Yield[b] (%)	T_m (°C)	ΔH (J/g)
C1	0.6	15	73	–	–
C2	0.8	24	71	56.4	70.3
C3	1.2	37	68	56.8	73.3
C4	1.6	42	69	59.2	74.2

[a] Degree of substitution of PEG to chitosan determined by ^{1}H NMR.
[b] Calculated from the amount of recovered chitosan in the copolymer by DS.

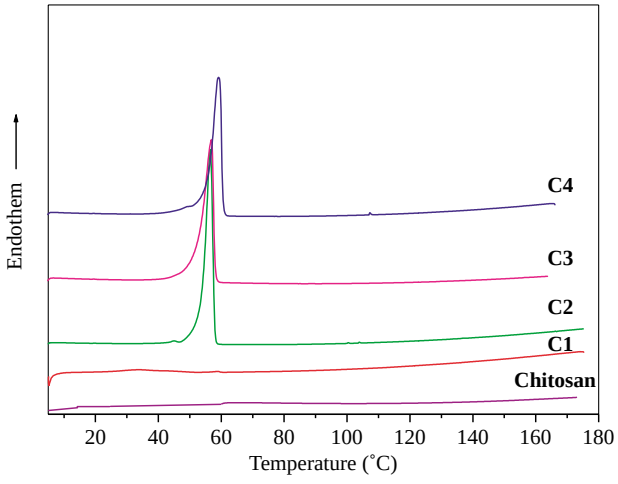


Fig. 5. DSC curves of chitosan and the copolymers obtained from the second run at a heating rate of 10 °C/min.

Table 2
Solubility of PEG-*g*-chitosan

Sample No.	DS[a] (%)	Solubility[b]				
		0.1 M PBS (pH = 7.4)	0.1 M Na_2CO_3/$NaHCO_3$ buffer (pH = 10.0)	$CHCl_3$	DMF	DMSO
C1	15	+	+	−	−	−
C2	24	+	+	−	±	±
C3	37	+	+	−	+	+
C4	42	+	+	−	+	+

[a] Determined by ^{1}H NMR.
[b] Evaluated at the concentration of 5 mg/ml and observed 24 h later. +, soluble; ±, partially soluble or swelling, and becoming soluble by ultrasonic; −, insoluble.

signals at 3.29 and 58.3 ppm were assigned to -OCH_3 of PEG units. The signals at 98.4 (C1), 76.9 (C4), 75.0 (C5), 71.9 (C3), 60.6 (C6) and 56.2 (C2) ppm were attributed to the polysaccharide structures. Because the peak of PEG methylene was overlapped with those of H-3, 4, 5, 6 and 6? of the glucosamine units, the DS value was evaluated by the relative peak intensities between –NH–CH_2CH_2O- (2.8 ppm) and H-2 (2.9–3.1 ppm) in the ^{1}H NMR spectra (Table 1), which was dependent on the molar ratio of PEG iodide to chitosan.

Several researchers have observed the T_g of chitosan to be around 150–160 °C using the dynamic mechanical analysis technique (Ahn, Choi, & Cho, 2001; Ko, Jo, Lee, & Kim, 1997; Ogura et al., 1980). At the same time, Sakurai et al. (2000) reported a much higher temperature value of T_g around 200 °C. Recently, Dong et al. (2004) considered 140–150 °C as the T_g of chitosan by means of four techniques. It is difficult to study the glass transition temperature (T_g) of chitosan since its tendency to absorb moisture and moreover its thermal degradation begins at about 250 °C. Fig. 5 shows the DSC curves of the chitosan and its derivatives obtained from the second heating run in the present study. To eliminate the effect of moisture, two cycles of heating and cooling runs were adopted. The first heating run of initial chitosan gives a single endothermic peak at about 95 °C, attributed to absorbed moisture. However, there was no evident baseline step on the second heating curves of both chitosan and its derivatives in the measured temperature range. It might be due to the fact that DSC measurement is not sensitive enough to detect the relaxation temperature of polysaccharides. Sharp endothermic peaks of C2, C3 and C4 appeared at 56.4 °C (ΔH 70.3 J/g), 56.8 °C (ΔH 73.3 J/g), 59.2 °C (ΔH 74.2 J/g), respectively, corresponding to the melting transition of PEG segments. But for copolymer C1, the melting peak was too weak to be seen clearly in Fig. 5, implying the poor crystallinity of PEG units due to its lower content.

3.3. Solubility of the copolymer

The results of the solubility tests at room temperature are summarized in Table 2. Introduction of hydrophilic PEG chain on amino groups destructed the inherent crystalline structure of chitosan, thus disturbed the intra- and intermolecular hydrogen bonding in chitosan. As a result, all the copolymers were soluble in water of wide pH range within short time and the viscosities of the solution were very low, consistent with the report of Sugimoto et al. (1998), who found that grafting PEG (M_n 2000) onto chitosan could obtain full water-solubility as the DS reach 12%. Moreover, the copolymers C3 and C4 could dissolve in DMF and DMSO, so could C2 after ultrasonification. The organosolubility of the hybrids in DMF and DMSO would extend the applications of chitosan in biomedical engineering.

3.4. Enzymatic degradation of PEG-g-chitosan

To evaluate the influence of PEG branches on the biodegradation of chitosan in aqueous neutral medium, the graft copolymers were subjected to enzymatic degradation by lysozyme. Because the original chitosan was insoluble in neutral medium, it was not possible to compare directly the degradation of chitosan and its

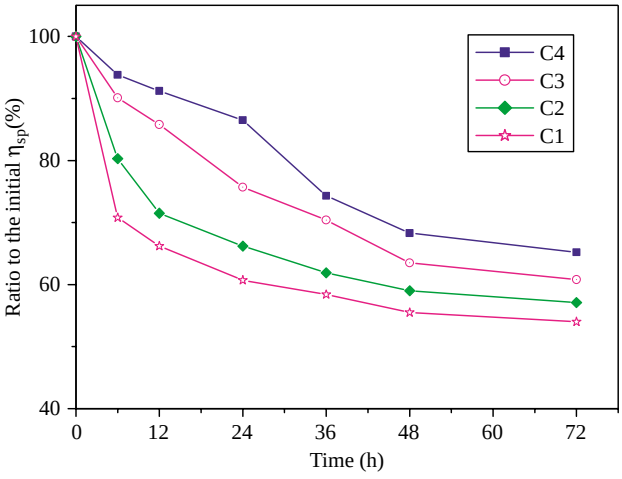


Fig. 6. Enzymatic degradation profiles of PEG-*g*-Chitosan in 0.1 M PBS at pH 7.4 at 37 °C by lysozyme.

copolymers. As shown in Fig. 6, the degradation rate of the copolymer was decreased with the increasing DS value. It was probably owing to the relative stability of PEG to lysozyme. Furthermore, the large 'exclusion volume' of PEG molecule, resulting from its outstanding hydratability and rapid motion in aqueous solution (Harris & Zalipsky, 1997), which can disturb the reaction between chitosan and lysozyme, also should be responsible for this phenomenon. The solution of the copolymers during the observation period showed no appreciable insoluble substance, suggesting the depolymerization mainly occurred in the backbone of chitosan molecules.

4. Conclusions

PEG-*g*-chitosan with different DS was prepared by N-substitution of triphenylmethyl chitosan with MPEG iodide in organic medium and subsequent removal of triphenylmethyl groups. All the copolymers were soluble in water of wide pH range and the viscosities of the solution were lower. Furthermore, organosolubility of the hybrids in DMF and DMSO were also achieved when the DS value more than 24% and it would extend the applications of chitosan in biomedical and other fields.

Acknowledgements

We are indebted to the National Natural Science Foundation of China for their support of this work (Project No. 20304013).

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