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Effects of quaternization and PEGylation on the biocompatibility, enzymatic degradability and antioxidant activity of chitosan derivatives

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ABSTRACT

Chitosan-N-trimethylaminoethylmethacrylate chloride (CS-TM) copolymers with different quaternization degrees (DQ, 30 and 50%) were synthesized and further modified with methoxypoly(ethylene glycol) (mPEG) of different molecular weights (MW, 2 and 5 kDa). The hydrophilicity of the resulting copolymers was significantly increased as evidenced by decreased contact angles. PEGylation with higher mPEG MW could significantly reduce the hemolytic potential, protein adsorption, cytotoxicity and intestinal mucosal damage of CS-TM (DQ of 50%, CS-TM50). PEGylation resulted in a considerable increase in the release of reducing sugars following 84-day lysozyme-catalyzed degradation, and an increase in mPEG MW led to a faster degradation of CS-TM50. The antioxidant activity of CS-TM50 was superior to that of PEGylated CS-TM50, exhibiting dose-dependent reducing power and lipid peroxidation inhibition effect. In conclusion, quaternization and subsequent PEGylation of CS with rational modification degree of its free amino group will be a potential strategy for the development of biocompatible and biodegradable CS derivatives.

1. Introduction

Biocompatibility and biodegradability are two main indispensable considerations when polymers are applied as drug delivery systems because these properties are critically related to the safety. Chitosan (CS), a naturally occurring polysaccharide, is highly stable, hydrophilic, non-toxic, biocompatible and biodegradable. Due to these outstanding merits, CS has received more and more attention in the field of drug delivery system (Kumar, Muzzarelli, Muzzarelli, Sashiwa, & Domb, 2004). However, its applications have only restricted to acidic medium because of its poor solubility in neutral and basic pH. To address such problem and also to endow CS with other physicochemical as well as biological properties, various CS derivatives have been synthesized following chemical modification of their functional groups (amine and hydroxyl) (Amidi, Mastrobattista, Jiskoot, & Hennink, 2010).

Trimethyl chitosan (TMC), a partially quaternized CS derivative with good water solubility, mucoadhesion, and penetration enhancing properties at neutral pH, has been widely applied to delivery of hydrophilic macromolecules, such as peptides and proteins (Sandri et al., 2005). It has been shown that TMC with higher quaternization degree (DQ) would display stronger mucoadhesive and penetration enhancing properties. However, an increase in DQ also leads to an elevation in the cytotoxicity of TMC. For the practical use of TMC as a safe mucoadhesive excipient and permeation enhancer, biocompatibility studies are required to guarantee the absence of cytotoxicity and tissue damage (Amidi et al., 2006; Jintapattanakit, Mao, Kissel, & Junyaprasert, 2008; Kean, Roth, & Thanou, 2005; Verheul et al., 2008).

Polyethylene glycol (PEG) as a hydrophilic polymer, has been frequently used to modify polycations to potentially reduce the cytotoxicity and hemolytic toxicity. In general, PEGylation could improve solubility (Kichler, Chillon, Leborgne, Danos, & Frisch, 2002), shield surface charges (Petersen et al., 2002), prolong circulation time (Ogris, Brunner, Schuller, Kircheis, & Wagner, 1999), and reduce complement activation (Finsinger, Remy, Erbacher, Koch, & Plank, 2000), hence reducing the interactions with plasma proteins and cells. PEG molecular weight (MW) and grafting ratio are two main factors that dominate the PEGylation effect. In the study of Mao et al. (2005), PEG with MW of 550 Da and 5 kDa was grafted onto TMC (DQ 40%) where PEGylation was shown to substantially reduce the *in vitro* cytotoxicity of TMC and in such concern PEG 5 kDa notably outperforms PEG 550 Da. In the cytotoxicity evaluations of different PEGylated poly(ethylene imine) (PEI), it was suggested that the PEGylation degree correlated with both cytotoxicity and oxidative stress in that modification with higher PEGylation degrees and lower PEG chain length was favorable for reduced cytotoxic and oxidative stress response in lung cells (Beyerle, Merkel, Stoeger, & Kissel, 2010).

Besides suitable biological properties and biocompatibility, the degradation behavior should be observed with biomaterials served as drug delivery carriers. CS is thought to be degraded efficiently

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by lysozyme which is known to exist in mammalian bodies (Kean & Thanou, 2010). *In vitro* enzymatic degradation studies on CS suggested that the degradation rate can be affected by the MW of polymer and degree of deacetylation (DD) (Aggarwal & Matthew, 2009). CS of lower MW and lower DD was more susceptible. Other modifications, such as covalent crosslinking and graft copolymerization have been shown to alter degradation profiles of CS (Lu et al., 2009; Prashanth, Lakshman, Shamala, & Tharanathan, 2005).

In our previous investigation, chitosan-N-trimethylaminoethylmethacrylate chloride-PEG (CS-TM-PEG) copolymer (DQ of 44% and PEGylation degree of 34%) with good solubility was designed, and nanoparticles based on CS-TM-PEG have demonstrated desired efficacy for delivery of therapeutic protein as well as nucleic acids (Zhu, Qian, Zhang, Tang, & Yin, 2007). In the present work, CS-TM with different DQs and methoxypoly(ethylene glycol) (mPEG) MWs used for PEGylation was prepared and evaluated in terms of biocompatibility and biodegradation, thereby providing guidelines for the rational design of CS derivatives for effective and safe drug delivery.

2. Materials and methods

2.1. Materials

Chitosan (deacetylation degree 85%) was purchased from Sigma Chemicals (St. Louis, MO, USA). mPEG (MW 2 and 5 kDa) and N-trimethylaminoethylmethacrylate chloride (TMAEMC) were obtained from Fluka (Buchs, Switzerland). Bovine serum albumin (BSA) and lysozyme (20,000 U/mg) were purchased from Shanghai Yuanju Biotech Co. Ltd (Shanghai, China). All other reagents were of analytical grade.

Chitosan-N-trimethylaminoethylmethacrylate chloride (CS-TM) copolymers with different DQs of 30% and 50%, designated as CS-TM30 and CS-TM50, respectively, were obtained through quaternization of CS with TMAEMC by free radical polymerization as described (Qian, Cui, Ding, Tang, & Yin, 2006). CS-TM50 was further grafted with active ester of mPEG of different MWs (2 and 5 kDa) according to the previous report (Zhu et al., 2007), and a constant PEGylation degree of 50% was maintained. The resulting polymers were named as CS-TM50-PEG2K and CS-TM50-PEG5K, respectively.

Chinese hamster ovary (CHO) cells were obtained from the American Type Culture Collection (Rockville, MD, USA) and human hepatocarcinoma cells (SMMC-7721) were obtained from Cell Bank of Shanghai Institute of Cell Biology (Shanghai, China). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Grand Island, NY, USA) containing 10% (v/v) fetal bovine serum at 37 °C in a humidified environment of 5% CO_2.

Male Sprague-Dawley rats weighing 200–250 g and male New Zealand rabbits weighing 2.0–2.5 kg were obtained from the Animal Centre of Fudan University, and were housed at a temperature of 24 ± 2 °C and a relative humidity of 50–70% with a 12-h light/dark cycle. Animal study protocol was reviewed and approved by the Institutional Animal Care and Use Committee, Fudan University, China.

2.2. Characterization of CS-TM-PEG

The degrees of amino substitution of CS and its derivatives were estimated by elemental analysis on a VARIO EL III elemental analyzer (Elemen Tar, Germany).

For the evaluation of the crystallinity of each polymer, X-ray diffraction (XRD) analysis was performed on an X-ray diffractometer (D/max-γB, Rigaku, Japan) at a voltage of 40 kV and a current of 60 mA, employing Cu-Kα radiation (λ = 1.54 Å). The scanning rate was 5°/min and the scanning scope of 2θ was from 2.5° to 50° at room temperature.

Wettability of CS and CS derivatives was explored in terms of contact angle. CS derivative film was firstly prepared by casting 1 mL of CS derivative solution (10 mg/mL) on glass slide followed by vacuum drying at room temperature for 24 h. As for CS film, 1% (w/v) CS solution in 1% (v/v) acetate acid was placed on the glass slide, vacuum-dried, neutralized in a solution containing 1 M sodium hydroxide and 50% (v/v) ethanol, and extensive washed with water before drying. Contact angle measurements were performed using JC 2000A Goniometer (Zhongchen Digital Technology Co. Ltd, Shanghai, China) in sextuplicate for each sample.

2.3. Biocompatibility

2.3.1. Hemolysis

The hemolytic potential of CS derivatives was investigated according to the reported procedures (Zhang, Qu, et al., 2008). Briefly, 10 mL of whole blood from rabbit was depleted of fibrinogen and centrifuged at 3000 rpm for 5 min. The erythrocyte pellets were washed with saline, and resuspended in saline to achieve 2% (v/v) erythrocyte dispersion.

CS was dissolved in 1% (v/v) acetic acid solution at 10 mg/mL, and the pH was adjusted to 6.0 with sodium hydroxide. CS derivatives were dissolved in saline at 10 mg/mL. Different volumes of polymer solution (0.1, 0.5 and 2.5 mL) were added into 2.5 mL of 2% erythrocyte dispersion, and saline was added to obtain a total volume of 5 mL. Negative and positive controls were prepared by adding 2.5 mL of water or saline into 2.5 mL of 2% erythrocyte dispersion. Samples were incubated at 37 °C for a period of time ranging from 30 min to 3 h before microscopic observation.

2.3.2. Dynamic blood clotting

Whole blood from rabbit was diluted with 3.8% sodium citrate solution at a volume ratio of 9:1 to achieve the anticoagulant blood. Two hundred microliters of anticoagulant blood was dropped on polymer films pre-casted on the glass dishes (45 mm in diameter). The clotting reaction was activated upon addition of 20 μL $CaCl_2$ solution (0.2 M) to the blood. Following incubation at room temperature for 10, 20, 40, 60, 80 and 110 min, respectively, 50 mL of water was flooded on glass dishes to lyse the red blood cells which were not trapped in a thrombus. The concentration of hemoglobin dispersed in the water was measured in terms of absorbance at 540 nm by spectrophotometer (Hitachi, Japan). Two hundred microliters of anticoagulant blood in 50 mL of water served as a control. The blood clotting index (BCI) was calculated as follows (Shih et al., 2006):

$$\text{BCI}\ \ (\%) = \frac{A_s}{A_c} \times 100$$

where A_s and A_c refer to absorbance of sample and control, respectively.

2.3.3. Protein adsorption

CS derivative films were prepared by casting 0.4 mL of polymer solution (10 mg/mL) to each well in 24-well plates followed by vacuum drying. CS films were prepared by casting 1% (w/v) CS solution in 1% (v/v) acetate acid and neutralization by Tris–HCl solution (pH = 8.0) before vacuum drying. Polymer films were soaked in 1 mL of BSA solution (4 mg/mL) for different times at 37 °C. The amount of adsorbed protein was calculated by evaluating the decrease of the BSA content in the solution with the Lowry method.

2.3.4. In vitro cytotoxicity (MTT assay)

CHO and SMMC-7721 cells were seeded in 96-well plates at a density of 1×10^4 cells per well and subsequently cultured in a

humidified atmosphere of 5% CO_2 at 37 °C for 24 h. CS and its derivatives at concentrations of 0.08, 0.4 and 2.0 mg/mL were added. Cell viability was monitored after incubation for 6 h using the MTT assay (Yin et al., 2009).

2.3.5. In situ cytotoxicity (lactate dehydrogenase (LDH) assay)

Male rats were anesthetized by an intraperitoneal injection of 350 mg/kg body weight of chloral hydrate solution. Following a midline incision in the abdomen, a 10-cm jejunum segment was isolated and washed gently with pre-warmed saline to remove the intestinal content. One milliliter of polymer solution (5 mg/mL) in saline was injected to the jejunum loop. Saline with no polymer and 1 mL of 1% (w/v) sodium tauroglycocholate were used as negative and positive control, respectively. Following 2-h incubation in the abdominal cavity, the intestinal fluid was collected and centrifuged at 3000 rpm for 10 min. The LDH content in the supernatant was determined using an LDH kit (Changchun Huili Biotech Co., Jilin, China).

2.4. In vitro lysozyme degradation

The enzymatic degradation of CS and its derivatives was performed in acetic acid/sodium acetate buffer solution (0.1 M, pH 5.0) at 37 °C containing 40 μg/mL of lysozyme and 2 mg/mL polymer. The content of reducing sugars in the medium was estimated by the modified Schales method at predetermined time intervals. Briefly, 0.5 mL of samples were transferred to 1.0 mL of water and 2.0 mL of Schales solution (0.5% (w/v) potassium ferricyanide in 0.5 M sodium carbonate solution), boiled for 15 min. After cooling to room temperature, the absorbance at 420 nm of each sample was detected by spectrophotometer (Hitachi, Japan). The content of reducing sugars was calculated according to the calibration curve which was obtained using N-acetyl-glucosamine as a standard. Degradation ratio of CS and each derivative was quantified by the following equation:

$$\text{Degradation ratio}\ (\%) = \frac{S_r}{S_0} \times 100$$

where S_r and S_0 refer to the content of reducing sugar (μmol) and the total sugar units of initial CS (μmol), respectively.

2.5. Antioxidative activities

2.5.1. Measurement of reducing power

The reducing power of CS and its derivatives was determined according to the reported procedures (Feng, Du, Li, Wei, & Yao, 2007). One milliliter of polymer solution (2.5, 5 and 10 mg/mL) was mixed with 2.5 mL of phosphate buffer (0.2 M, pH 6.6) and 2.5 mL of potassium ferricyanide (1%, w/v). Following incubation at 50 °C for 20 min, 2.5 mL of 10% (w/v) trichloroacetic acid (TCA) was added into the mixture and centrifuged at 12,000 rpm for 3 min. The supernatant (2.5 mL) was mixed with 2.5 mL of water and 1 mL of ferric chloride. The absorbance was measured at 700 nm, higher absorbance indicating greater reducing power.

2.5.2. Lipid peroxidation in rat liver homogenate

The liver obtained from exsanguinated male rat was homogenized in saline at 4 °C, achieving a final concentration of 10% (w/v). One milliliter of polymer solution (2.5, 5 and 10 mg/mL) was incubated with 1 mL of liver homogenate at 37 °C for 2 h before addition of 2 mL of 10% (w/v) TCA and 1 mL of 0.67% (w/v) thiobarbituric acid and subsequent boiling at 100 °C for 15 min. After cooling at room temperature, samples were centrifuged at 3000 rpm for 15 min and the absorbance of the supernatant was measured at 532 nm by spectrophotometer (Hitachi, Japan). The lipid peroxidation inhibition effect was calculated as follows:

$$\text{Inhibition effect}\ (\%) = \frac{A_c - A_s}{A_s} \times 100$$

where A_s and A_c refer to absorbance of sample and control (water instead of samples), respectively.

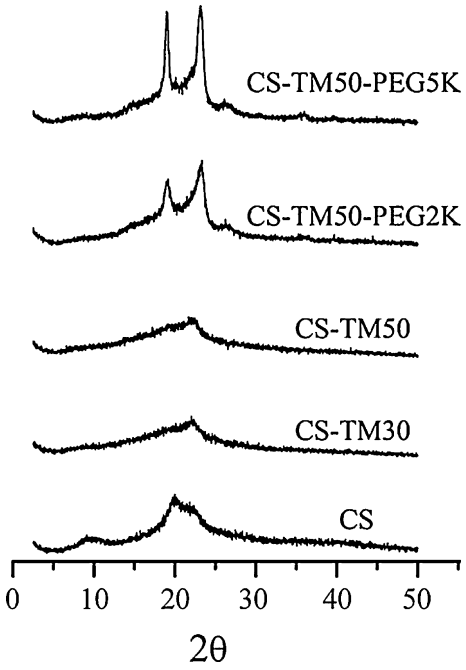

Fig. 1. X-ray diffraction patterns of CS and its derivatives.

2.6. Statistical analysis

Statistical analysis was performed using Student's *t*-test and differences were judged to be significant at $p < 0.05$.

3. Results

3.1. Characterization of CS derivatives

The element analysis results of CS derivatives are shown in Table 1. The amino substitution of CS-TM30 and CS-TM50 was 23.7% and 49.5%, respectively, which was in accordance with the feed ratio (molar ratio) of TMAEMC to CS. A higher carbon to nitrogen molar ratio was observed in CS-TM50-PEG than in CS-TM50, confirming grafting of the mPEG moiety. The degree of PEGylation was calculated to be about 50% based on the carbon to nitrogen molar ratio (Miwa et al., 1998).

As shown in Fig. 1, the XRD pattern of CS showed three characteristic peaks at about 11°, 20° and 22°, indicating a high degree of crystallinity of CS (Zhang, Yang, & Nie, 2008). However, for CS-TM30 and CS-TM50, only one broad peak at 22° remained. The disappearance of signal at 11° belonging to polysaccharide sequences indicated that grafting of TMAEMC destroyed the crystallization of CS possibly due to the weakening of hydrogen bonding between the amino and hydroxyl groups. Following PEG modification, two narrow peaks at 19° and 24° appeared in CS-TM50-PEG2K and CS-TM50-PEG5K, which coincided with the diffraction peaks of PEG crystals and thereby demonstrated the crystalline phases of PEG in the copolymers.

The results of the contact angles are as follows: CS (66.3 ± 1.8°), CS-TM30 (58.5 ± 8.9°), CS-TM50 (53.7 ± 2.4°), CS-TM50-PEG2K

Table 1
Quaternization degree (DQ) and PEGylation degree (DP) of CS derivatives as evaluated by elemental analysis.

Sample	Element (%)			C/N[a]	DQ (%)	DP (%)
	C	H	N			
CS	43.50	6.49	7.92	6.41	–	–
CS-TM30	35.95	8.21	6.08	6.90	23.7	–
CS-TM50	37.87	9.72	6.08	7.26	49.5	–
CS-TM50-PEG2K	50.46	8.79	1.29	45.74	49.5	47.8
CS-TM50-PEG5K	51.90	10.72	0.70	86.87	49.5	57.7

[a] Molecular ratio of carbon to nitrogen.

(48.8 ± 4.4°), CS-TM50-PEG5K (45.4 ± 2.7°). Contact angle is an indicator for the hydrophilicity of film surfaces, and lower contact angle correlates to higher hydrophilicity of the polymer. Compared to that of CS, the contact angles of CS-TM30 and CS-TM50 were decreased to some extent by TMAEMC grafting. A further decrease of contact angle was observed for CS-TM50 following PEGylation ($p < 0.05$ vs CS-TM50), which suggested that modification of mPEG groups may provide a more wettable surface. Nevertheless, no significant difference was observed between mPEG 2 kDa and 5 kDa.

3.2. Biocompatibility

3.2.1. Hemolysis

Table 2 shows the response of red blood cells to various CS derivatives. Hemolysis and excessive amounts of clumping and aggregation were observed following incubation with CS-TM30 and CS-TM50 at 5.0 mg/mL for 1 h. As for PEGylated CS-TM50 at such concentration, the erythrocytes precipitated at the bottom and dispersed after shaking and the supernatant was achromatic and transparent within the observation period for 3 h, indicating the absence of hemolysis and erythrocyte aggregation.

3.2.2. Dynamic blood clotting

The effect of quaternization and PEGylation on the blood thrombus formation is illustrated in Fig. 2. Compared to that of the unmodified CS film, the blood clotting profiles of CS-TM50-PEG2K and CS-TM50-PEG5K films showed higher absorbance of hemoglobin solution during the 110-min incubation period, indicating better clot resistance and improved blood compatibility. The relatively higher absorbance observed for the whole blood incubated with CS-TM30 and CS-TM50 might be attributed to hemolysis induced by cationic polymers, indicating that the quaternized derivatives of CS did not exhibit the desired blood compatibility.

3.2.3. Protein adsorption

Fig. 3 illustrates the protein adsorption profiles in the presence of CS and its derivatives. CS-TM copolymers showed notable BSA adsorption at pH 7.4 and the adsorption ratio further increased with the increase of DQ and incubation time. Compared to the unmodified CS-TM50, PEGylated CS-TM50 copolymers adsorbed less BSA with a relatively low level even after polymer-protein contact for 6 h. Such low protein adsorption characteristic of CS-TM50-PEG5K may also contribute to its low coagulation activity, which was essential for blood contacting applications.

3.2.4. Cytotoxicity

Cell metabolic activity following treatment with CS and its derivatives was analyzed in CHO and SMMC-7721 cells by MTT assay. The viability of both cells was found to be dose-dependent within the concentrations from 0.08 to 2 mg/mL (Fig. 4). The viability of both cells treated with CS at 2 mg/mL was over 80%, while in contrast cell viability significantly decreased to 20–30% following CS-TM50 treatment. Such result confirmed the appreciable toxicity of quaternized polymer with high positive charge density. Cytotoxicity of CS-TM50 was remarkable decreased upon PEGylation, leading to cell viability of approximately 70% after polymer treatment. Besides, it was noted that the higher mPEG MW corresponded to higher cell viability, pointing out that CS-TM50-PEG5K might a more favorable copolymer for further *in vivo* investigation with regard to the reduced impact on cell viability.

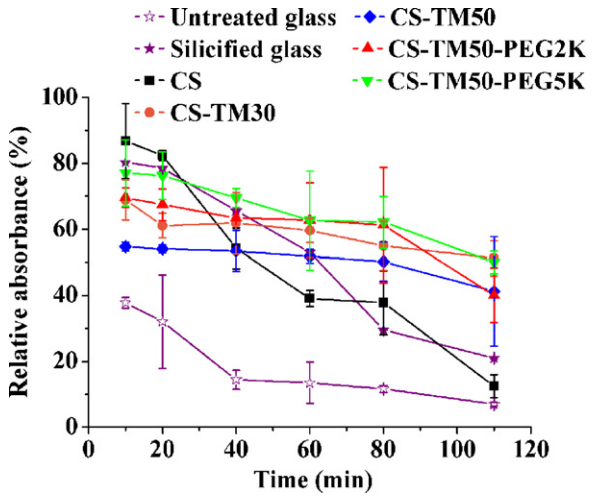

Fig. 2. Dynamic blood clotting curves for CS, CS-TM30, CS-TM50, CS-TM50-PEG2K and CS-TM50-PEG5K. Untreated glass and silicified glass were used as controls of thrombogenic and anticoagulated materials, respectively. Indicated values were mean ± SD ($n = 3$).

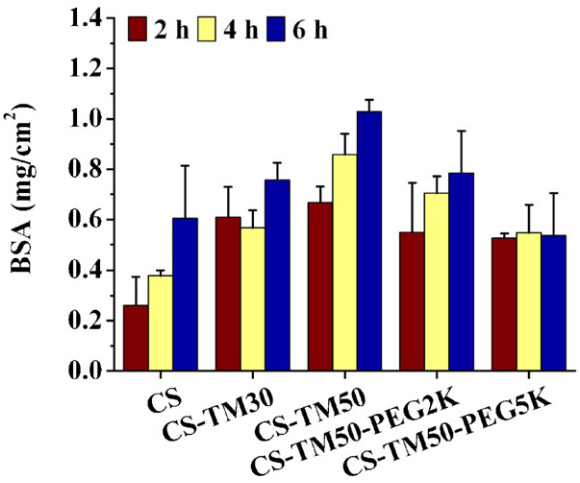

Fig. 3. BSA adsorption on CS, CS-TM30, CS-TM50, CS-TM50-PEG2K and CS-TM50-PEG5K films casted in the 24-well culture plate. Indicated values were mean ± SD ($n = 3$).

Table 2
Hemolysis and erythrocyte aggregation induced by CS and its derivatives.

Sample (mg/mL)	Hemolysis				Erythrocyte aggregation			
	0.5 h	1 h	2 h	3 h	0.5 h	1 h	2 h	3 h
CS (0.2)	–	–	–	–	–	–	–	–
CS (1.0)	+	+	+	+	+	+	+	+
CS (5.0)	+	+	+	+	+	+	+	+
CS-TM30 (0.2)	–	–	–	–	–	+	+	+
CS-TM30 (1.0)	–	–	–	–	–	+	+	+
CS-TM30 (5.0)	+	+	+	+	–	+	+	+
CS-TM50 (0.2)	–	–	–	–	–	+	+	+
CS-TM50 (1.0)	–	–	–	+	–	+	+	+
CS-TM50 (5.0)	+	+	+	+	–	+	+	+
CS-TM50-PEG2K (0.2)	–	–	–	–	–	–	–	–
CS-TM50-PEG2K (1.0)	–	–	–	–	–	–	–	–
CS-TM50-PEG2K (5.0)	–	–	–	–	–	–	–	–
CS-TM50-PEG5K (0.2)	–	–	–	–	–	–	–	–
CS-TM50-PEG5K (1.0)	–	–	–	–	–	–	–	–
CS-TM50-PEG5K (5.0)	–	–	–	–	–	–	–	–

+, the occurrence of hemolysis or erythrocyte aggregation; –, no hemolysis or erythrocyte aggregation was observed.

3.2.5. Intestinal damage

In addition to the influence of metabolic activity, the membrane damage effects caused by the CS derivatives were analyzed by LDH assay. LDH release in rat intestine following treatment with saline, CS, CS-TM30, CS-TM50, CS-TM50-PEG2K, CS-TM50-PEG5K, and a sodium tauroglycocholate solution for 2 h was 1.29 ± 0.45, 3.90 ± 0.28, 3.15 ± 0.60, 3.75 ± 0.88, 2.00 ± 0.22, 1.88 ± 0.30, and 3.26 ± 0.37 U ($n = 4$), respectively. LDH leakage was dramatically induced following 2-h administration of typical penetration enhancer, 1% (w/v) sodium tauroglycocholate, as compared to the negative control ($p < 0.001$), demonstrating the occurrence of epithelium membrane damage and cell death. For CS-TM30 and CS-TM50, the LDH leakage increased by 2.4-fold and 2.9-fold, respectively, compared to the negative control. Nevertheless, an appreciable decrease in cell damage was observed for the two PEGylated CS-TM50 copolymers, and for CS-TM50-PEG5K with high MW, LDH was negligibly leaked into the jejunum loop, which was similar to the negative control ($p > 0.05$), indicating that the copolymer induced no mucosal damage and the intestinal epithelia remained intact throughout. The higher MW of mPEG led to the lower cytotoxic effect, which coincided well with the results of MTT assay.

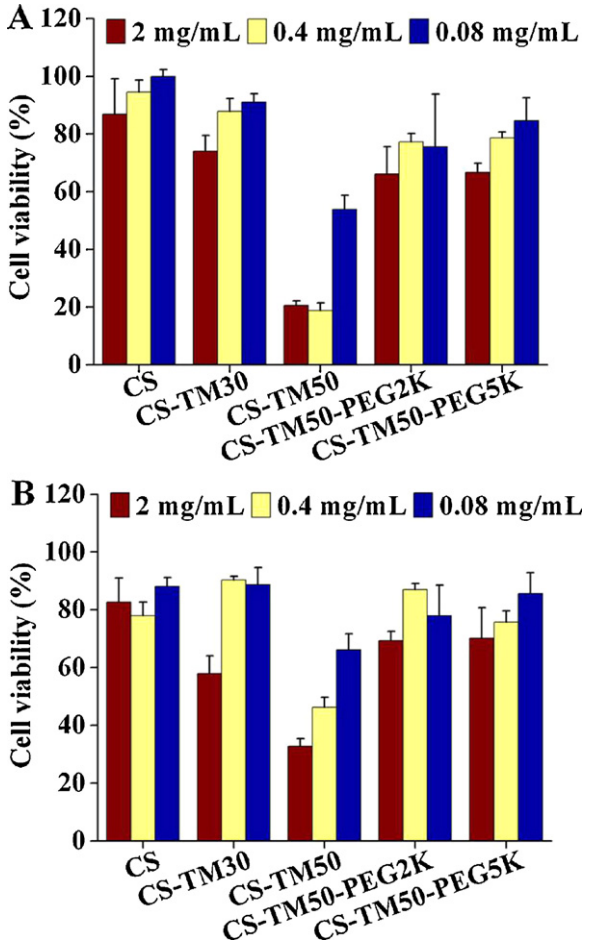

Fig. 4. Viability of CHO (A) and SMMC-7721 (B) cells measured by MTT following incubation with CS and its derivatives at different concentrations for 6 h. Indicated values were mean ± SD ($n = 4$).

3.3. In vitro lysozyme degradation

As shown in Fig. 5, the content of reducing sugars released from CS and its derivative was increased within the 84-day incubation period. The molar percent of reducing sugars to the total sugar units of initial CS was calculated to be 1%, 1.5%, 1.7%, 4.8%, and 7.4% for CS, CS-TM30, CS-TM50, CS-TM50-PEG2K, and CS-TM50-PEG5K, respectively. It was suggested that the amino substitution affected the enzymatic degradation of CS with higher DQ or mPEG MW

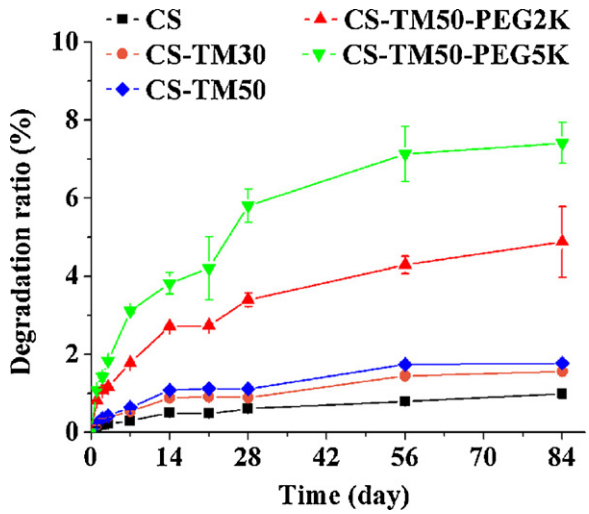

Fig. 5. Degradation of CS and its derivatives by lysozyme (40 μg/mL) at pH 5.0 and 37 °C. Indicated values were mean ± SD ($n = 3$).

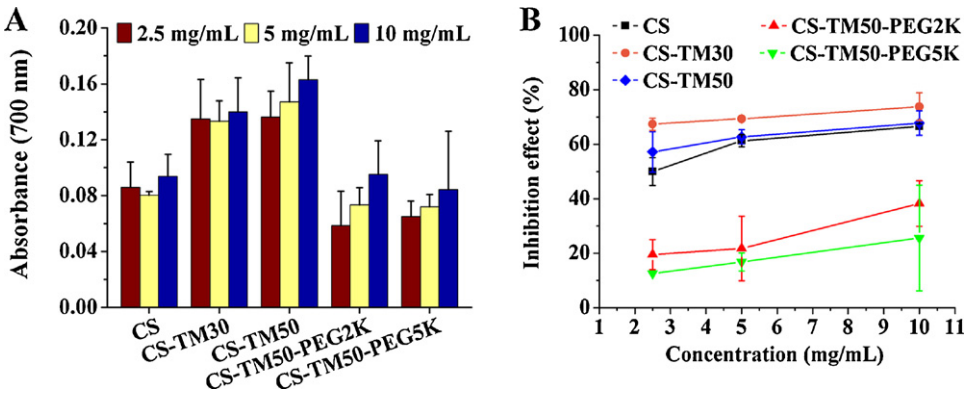

Fig. 6. Antioxidant activity of CS and its derivatives. (A) Reducing power of CS and its derivatives at different concentrations as indicated by the absorbance at 700 nm. (B) Inhibition effects of CS and its derivatives on rat liver lipid peroxidation. Indicated values were mean ± SD ($n = 3$).

leading to faster degradation rate. Besides, PEGylation with high MW was especially remarkable to accelerate the degradation process.

3.4. Antioxidative activities

Fig. 6A shows that the reducing power of CS and its derivatives was elevated with an increase in concentration from 2.5 to 10 mg/mL. Among all the tested polymers, CS-TM50 showed the most pronounced reducing power, while the two PEGylated copolymers exhibited significantly decreased reducing power. In addition, reducing power of CS seemed to be irrelevant with DQ or mPEG MW.

Fig. 6B presents the inhibition of lipid peroxidation in rat liver homogenate by CS and its derivatives. Treatment with CS-TM30 and CS-TM50 at 2.5 mg/mL resulted in a 50% increase in the inhibition of lipid peroxidation, suggesting high protection of liver from peroxidative damage. A notable reduction in the level of lipid peroxidation was observed for CS-TM50-PEG2K and CS-TM50-PEG5K with their inhibition ratio less than 50% even at the maximal concentration of 10 mg/mL.

4. Discussion

Aggregation, crenation, hemolysis, and coagulation of red blood cells induced by polymer treatment would largely restrict the safety application of polymers as drug carriers. Therefore analyses on the blood compatibility are deemed highly necessary. Quaternized CS derivatives possessed high positive charge density and would thus easily interact with negatively charged blood corpuscles, resulting in hemolysis and toxicity. PEG could partly shield the positive charges on polymer backbones, thereby obviously decreasing the haemolytic activity of CS-TM50.

When blood contacts foreign materials, plasma proteins would always be adsorbed onto the material surfaces, and provoke the adhesion of platelets, white blood cells, and some red blood cells onto the plasma protein layer, and ultimately lead to the formation of fibrin (Liu, Lin, Huang, Chen, & Yang, 2005). The reduction of protein adsorption onto the material surface could effectively reduce activation of the immune system, which could consequently prevent or delay the initial coating of foreign materials with components that may lead to phagocytosisacute and clotting. Protein adsorption is dependent on the chemistry, wettability (hydrophilicity/hydrophobicity), energy and topography of the polymeric surface. Among them surface charge as well as hydrophobicity is likely to be most important.

In this investigation, BSA (pI = 4.7), an amphiphilic protein due to the presence of NH_2 and COOH groups in its molecular structure, was selected as a model protein to simulate the protein adsorption. Under the experimental condition (pH = 7.4), BSA carried negative charges. Charge–charge attraction could be responsible for the increased BSA adsorption on CS-TM30 and CS-TM50, and the adsorption amount increased with the increase of DQ and incubation time. It was reported that a hydrophilic surface was good for anti-non-specific protein adsorption (Tangpasuthadol, Pongchaisirikul, & Hoven, 2003). Grafting the hydrophilic PEG to the surface of quaternized CS derivatives could produce a nonspecific barrier to repel the adsorption of proteins. The studies with CS also showed that an increase in hydrophobicity led to increased protein adsorption compared to the more hydrophilic non-modified surfaces (Tangpasuthadol et al., 2003).

Also, the PEG chain length and surface density may influence the interactions between materials and blood components (Finsinger et al., 2000; Ogris et al., 1999). In current investigation, mPEG with 5 kDa MW was preferable to mPEG with 2 kDa MW in reducing the polymer-induced hemolysis, cytotoxicity, and intestinal damage, because its comparatively long chain could more efficiently shield the positive charge of CS-TM50. Collectively, it was suggested that modification of quaternized CS derivatives with high PEG chain length could improve the biocompatibility.

The degradability of CS derivatives will also play an important role when subjected to pharmaceutical applications. Ideally, the carriers will degrade after delivery at the target site, thereby enabling drug release and absorption to the body accumulation. To evaluate the degradation profiles of quaternized and PEGylated CS derivatives, the *in vitro* simulated conditions were adopted. Lysozyme, commonly existing in various human body fluids and tissues, was selected because CS had been found to be degraded predominantly by lysozyme through cleavage of β-(1 → 4)-glycosidic linkages. The degradation products include CS with lower molecular weight, chitooligomers and reducing sugars (glucosamine residues and N-acetyl-glucosamine residues with reducing ends) (Kean & Thanou, 2010). Therefore, the formation of reducing sugars in lysozyme-containing sodium acetate buffer was monitored as an indicator of chitosan degradation. A lysozyme concentration of 40 μg/mL was chosen in correspondence to human serum concentration, and pH 5.0 was chosen to maintain its optimal enzyme activity. The substrate specificity of lysozyme was mainly determined by short range interactions inside the active cleft of lysozyme including hydrogen bonding, van der Waals interactions and electrostatic forces (Li, Xie, Lin, Xie, & Ma, 2009). The higher degradation ratio of CS-TM50-PEG5K was probably attributed to the loose structure of the polymer backbone following quaternization and PEGylation, and subsequent increase in the accessibility of the active sites by the enzyme. In addition, the high hydrophilicity of CS-TM50-PEG5K

may also account for a significant degree of enzymatic susceptibility.

Because a direct correlation between antioxidant activities and reducing power has been previously observed (Xing et al., 2008), CS and its derivatives were also evaluated for their inhibition upon lipid peroxidation. The attained results of reducing power suggested that the antioxidant effect of CS derivatives was relevant to DQ or mPEG MW. The antioxidant property of CS was generally associated with the presence of active hydroxyl and amino groups, which showed antioxidant activity by reacting with free radical and certain precursors of peroxide to prevent peroxide formation (Xie, Xu, & Liu, 2001). The stronger positive charges in the quaternized CS derivatives were believed to be helpful to scavenging the free radicals, thus explaining the result that the antioxidant property significantly increased with the increase of DQ. In comparison, PEGylated CS-TM50 was deficient in free amino groups and its positive charge was greatly shielded, which would account for its impotent reducing power and lipid peroxidation inhibition effect. However, PEGylation could induce to some extent protect the cells from lipid peroxidation (Beyerle et al., 2010), in that modification of PEI with high degree of PEGylation and low PEG chain length reduced lipid peroxidation via the non enzymatic pathway and cyclooxygenase mediated pathway. Accordingly, the detailed mechanism underlying the PEGylation associated lipid peroxidation inhibition effect should be considered in the further study.

5. Conclusions

The effects of quaternization and subsequent PEGylation of CS on its biocompatibility, biodegradability, and antioxidant properties were investigated to provide instructions for the rational design of CS derivatives as desired pharmaceutical excipients. The hydrophilicity of CS was increased significantly after quaternization and PEGylation. Quaternization caused significantly increased hemolytic potential, protein adsorption effect, cytotoxicity and tissue damage of CS, while subsequent PEGylation resulted in improved safety profiles, among which mPEG with higher MW was more favorable for the improved biocompatibility and facilitated lysozyme mediated degradation of CS-TM50. Additionally, quaternized CS derivatives showed dose-dependent reducing power and lipid peroxidation inhibition effect which were weakened following PEGylation. In conclusion, quaternization and PEGylation of CS with rational modification degree of its free amino group will be a potential strategy for the development of biocompatible and biodegradable CS derivatives.

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