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Oligoamines conjugated chitosan derivatives: Synthesis, characterization, *in vitro* and *in vivo* biocompatibility evaluations

Yi Jia[a], Yingqian Hu[b], Yuxuan Zhu[a], Ling Che[a,c], Qiying Shen[d], Jianxiang Zhang[a,*], Xiaohui Li[a,*]

[a] Department of Pharmaceutics, College of Pharmacy, Third Military Medical University, 30 Gaotanyan Zhengjie, Chongqing 400038, China
[b] Key Laboratory of Organosilicon Chemistry and Material Technology of Ministry of Education, Hangzhou Normal University, Hangzhou 310012, China
[c] Department of Pharmacy, Hospital 309 of PLA, Beijing 100091, China
[d] College of Medicine and Hygiene Administration, Hangzhou Normal University, Hangzhou 310036, China

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ABSTRACT

The aim of this study was to synthesize water-soluble chitosan derivatives with good compatibility and multiple amine functionalities for delivery applications. Periodate mediate oxidation was employed to give rise to chitosan with flanking aldehyde groups, onto which oligoamines were conjugated. The polymer structure was confirmed by FT-IR and ^{1}H NMR spectra. These newly synthesized chitosan derivatives displayed high water-solubility. *In vitro* cell culture experiments using both normal and tumor cell lines indicated that some polymers synthesized herein possess extremely low cytotoxicity even at concentrations as high as 5.0 mg/mL. Depending on amine type, the IC_{50} values of newly synthesized chitosan derivatives for both cell lines are 3–4 order of magnitude larger than those of branched polyethyleneimine (25 kDa). Concentration/structure-dependent toxicity was observed for both cell lines as well. *In vitro* hemolysis test using ethylenediamine-conjugated chitosan suggested that this polymer did not elicit any hemolysis at concentrations up to 200 μg/mL. *In vivo* local inflammation evaluation also revealed the compatibility of this polymer post-intramuscular injection. These functionalized chitosan derivatives are candidate materials to construct particulate or hydrogel formulations for biomedical applications.

1. Introduction

During the past two decades, considerable progress has been made in the development of biodegradable polymers for drug delivery, gene therapy, regenerative medicine as well as other biomedical applications (Nair & Laurencin, 2007). A broad spectrum of natural or synthetic polymers capable of undergoing *in vivo* degradation have been extensively studied as biomaterials. Among them, chitosan is one of the most intensively studied materials, mainly due to its excellent biological properties such as biodegradability, biocompatibility, low toxicity, antimicrobial as well as wound-healing activity (Duarte, Mano, & Reis, 2009; Jayakumar et al., 2010; Kim et al., 2007; Kweon, Song, & Park, 2003; Muzzarelli, 2009; Rabea, Badawy, Steurbaut, & Stevens, 2009; Sashiwa & Aiba, 2004). Despite its interesting biological properties, commercial or practical utilization of unmodified chitosan has been limited to some extent, partly resulting from its poor solubility in non-acidic media including aqueous solutions and organic solvents, which confines the processability.

Chitosan, a cationic polysaccharide composed essentially of β(1→4) linked glucosamine units (GlcN) together with some N-acetylglucosamine units (GlcNAc), is the deacetylated form of chitin. In nature, chitin-chitosan exists in the shells of crustaceans, mollusks, the cuticles of insects and the cell walls of fungi, and it is the second most abundant naturally occurring polysaccharide (Rinaudo, 2006). Great attention has been paid in recent years to synthesize chemically modified chitosan derivatives to endow chitosan with diverse physicochemical characteristics and functionalities for various applications (Jayakumar et al., 2010; Kim et al., 2007; Kumar, Muzzarelli, Muzzarelli, Sashiwa, & Domb, 2004; Muzzarelli, 2009; Peniche, Arguelles-Monal, Peniche, & Acosta, 2003; Sajomsang, Gonil, & Saesoo, 2009; Sashiwa & Aiba, 2004). Conjugation of hydrophobic entities such as aliphatic alkyl groups (Sashiwa, Kawasaki, Nakayama, Muraki, Yamamoto, Zhu, et al., 2002), deoxycholic acid (Chae, Son, Lee, Jang, & Nah, 2005), N-dodecyl group (Liu et al., 2003), 5β-cholanic acid (Nam et al., 2009), stearic acid (Hu et al., 2006), poly(ε-caprolactone) (PCL) (Cai et al., 2009a), poly(D, L-lactic acid) (Qu, Wirsen, & Albertsson, 1999), as well as poly(methyl methacrylate) was performed to synthesize organosoluble or amphiphilic chitosan (Kurita, Inoue, & Harata, 2002). In addition, water-soluble chitosan derivatives have been prepared by simple N,O-acetylation in methanesulfonic acid (Sashiwa, Kawasaki, Nakayama, Muraki, Yamamoto,

* Corresponding authors.
E-mail addresses: jxzhang1980@gmail.com (J. Zhang), lpsh008@yahoo.com.cn (X. Li).

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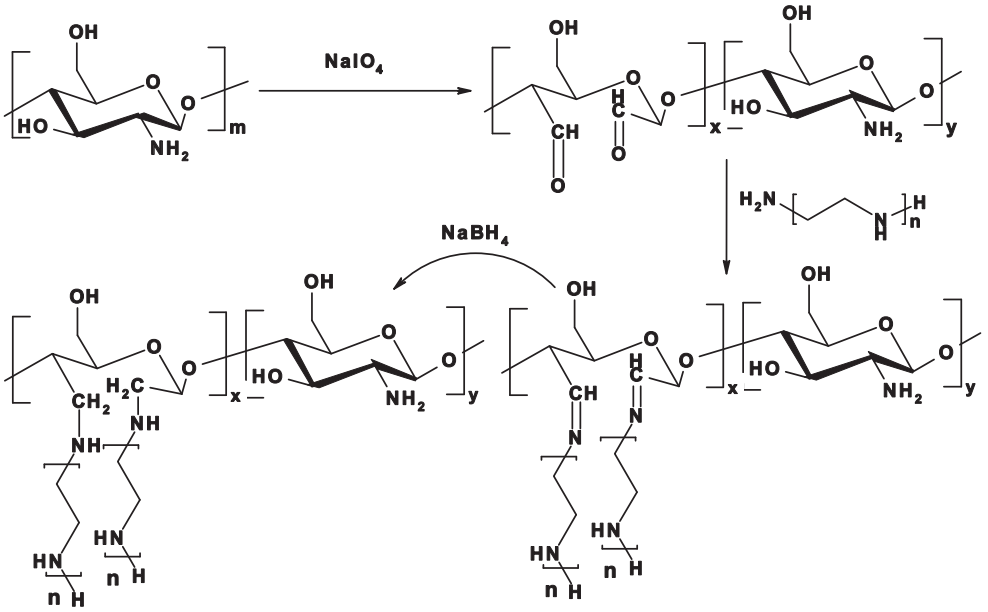


Fig. 1. Schematic illustration of the synthesis of oligoamines conjugated chitosan. $n = 1$, CS–EDA; $n = 2$, CS–DETA; $n = 3$, CS–TETA; $n = 4$, CS–TEPA; $n = 5$, CS–PEHA.

& Aiba, 2002), N-alkylation (Sashiwa, Shigemasa, & Roy, 2000) and Michael reaction (Sashiwa, Yamamori, Ichinose, Sunamoto, & Aiba, 2003). Other hydrophilic components including sugars (Morimoto, Saimoto, & Shigemasa, 2001), crown ethers (Tang, Tan, & Wang, 2002), cyclodextrins (Aoki, Nishikawa, & Hattori, 2003), and polymers such as polyethylene glycol (PEG) (Cai, Jiang, Tu, Wang, & Zhu, 2009b; Sugimoto, Morimoto, Sashiwa, Saimoto, & Shigemasa, 1998), polyamidoamine (PAMAM) dendrimers (Sashiwa, Shigemasa, & Roy, 2002), polyethyleneimine (PEI) (Jiang et al., 2007), were also conjugated onto chitosan to fabricate functionalized water-soluble derivatives for molecular recognition, cell targeting, bioengineering, drug delivery and gene therapy (Pedro, Cabral-Albuquerque, Ferreita, & Sarmento, 2009; Sajomsang, 2010). These studies have significantly increased the number of available polymeric biomaterials based on chitosan. However, there are still increasing demands on chitosan derivatives with desirable properties such as functionality, water-solubility and low toxicity for efficient and safety drug/gene delivery.

The aim of this study is to synthesize a series of oligoamines conjugated chitosan derivatives that possess oligoamine functionality, excellent water solubility in aqueous solutions. The cytotoxicity of synthesized polymers was evaluated by *in vitro* cell culture using both normal and tumor cell lines. *In vitro* hemolysis test and *in vivo* local inflammation evaluation were carried out as well.

2. Materials and methods

2.1. Materials

Chitosan with a deacetylation degree of ≥85% (M_v determined by viscosity method is 230 kDa) and sodium borohydride (granular, 10–40 mesh, 98%) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Sodium periodate (99%) was obtained from Fisher (USA). Ethylenediamine (EDA), diethylenetriamine (DETA), triethylenetetramine (TETA), tetraethylenepentamine (TEPA) and pentaethylenehexamine (PEHA) were purchased from Sigma (St. Louis, USA). All other reagents were commercially available and used as received.

2.2. Oxidation of chitosan

1.0 g chitosan (containing about 6 mmol GlcN monomer) was dissolved into 80 mL acetate buffer (pH 5.0) overnight, and the obtained clear solution was degassed by purging N_2 for 30 min. Thereafter, 0.664 g sodium periodate (3 mmol) was added. The oxidation was then performed at 20 °C. After 12, 24 or 48 h of reaction, the oxidation was ended by adding 10 mL ethylene glycol. The oxidized product was initially precipitated from acetone, and a subsequent purification was performed by dialyzing against deionized water (pH 4.5) for 2 d using dialysis tubing with a molecular weight cutoff (MWCO) of 3500. After being filtered through a syringe filter (0.22 μm), the dialysate was lyophilized. To trace the oxidation process, a similar reaction was performed. At predetermined time points, 6 mL of reaction solution was withdrawn, and purified according to the same procedure.

2.3. Conjugation of oligoamines onto oxidized chitosan (O-CS)

The synthetic route is schematically shown in Fig. 1. Specifically, 400 mg oxidized chitosan dissolved in 40 mL deionized water was slowly added to a Na_2CO_3–borate buffered solution (0.1 M, pH 11) containing excess amount of oligoamine (20 equimolar amount of aldehyde groups). The mixture thus obtained was gently stirred at room temperature for 96 h, and 5 equimolar $NaBH_4$ was then added. After continued stirring for 48 h under the same conditions, the resulting light-yellow solution was poured into dialysis tubing (MWCO: 7000) and dialyzed against deionized water at room temperature for 2 d. The dialysate was filtered through a syringe filter (0.22 μm) to remove any insoluble substance. A straw yellow powder was obtained after lyophilization.

2.4. Measurements

^{1}H NMR spectra were recorded on a Varian INOVA-400 spectrometer operating at 400 MHz. FT-IR spectra were acquired on a Perkin-Elmer FT-IR spectrometer (Spectrum GX). Combustion analysis (C, H, N) was performed on a Perkin-Elmer 2400 Series II Analyzer. Before elemental analysis, polymer samples were thoroughly dried by lyophilization for 3 d.

Table 1
The scoring criteria for hemolysis test.

Reaction items	Scores
Non-hemolysis (colorless supernatant, all the erythrocytes precipitated)	–
Partial hemolysis (red supernatant, partial erythrocytes precipitated)	±
Hemolysis (red supernatant, no erythrocytes precipitated)	+

2.5. Potentiometric titration

Titration experiments were performed to determine the p*K*a values of oxidized chitosan based on pH dependent protonation curves. In brief, polymers were dissolved in 20 mL 0.01 N HCl and titrated with 0.01 N NaOH. The pH values of the solution were determined by a pH meter (Orion 320, Thermo Electron Corporation).

2.6. Evaluation of in vitro cytotoxicity

Hela and RAW264.7 cells were cultured in 96-well plates with 1640 medium (GIBCO, Invitrogen Corporation, USA), containing 10% (v/v) fetal calf serum (GIBCO), 100 U/mL of penicillin per mL and 100 μg/mL of streptomycin, and incubated at 37 °C in a humidified atmosphere of 5% CO_2 for 24 h before the introduction of polymer samples. Cells were then treated with the medium containing polymers at different concentrations for 24 h. After the medium was discarded and cells were washed with buffer solutions for three times, the cells were then incubated with MTT solution (5 mg/mL, 20 μL/well) for another 4 h. Thereafter, MTT solution was removed, 150 μL DMSO was added into each well. The absorbance at 490 nm was measured (Bio-Rad Model 680) after the plates were gently oscillated for 10 min. The cell viability was calculated based on the determined absorbance data.

2.7. Hemolysis test

2% erythrocytes suspension in saline was prepared using freshly harvested rabbit blood. 2.5 mL aliquot of the erythrocyte suspension was then mixed with EDA conjugated chitosan (CS–EDA) at different concentrations, with the total volume of 5 mL. The final concentration of CS–EDA ranged from 12.5, 25, 50, 100, to 200 μg/mL. In positive control, deionized water was added, while saline solution was added for negative group. All the mixed suspensions were incubated at 37 °C, observed each hour for four times. Scoring was made according to the criteria listed in Table 1.

2.8. Local irritation and inflammation

For local irritation and inflammation test in muscle, 1 mL CS–EDA at 25 and 50 mg/mL was injected into left quadriceps femoris muscle of Japanese white rabbits, while saline solution was injected into the contralateral one for control. At day 1 and 7 post-injection, rabbits were sacrificed by carbon dioxide asphyxiation and the longitudinally incised quadriceps femoris muscles were observed. For histological evaluation, muscular tissues were harvested and fixed in formalin, and pathological sections were made and stained with hematoxylin–eosin (H&E).

All the animal care and experimental protocols were performed in compliance with the Animal Management Rules of the Ministry of Health of the People's Republic of China (No. 55, 2001) and the guidelines for the Care and Use of Laboratory Animals of Third Military Medical University.

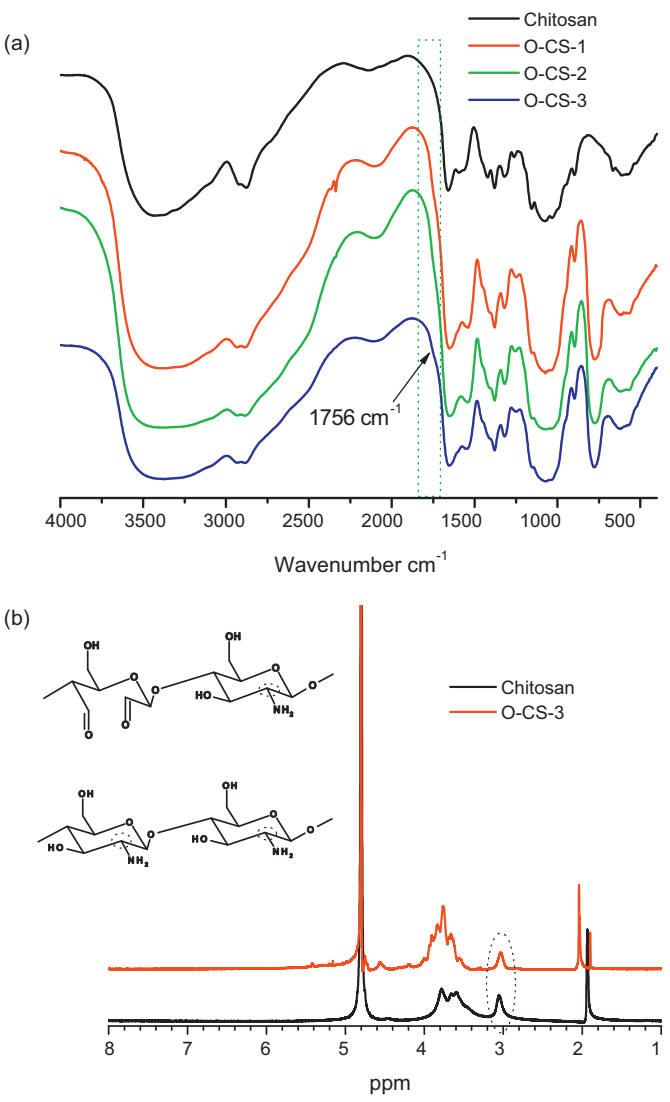


Fig. 2. (a) FT-IR spectra of oxidized chitosans synthesized with different oxidation times: chitosan, un-oxidized sample; O-CS-1, 12 h; O-CS-2, 24 h; O-CS-3, 48 h. (b) ^{1}H NMR spectra of chitosan before and after oxidation.

3. Results and discussion

3.1. Oxidation of chitosan

As well known, periodate mediated oxidation of diols has been widely employed as a routine method for elucidating structures of carbohydrates or polysaccharides including cellulose, starch, glycogen and xylan, among them. During this oxidation reaction, the periodate ion splits the carbon–carbon bond of vicinal diols to give the dialdehyde group (Perlin, 1980). Study by Nicolet and Shinn suggested that this oxidation can be extended to cases in which hydroxyl is replaced by primary or secondary amines (Nicolet & Shinn, 1939).

Herein sodium periodate was employed as the oxidant since it exhibits significantly higher water-solubility compared with that of potassium periodate (Fig. 1). The molar ratio of GlcN to IO_4^- was kept at 1:0.5. Fig. 2a shows FT-IR spectra of oxidized chitosans (O-CS) synthesized with various reaction times. One can observe a shoulder-peak with absorption at about 1756 cm^{-1} appeared for oxidized samples, compared with the un-oxidized chitosan. This suggested the appearance of aldehyde groups by periodate mediated oxidation, since the absorption at 1756 cm^{-1} is a characteristic

Table 2
Aldehyde content and molecular weight of oxidized chitosan.

Polymer	Oxidation time (h)	The degree of oxidation (mol%)		M_v(kDa)[a]
		^{1}H NMR	Elemental analysis	
O-CS-1	12	30	32	31.2
O-CS-2	24	39	37	25.8
O-CS-3	48	54	52	18.9

[a] The degree of oxidation was expressed as percentages of dialdehyde groups per 100 GlcN units. The M_v was determined by viscosity method.

band of the carbonyl group in aldehyde. In addition, this shoulder peak was intensified as reaction time was increased, indicating the enhanced aldehyde groups as time increased. ^{1}H NMR spectra of chitosan before and after oxidation are shown in Fig. 2b. It can be observed that the chemical shift at δ = 3.0–3.1 (due to proton signal from the 2-carbon of GlcN) was significantly attenuated for the O-CS-3, indicating the oxidation of vicinal C–C bond substituted with respective hydroxyl and amine groups, and therefore the formation of dialdehyde units. The oxidation degree of chitosan was quantified based on ^{1}H NMR as well as elemental analysis. As listed in Table 2, comparable results were obtained by these two measurements.

Further information on the oxidation of chitosan was provided by tracing the reaction during a period of 96 h. As can be seen from Fig. 3a, the molecular weight of oxidized products dramatically decreased within the first 24 h. This may be attributed the extensive depolymerization during the oxidation. Similar effect has been reported for chitosan as well as other polysaccharides such as dextran, arabinogalactan and pullulan (Azzam et al., 2002; Jiang et al., 2007; Vold & Christensen, 2005). Further increase in reaction time only led to slight decrease of molecular weight. A simple curve fitting suggested that the time-dependent changes of molecular weight excellently matched an equation of exponential decay (R^2 = 0.989). This result is consistent with the previous study by Vold and Christensen (2005). FT-IR spectra of polymers at various time points are illustrated in Fig. 3b. Clearly, no significant qualitative discrimination could be found from the FT-IR spectra of O-CS samples post-oxidation, even after 0.5 h, whereas the difference was clearly shown for the spectra before and after oxidation (Figs. 2a and 3b). Comparison of the absorption intensity at 1756 and 1631 cm^{-1} corresponding to the characteristic bands of carbonyl in aldehyde and amide groups, respectively, indicated that the quantity of aldehyde groups did not significantly increase after 48 h of oxidation. Similar trend was observed from the time-dependent ^{1}H NMR spectra illustrated in Fig. 3c. Again, we did not observe the appearance of new proton signals for polymers obtained at various time points. Determination on the polymer yield showed that the marked reduction occurred within the first 20 h (Fig. 3a). After that, a gradual decrease could be observed. This decreased polymer yield should be resulted from the removal of low molecular weight product during dialysis.

Above results suggested that the oxidation and aldehydes production mainly occurred within the first 24 h of oxidation. Further increasing reaction time would decrease the molecular weight of oxidized products, while only slightly increases the quantity of aldehyde groups produced. The molecular weight change displayed a nearly exponential decay profile. In addition, the polymer yield decreased concomitant with the decrease in molecular weight.

3.2. Synthesis of oligoamines conjugated chitosan

Studies based on polymers with a polypeptide backbone suggested that copolymers functionalized with different amine groups can be employed as efficient non-viral delivery vectors, and they

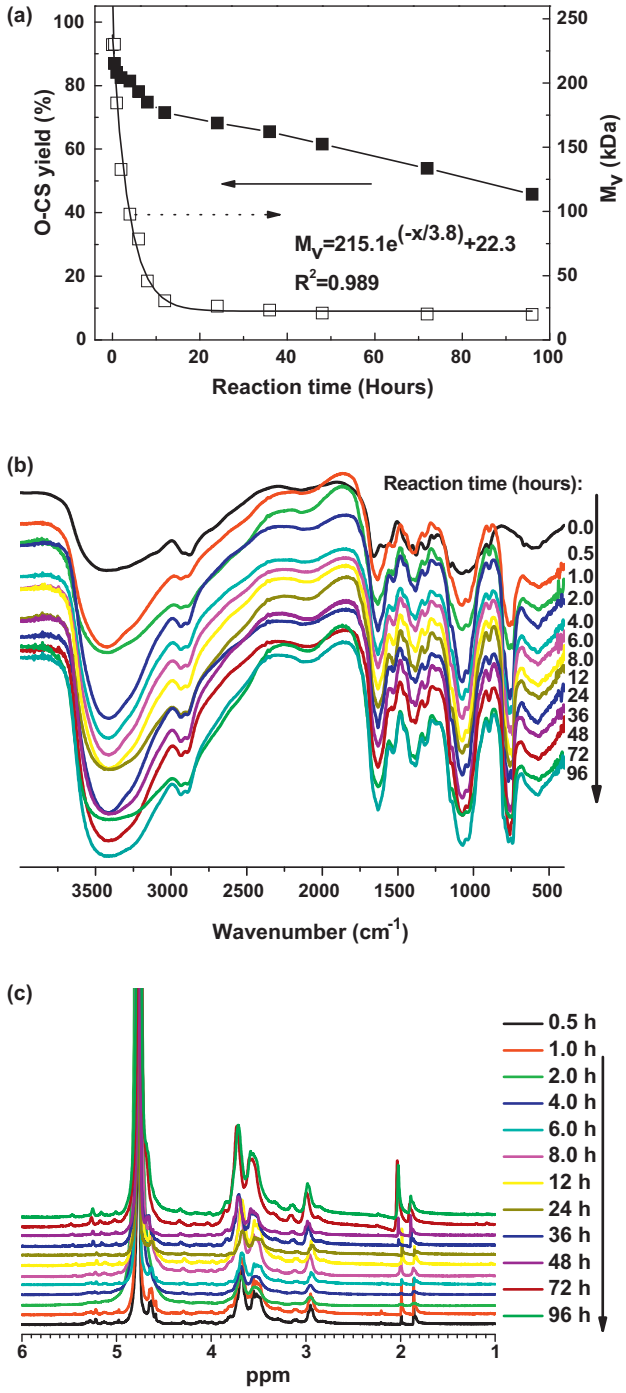


Fig. 3. (a) The effect of reaction time on the yield and molecular weight of oxidized product. (b) Time-dependent FT-IR spectra of oxidized products during the periodate oxidation of chitosan. (c) ^{1}H NMR spectra of O-CS polymers as a function of reaction time.

generally exhibit low cytotoxicity (Kanayama et al., 2006; Zhang, Liu, Xue, Huang, & Zhuo, 2009). The amines modified dextrans were also found to be active for transfecting cells (Azzam et al., 2002). In addition to gene therapy, polyelectrolytes derived from polysaccharides possess great potentials for the delivery of other pharmacologically active macromolecules such as peptides and proteins (Berger et al., 2004; Bin, Cheng, Choi, & Kim, 2008; Hennink & van Nostrum, 2002; Lin & Metters, 2006).

In this study, oligoamines including EDA, DETA, TETA, TEPA and PEHA were conjugated to chitosan to synthesize positively charged polyelectrolytes based on chitosan. A same batch of oxidized chi-

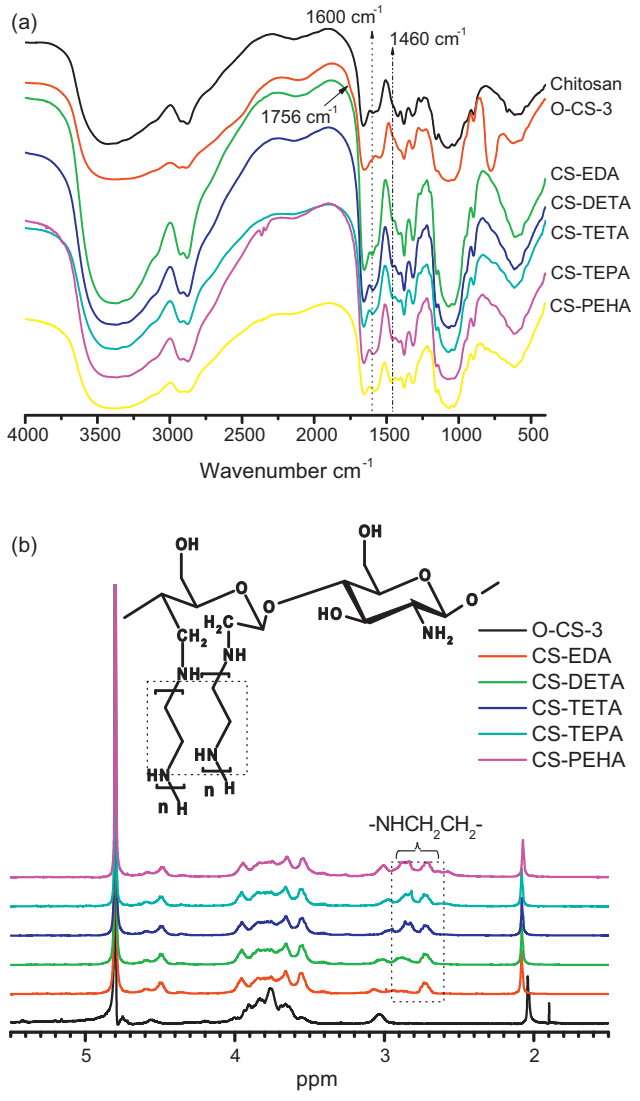


Fig. 4. FT-IR (a) and ^{1}H NMR (b) spectra of raw chitosan and oligoamines–conjugated chitosan derivatives. The spectrum of chitosan was acquired in D_2O containing acetic acid-d_6, while those of other polymers were recorded in pure D_2O.

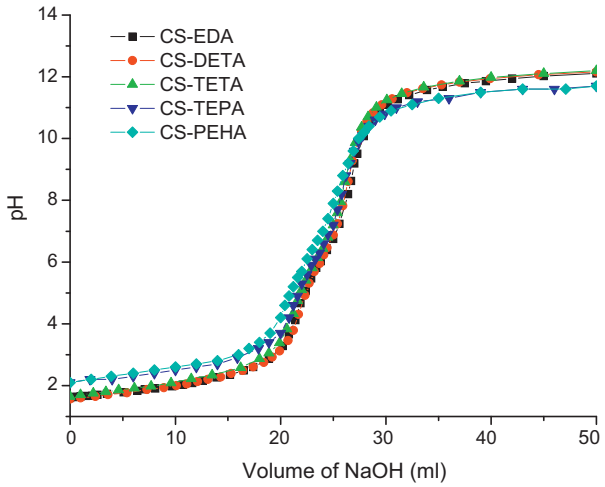


Fig. 5. Titration of aqueous solutions of chitosan derivatives in 0.01 N HCl with 0.01 N NaOH solution.

tosan with flanking aldehyde groups, which was synthesized under the same conditions as those of O-CS-3, was employed to conjugate oligoamines. The degree of oxidation and remaining amino groups in this oxidated chitosan was 51% and 52%, respectively, which were quantified by elemental analysis. As illustrated in Fig. 1, a two-step procedure was adopted to synthesize oligoamine-containing chitosans. Firstly, Schiff base (carbon–nitrogen double bond) linked oligoamine–chitosan polymers were synthesized by a nucleophilic addition and a subsequent dehydration between amine and aldehyde groups. Since the Schiff base is unstable, a reduction process is necessary to convert the carbon–nitrogen double bond to carbon–nitrogen single bond. Accordingly, a reduction using $NaBH_4$ was carried out in the second step to give rise to the target polymers. For the successful conjugation, the main challenge is the cross-linking of aldehyde-containing O-CS in the presence of oligoamine. Preliminary experiments suggested that process conditions like the concentration of O-CS, oligoamine concentration, the molar ratio of oligoamine to aldehyde group, and the dropping rate of O-CS into oligoamine solution are main factors that influence the reactions between aldehyde and amine. It was found that higher concentration of O-CS, lower concentration of oligoamine, lower molar ratio of oligoamine to aldehyde, and faster dropping of O-CS solution favored the cross-linking side reaction. Accordingly, by using lower concentration of O-CS, higher concentration of oligoamine, excess amount of oligoamines, and decreasing the dropping rate of O-CS solution, the side reaction of cross-linking can be largely suppressed. For the synthesis of all the oligoamine-conjugated chitosan polymers, 10 mg/mL of O-CS, 12 mmol of oligoamine, a molar ratio of 20 of oligoamine to aldehyde, and 20 mL/h of dropping rate, were employed.

Fig. 4a shows the FT-IR spectra of chitosan derivatives with various oligoamines as side groups. As can be seen, the shoulder carbonyl peak corresponding to aldehyde disappeared after the amine conjugation. In addition, the absorption at 1600 and 1560 cm^{-1} resulting from the vibration bands of –C–N–H– and $–CH_2–$, respectively, was enhanced. This is especially true for those polymers with relative long amine chains such as CS–DETA, CS–TETA, CS–TEPA, and CS–PEHA. ^{1}H NMR spectra of chitosan and its derivatives are illustrated in Fig. 4b. The selected assignment due to the ethylene units of amines also suggested the introduction of amine side groups. FT-IR together with ^{1}H NMR measurements pointed to the conclusion that amines with different chain length can be successfully conjugated onto the chitosan via a condensation and subsequent reduction reaction. In addition, the final polymer yield (relative to the initial chitosan) was 52.9%, 53.4%, 51.7%, 48.9%, and 49.6% for CS–EDA, CS–DETA, CS–TETA, CS–TEPA, and CS–PEHA, respectively.

Titration experiments were then carried out to determine the p*K*a values of chitosan derivatives based on pH dependent protonation curves. As presented in Fig. 5, similar two-stage protonation profiles could be observed for all the chitosan derivatives independent of their different amine functionalities. From these protonation curves the p*K*a values of the primary and secondary amino groups were determined. The results listed in Table 3 are consistent with those based on polyaspartamides with DETA as side

Table 3
p*K*a values of chitosan derivatives with various oligoamines as side groups determined by titration experiments.

Polymer	pK_a	
	pK_{a1}	pK_{a2}
CS–EDA	5.7	9.5
CS–DETA	5.7	9.5
CS–TETA	5.9	9.6
CS–TEPA	5.9	9.7
CS–PEHA	6.1	9.6

groups (Itaka et al., 2004; Kanayama et al., 2006). This also supports the desirable structure of oligoamine–conjugated chitosan polymers.

3.3. In vitro cytotoxicity study

For the polymers to be used as delivery carriers, the low cytotoxicity is a crucial aspect, particularly for the successful nonviral gene delivery. As well known, the high cytotoxicity of high molecular weight polyethyleneimines (PEI) has markedly limited their application for safety gene delivery, although they are among the polymers with the highest transfection efficiency. As a preliminary biologic evaluation, both normal and tumor cell lines, i.e. RAW264.7 and Hela cells were selected for cell culture experiments in this study. RAW264.7 is a murine macrophage cell line. Fig. 6a shows the effect of polymer concentration on the cell viability of RAW264.7. Generally, concentration-dependent cell viability could be observed. The higher the polymer concentrations, the lower the cell viability will be. This trend is independent of polymer structure. This concentration-related cytotoxicity is well consistent with the results by other researchers (Jiang et al., 2007; Zhang et al., 2009). However, even at a high concentration up to 5000 μg/mL, the viability of cells treated with oligoamine–conjugated chitosan derivatives such as CS–EDA, CS–DETA and CS–TETA was still above 60%. Interestingly, CS–TEPA and CS–PEHA possessed a relatively high cytotoxicity, especially when their concentrations were above 1000 μg/mL. These results suggested that the cytotoxicity of oligoamines–conjugated chitosan derivatives to RAW264.7 was associated with their concentration as well as the chain length of oligoamines. Increasing polymer concentration or the chain length of oligoamines may result in enhanced cell toxicity. As a clear contrast, branched PEI (molecular weight: 25 kDa), which is a classic polymer used for gene delivery, exhibited a significantly higher cytotoxicity against RAW264.7 cells (Fig. 6c).

Hela cell, an immortal cell line derived from cervical cancer cell, was also employed as a cell model for cytotoxic study. As shown in Fig. 6b, a concentration-dependent toxic behavior was observed as well. Except CS–EDA, all the other derivatives displayed low cytotoxicity (cell viability > 70%) against Hela cells when the polymer concentration was below 2250 μg/mL. In comparison with other derivatives, CS–TETA expressed relatively high toxicity at higher concentrations. As far as the CS–TEPA and CS–PEHA were concerned, they were relatively low toxic in the concentration range examined, and the cell viability was above 70% even when their

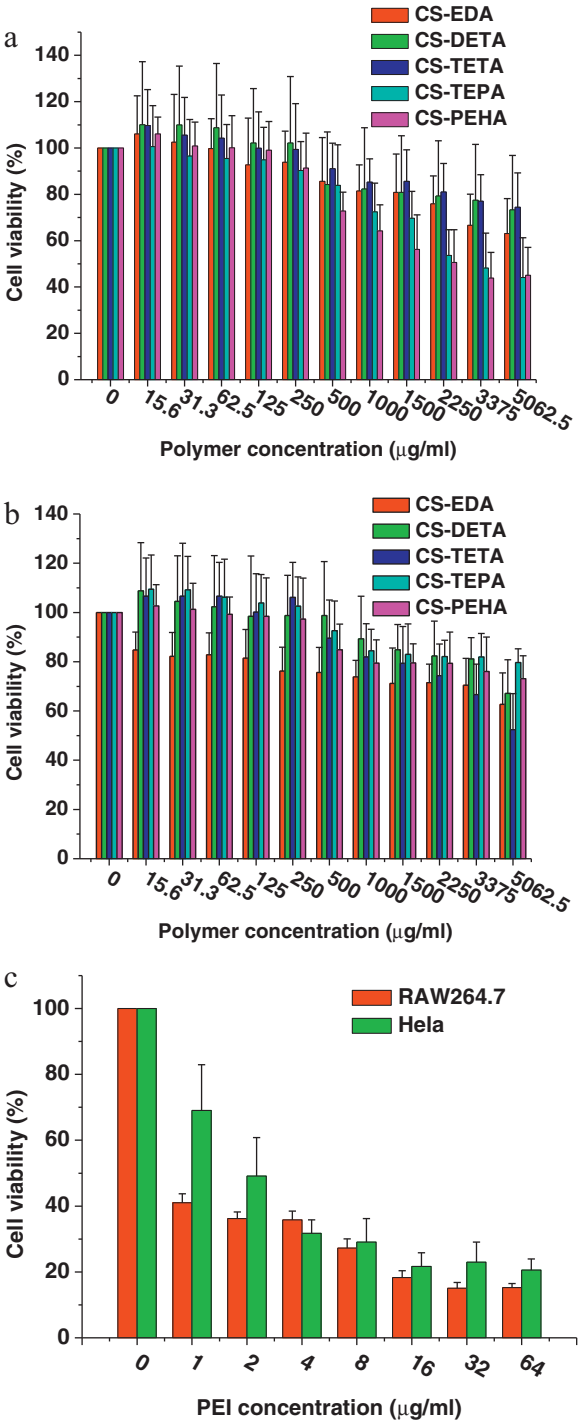
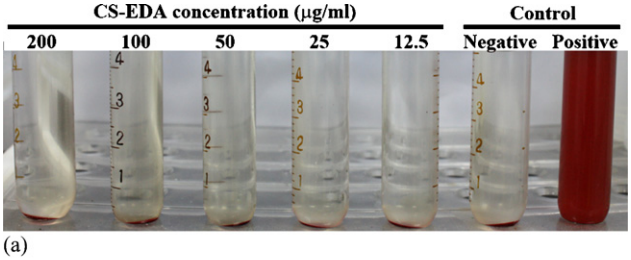


Fig. 6. *In vitro* cytotoxicity to RAW264.7 (a) and Hela (b) after 24 h of incubation with chitosan derivatives and branched PEI (c) with a molecular weight of 25 kDa. Each polymer was dissolved in cell culture medium at various concentrations, and was applied to cultured cells ($n = 5$). Twenty-four hours later, cell viability was assessed by MTT assay. Values are shown as mean ± s.e.m.



(a)

The scores of hemolysis test based on rabbit red blood cells

Groups		Time (h) 1	2	3	4
CS-EDA (μg/mL)	200	-	-	-	-
	100	-	-	-	-
	50	-	-	-	-
	25	-	-	-	-
	12.5	-	-	-	-
Normal saline		-	-	-	-
Deionized water		+	+	+	+

(b)

Fig. 7. Hemolysis evaluation of CS–EDA using erythrocytes freshly harvested from rabbit blood. After the blood cells were treated with CS–EDA at various concentrations, they were incubated at 37 °C for 4 h and then centrifuged at 2000 rpm for 5 min to give a clear presentation. (a) Photos showing erythrocytes that were mixed with CS–EDA, saline (negative control), or pure water (positive control) for 4 h. (b) Scores of various groups based on the criteria listed in Table 1.

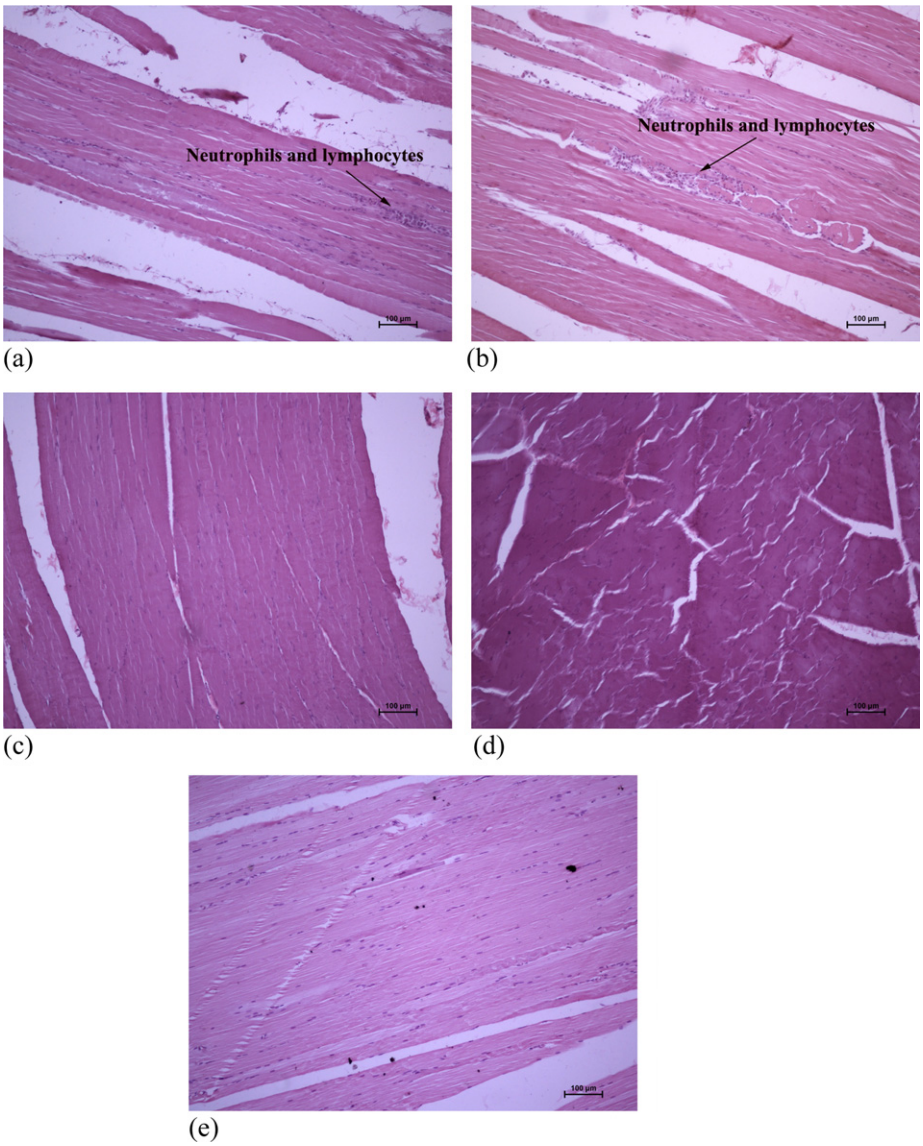


Fig. 8. CS–EDA aqueous solutions (1.0 mL) with various concentrations (either 25 or 50 mg/mL) were injected into quadriceps femoris muscle, the muscular tissues of interest were incised and harvested at day 1 and 7. Histological sections were made and stained with H&E. (a) 25 mg/mL at day 1; (b) 50 mg/mL at day 1; (c) 25 mg/mL at day 7; (d) 50 mg/mL at day 7; and (e) control with saline injection.

concentration was 5062.5 μg/mL. On the contrary, severe cytotoxicity against Hela cell line was also found for PEI. The IC_{50} of PEI to Hela cell is about 2 μg/mL as shown in Fig. 6c. With a partial conclusion, besides polymer concentration, there should be an optimal polymer structure for which the cytotoxicity against Hela cell is minimal.

3.4. In vitro hemolysis test

For materials to be employed for intravenous delivery of therapeutics, such as in the case of gene therapy based on non-virus vectors, it is mandatory to examine their hemolytic character. For this purpose, we evaluated the hemolysis effect of CS–EDA. As shown in Fig. 7a, a thorough hemolysis could be observed in the case of positive control group. No hemolysis, however, was visible for CS–EDA solutions with concentrations ranging from 12.5 to 200 μg/mL, which is similar to the negative control group. The qualitative scores illustrated in Fig. 7b also suggested the extremely low hemolysis of CS–EDA at each time point of incubation in the concentration range examined. This result might, at least to some extent, suggest the good blood compatibility of oligoamine–conjugated chitosan derivatives, although further examinations are still necessary.

3.5. In vivo inflammation evaluation

Evaluation on the local irritation and inflammation was performed based on a rabbit model. After 1.0 mL aqueous solutions of CS–EDA at 25 and 50 mg/mL were injected into quadriceps femoris muscle of Japanese white rabbits, the host response of muscular tissues was examined following a one-week period. The histological sections were then made and observed at day 1 and 7. One day post-injection, only filtration of few neutrophils and lymphocytes was monitored independent of CS–EDA concentrations (Fig. 8a and b), indicating a minimal inflammation at this time point. This is a normal host response following the injection of extrinsic substances, while no swelling or necrosis was observable. At day 7, however, no inflammatory cells like neutrophils and lymphocytes could be observed for muscular tissues injected with either 25 or 50 mg/mL CS–EDA (Fig. 8c and d), which is comparable to the tis-

sues with saline injection (Fig. 8e). This suggested that the minimal inflammation largely disappeared 7 days post-injection, which in turn revealed the good tissue-compatibility of CS–EDA polymer.

4. Conclusions

With the attempt to synthesize water-soluble chitosan derivatives with multiple amine functionality, various oligoamines were conjugated onto the side chain of oxidized chitosan. To this purpose, chitosan was initially oxidized by periodate to give aldehyde groups. Oligoamines, including EDA, DETA, TETA, TEPA and PEHA were then conjugated under optimized conditions that can almost suppress the occurrence of undesirable cross-linking. Measurements based on FT-IR, ^{1}H NMR as well as potentiometric titration suggested the successful synthesis of oligoamines functionalized chitosan derivatives. These polymers are highly water-soluble. *In vitro* cell cytotoxicity evaluation using both normal and cancerous cells indicated that these chitosan derivatives display considerably low cytotoxicity even at concentration as high as 5062.5 μg/mL. In addition, concentration and structure dependent toxicity was observed for both cell lines. By selecting suitable amines, polymers with extremely low cytotoxicity can be obtained. Test based on freshly harvested rabbit erythrocytes demonstrated the low hemolysis character of newly synthesized polymers. Furthermore, local inflammation evaluation using a rabbit model suggested this type of chitosan derivatives exhibited a good compatibility in muscular tissues. These newly synthesized polymers with functional oligoamine groups can be potential carrier materials for the delivery of biomacromolecules such as proteins, vaccines and gene segments, owing to their functionality and good compatibility at cell, blood and tissue levels. Further study is undergoing in our lab to address their usefulness for gene delivery and the related results will be published elsewhere.

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