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Synthesis, Characterization, and Drug-Release Behavior of Amphiphilic Quaternary Ammonium Chitosan Derivatives

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ABSTRACT: A new type of amphiphilic quaternary ammonium chitosan derivative, 2-*N*-carboxymethyl-6-*O*-diethylaminoethyl chitosan (DEAE–CMC), was synthesized through a two-step Schiff base reaction process and applied to drug delivery. In the first step, benzaldehyde was used as a protective agent for the incorporation of diethylaminoethyl groups to form the intermediate (6-*O*-diethylaminoethyl chitosan). On the other hand, $NaBH_4$ was used as a reducing agent to reduce the Schiff base, which was generated by glyoxylic acid, for the further incorporation of carboxymethyl groups to produce DEAE–CMC. The structure, thermal properties, surface morphology, and diameter distribution of the resulting chitosan graft copolymers were characterized by Fourier transform infrared spectroscopy, ^{1}H-NMR, thermogravimetric analysis, differential scanning calorimetry, X-ray powder diffraction, scanning electron microscopy, and laser particle size analysis. Benefiting from the amphiphilic structure, DEAE–CMC was able to be formed into microspheres in aqueous solution with an average diameter of 4.52 ± 1.21 μm. An *in vitro* evaluation of these microspheres demonstrated their efficient controlled release behavior of a drug. The accumulated release ratio of vitamin B_{12} loaded DEAE–CMC microspheres were up to 93%, and the duration was up to 15 h. The grafted polymers of DEAE–CMC were found to be blood-compatible, and no cytotoxic effect was shown in human SiHa cells in an MTT [3-(4, 5-dimethyl-thiazol-2-yl)-2, 5-diphenyltetrazolium bromide] cytotoxicity assay. These results indicate that the DEAE–CMC microspheres could be used as safe, promising drug-delivery systems. © 2013 Wiley Periodicals, Inc. J. Appl. Polym. Sci. **2014**, *131*, 39890.

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INTRODUCTION

Chitosan is a natural polyaminosaccharide, and it has a repeating structural unit of 2-acetamido-2-deoxy-β-D-glucopyranose.[1,2] Because of its nontoxicity, biodegradability, and biocompatibility,[3,4] a range of biological applications have been reported for chitosan, including antibacterial fibrous membranes,[5,6] tissue engineering for cell cultures,[7] sorbents,[8,9] and drug delivery.[10] However, chitosan has shown some limitations because of its poor water solubility. To solve this problem, many chemical modifications of chitosan have been made.[11–14] Later on, extensive attention has been directed toward the introduction of functional groups into the definite sites of chitosan chains.[13,15,16] Ngah and Liang[17] prepared *N*-carboxymethyl chitosan by a Schiff base and its reduction reaction. Chen et al.[18] made *O*-carboxymethyl chitosan by introducing —CH_2COOH groups onto —OH along the chitosan chain. Prabaharan et al.[19] prepared carboxymethyl chitosan-*graft*-phosphatidylethanolamine amphiphilic matrices and further studied its controlled drug delivery. Holappa et al.[20] prepared various novel quaternary chitosan derivatives by reacting *N*-chloroacyl-6-*O*-triphenylmethyl chitosans with tertiary amines.

Generally, chitosan is a weak base, and a certain amount of acid is required to transform the glucosamine units into the positively charged, water-soluble form. Consequently, at a neutral pH, most chitosan molecules will lose their charge and precipitate from solution. This property implies that chitosan can be effective as an absorption enhancer only in a limited area of the intestinal lumen where the pH values are close to its pK. Chitosan derivatives with quaternary ammonium groups are thus interesting because permanent cationic charges are gained on the polysaccharide backbone.[20–22] Toward that end, these chitosan derivatives can become water-soluble at higher pH (>6.5)

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because quaternary ammonium cations remain positively charged across a wide range of pH. Quaternary ammonium chitosan has shown potential in various pharmaceutical applications, such as delivery vehicles of peptides or proteins,[23–25] novel mucoadhesive polymeric nanocomplex platforms,[26,27] and antimicrobial agents.[28,29]

The cationic character of chitosan has been suggested to be the main cause of the unique properties and bioactivity of chitosan.[30,31] For this reason, it is important to maintain the aminosaccharide unit for various specific functions, including biological activities and cationic polymer properties. In the drug-delivery systems, it is a powerful tool to control interaction of a polymer with drugs, enhance the drug-loading efficiency, and tailor the drug-release period.[32] Quaternary ammonium chitosan has been used in many different formulations for drug and gene delivery in the gastrointestinal tract. In addition, it appears to have potential as an absorption enhancer, promoting drug uptake across the mucosal barrier.[33–35] Junginger et al.[36] proposed that protonated chitosan derivatives can trigger the opening of intercellular tight junctions and thereby facilitate the paracellular uptake of macromolecular drugs. With this information taken into account, it is promising to combine chitosan with electropositive functional groups, such as quaternary ammonium groups, to increase its electropositivity. To further increase its solubility in aqueous solutions, we also incorporated carboxymethyl groups into the chitosan copolymer.

The major goal of this study was to demonstrate a new locating modification method in the chitosan backbone. We synthesized a novel, more highly cationic quaternary ammonium chitosan derivative, 2-*N*-carboxymethyl-6-*O*-diethylaminoethyl chitosan (DEAE–CMC), through a two-step Schiff base reaction procedure. The newly resulting copolymer and chitosan were used as carriers of a model drug, vitamin B_{12} (VB_{12}), and the drug-release profile in phosphate buffer solution (PBS, pH 7.4) and NaCl (pH 6.3) and HCl (pH 1.2) solutions was studied. The data suggested that DEAE–CMC is a promising material for drug delivery. Our new method should find many applications in the development of new chitosan-based biomedical materials to carry other drugs. In particular, our research may reveal the essence of the interactions between the quaternary ammonium chitosan derivatives and drug molecules.

EXPERIMENTAL

Materials

Chitosan [degree of deacetylation >92% and molecular weight calculated from the gel permeation chromatography (GPC) method $\approx 2.82 \times 10^5$) was purchased from Zhejiang Yuhuan Ocean Biochemistry Co., Ltd. (Zhejiang, China). VB_{12} and glutaraldehyde solution (25% v/v) were obtained from Sinopharm Group Chemical Reagent Co., Ltd. (Shanghai, China). 2-Diethylaminoethylchloride hydrochloride (DEAE·HCl; Shanghai Nanxiang Chemical Reagent Co., Ltd., China, ≥99.5%), glyoxylic acid, and sodium borohydrid (Schuchardt Merck Co., Germany, ≥99.5%) were used as received. The water used for all of the experiments was purified water obtained from a MilliQ Plus (Millipore, Schwalbach, Germany). All of the other reagents we used were commercially available and were analytical grade. The remaining materials were used from the indicated sources without further purification procedures.

Preparation of 6-*O*-Diethylaminoethyl Chitosan (DEAE–Chitosan)

DEAE–chitosan was prepared in accordance with a previously reported procedure (Scheme 1).[16,26] The benzaldehyde was used as a protective agent. It was mainly a protection–graft–deprotection process with benzaldehyde as the protective agent. In brief, 3.0 g of chitosan was dissolved in 100 mL of a 10 wt % acetic acid solution. The solution was diluted with 20 mL of methanol. The methanol prevented the gelation of the aminated support and increased the solubility of the benzaldehyde group. Benzaldehyde (9 mL) was gradually added in about 10 min and left to stir for 3 h. To obtain protection for the amino groups of the support, the pH of the homogeneous solution was adjusted to neutral with a sodium hydroxide solution (2*M*). The precipitate was collected by filtration and then washed completely by Soxhlet extraction with methanol to remove unreacted benzaldehyde. The methanol was removed by vacuum evaporation around 30°C to give the reaction intermediate, 2-*N*-benzylidene chitosan.

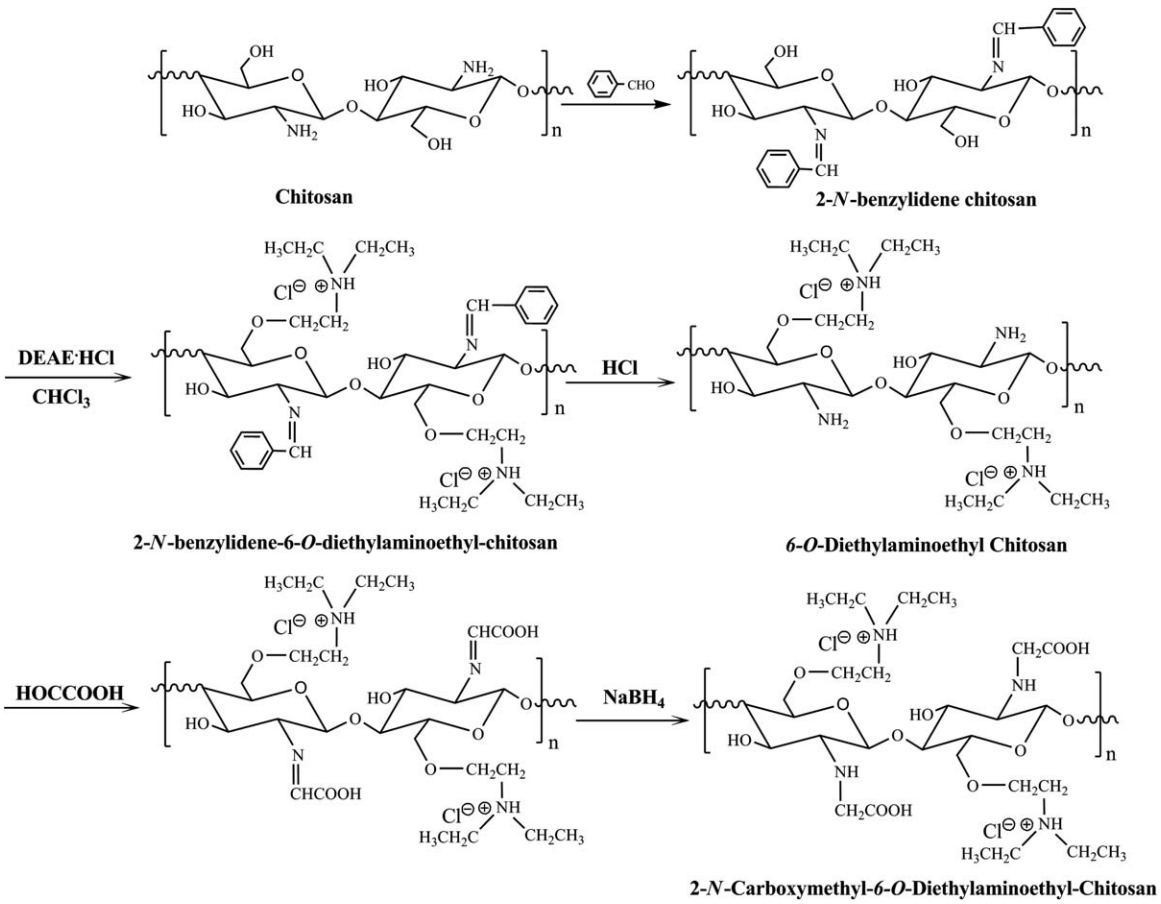
2-*N*-Benzylidene chitosan (3.0 g) was swollen in a chloroform/pyridine (2:1 v/v) solution and continuously stirred at 60°C for 12 h. DEAE·HCl (5.5 g) was added and left to react at 50°C for 12 h. Thus, we obtained 2-*N*-benzylidene-6-*O*-diethylaminoethyl chitosan. The obtained precipitate was suspended in an ethanolic solution of HCl (0.25*M*). With the deprotection of amino groups, acid hydrolysis was completed in 12 h at 25°C.[15] The solution was adjusted to neutrality with a sodium hydroxide solution (0.1*M*). The precipitate was filtered and washed several times with ethanol to remove excess DEAE·HCl. The synthesized DEAE–chitosan was dried at 50°C for 24 h. The GPC measurement gave a weight-average molecular weight (M_w) of 3.74×10^5 with M_w/number-average molecular weight (M_n) = 1.53.

Preparation of DEAE–CMC

DEAE–CMC derivatives were prepared by the introduction of carboxymethyl groups to NH_2 on C2 of glucosamine units in DEAE–chitosan (Scheme 1).[17] $NaBH_4$ was used as reducing agent to reduce the Schiff base, which was generated by glyoxylic acid. DEAE–chitosan (2 g) was dissolved in 200 mL of distilled water. With stirring, 0.85 g of glyoxylic acid was added and reacted for 4 h. Then, the resulting solution was reduced with 0.45 g of sodium borohydride at pH 6.5 and was insolubilized for 24 h. The precipitate was filtered and dried to obtain DEAE–CMC. After carboxymethylation, the molecular weight of the DEAE–CMC was estimated to be $M_w = 4.28 \times 10^5$ with $M_w/M_n = 1.68$.

Preparation of the Drug-Loaded DEAE–CMC Microspheres

VB_{12}-loaded DEAE–CMC microspheres were prepared by an emulsion–crosslinking technique.[37,38] Briefly, 0.2 g of DEAE–CMC and 40 mg of VB_{12} were dissolved in an acetic acid solution (3 wt %) and continuously stirred until a homogeneous solution was obtained. This solution was slowly added to 60 g of liquid paraffin, which contained 1% w/w Span-80, and was continuously stirred at 1000 rpm for 10 min. To this



Scheme 1. Synthetic procedure of DEAE–CMC.

water-in-oil emulsion, a glutaraldehyde solution (25% v/v) was added, and the mixture was stirred for 2 h. Hardened microspheres were separated by centrifugation and washed with sodium bisulfite solution (2 wt %) to eliminate excess glutaraldehyde. The solid residue was dehydrated with ethanol and then dried to obtain the VB_{12}-loaded DEAE–CMC microspheres.

Characterization

Fourier transform infrared (FTIR) spectra of chitosan and its derivatives were recorded with a double-beam Avatar-370 FTIR spectrometer (Shimadzu Co., Ltd., Japan) at 25°C in the region 4000–500 cm^{-1} with KBr pellets. ^{1}H-NMR spectra of the samples were recorded on a Varian Unity Plus 400-MHz spectrometer (Bruker Co., Ltd., Switzerland) in D_2O at 25°C. The powder X-ray powder diffraction (XRD) patterns were taken on a Bruker AXS D8-Advance X-ray diffractometer with Cu Kα radiation (1.5418 Å). Data were recorded in the range of $2\theta = 3$–50° at a scanning rate and step size of 6°/min and 0.02, respectively. The molecular weights were determined on an HLC-8320 GPC system (Tosoh, Japan) equipped with a low-angle laser-light-scattering photometer (LS-8000) and a differential refractometer (RI-8011). Each sample was dissolved in acetate buffer (1*M*, pH 4.8), which was the eluent, and filtered through a filter membrane (0.45 μm). The final concentration of the samples was 1.0 mg/mL. The column temperature was 40°C, the flow rate was 1.0 mL/min, and the injected sample size was 100 μL.[9,39]

Thermogravimetric analysis (TGA) was investigated with an STA PT-1000 instrument (Linseis Co., Ltd., Germany) with a heating rate of 10°C/min in a nitrogen flow (20 mL/min). Differential scanning calorimetry (DSC) was performed on a Q2000 instrument (TA Instruments, Inc., USA) at a heating rate of 10°C/min in a dry nitrogen atmosphere. The instrument was calibrated with an indium standard. The samples (ca. 10.0 mg in weight) were first heated up to 140°C and held at this temperature for 3 min to eliminate their thermal history; this was followed by quenching to −50°C. The melting temperature (T_m), glass-transition temperature (T_g), and heat of fusion (ΔH_m) were determined from the DSC curves.

The morphology of DEAE–CMC and its VB_{12}-loaded microspheres were analyzed with scanning electron microscopy (SEM; Hitachi Co., Ltd., Japan) at an accelerating voltage of 15 kV. The particle size distribution was determined with a laser particle size analyzer (LS13320, Beckman Coulter INC., USA) in the range 0.04–2000 μm.

Blood Compatibility Studies

A hemocompatibility test was performed according to a method described by Uchegbu et al.[33] Fresh human blood (6 mL) was centrifuged at 800 g for 5 min at 4°C to remove the plasma and the buffy coat. The erythrocyte pellet was isolated and washed twice with PBS (pH 7.4) by resuspension of the pellet in PBS followed by centrifugation. The pellet was dispersed in PBS (pH

7.4) to obtain an erythrocyte dispersion (3% w/w). Various concentrations of the DEAE–CMC solution (100 μL) were added to the erythrocyte solution (100 μL) in a 96-well plate. PBS and Triton X-100 (1 wt %) served as negative and positive controls, respectively. After incubation for 4 h, the well plate was centrifuged (1000 g for 10 min), and the supernatant (100 μL) was transferred to a new well plate. The absorbance was measured at 540 nm. The hemolysis given by the Triton X solution was considered to be 100%, whereas the hemolysis given by the PBS solution was taken as 0%. The results were thus expressed as hemolysis percentages.

In Vitro Cytotoxicity Studies

The cell cytotoxicity of chitosan and its derivatives was investigated on human SiHa cells. The cells were grown in Dulbecco's Modified Eagle's Medium supplemented with 50% F12 medium, 10% fetal calf serum, 2 m*M* glutamine, penicillin G sodium, streptomycin sulfate, and amphotericin B and were incubated at 10% CO_2 and 37°C.

Cells viability testing was carried out via the reduction of the MTT reagent [3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide]. MTT assay was based colorimetric assay for cell growth and chemosensitivity, provided a common and simple method for determining the comparative cell viability with a standard microplate absorbance reader.[40] The chitosan derivatives were placed at the bottom of a 96-well plate according to the standard procedure with minor modifications. Control experiments were carried out with the complete growth culture medium only (serving as a nontoxic control) and 1 wt % Triton X-100 (Sigma; serving as the toxic control). The cells were seeded at a density of 1.0×10^4 cells/well and incubated for 24 h. The MTT in PBS (0.5 mg/mL) was added to each well, and the cells were incubated for 4 h. The medium and MTT were then aspirated from the wells, and formazan crystal was solubilized with 200 μL of dimethyl sulfoxide. The absorbance was measured at 570 nm on a microplate reader. Six wells were tested for each type of chitosan derivative.

In Vitro Drug-Release Behavior

The release studies were performed in a glass apparatus at 37°C in phosphate buffer (pH 7.4), NaCl (pH 6.3), and HCl (pH 1.2) solutions. Drug-loaded microspheres (50 mg) were added into dialysis bags (molecular weight cut off = 8000–14000). The dialysis bags were immersed in 250 mL of release medium and stirred at 100 rpm and maintained at 37°C in a water bath. The VB_{12}-loaded chitosan microspheres were used as the control sample. The samples (3 mL) were periodically withdrawn, centrifuged, and subjected to assay immediately. The volume of each sample withdrawn was replaced by the same amount of fresh release medium.[18,41] The amount of released VB_{12} was determined by a UV spectrophotometer at 361 nm.

The total amount of VB_{12}-loaded into microspheres was determined by dissolution of an accurately weighed amount of microspheres in 1 mL of ethanol.[42] After the dissolution of the microspheres, the solutions were centrifuged at 10,000 rpm for 5 min, and the amount of VB_{12} in the supernatant was determined by the UV spectrophotometer at 361 nm. The loading efficiency of VB_{12} was calculated by the division of the amount of drug in the microspheres by the initial amount of drug added. The data represented in the graph show the mean plus or minus standard deviation of the three experiments.

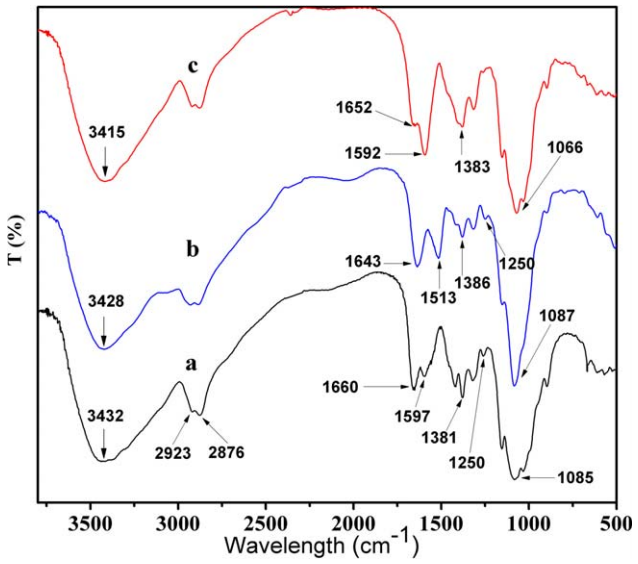


Figure 1. FTIR spectra of (a) chitosan, (b) DEAE–chitosan, and (c) DEAE–CMC. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

RESULTS AND DISCUSSION

FTIR Results

The FTIR spectra of chitosan and its derivatives are shown in Figure 1. The spectra of the chitosan derivatives were similar to that of the original chitosan. Chitosan [Figure 1(a)] showed a wide —OH stretching absorption band between 3450 and 3100 cm^{-1} and aliphatic C—H stretching between 2927 and 2876 cm^{-1}. Because the —OH stretching band and the aliphatic C—H were aligned, they appeared as a broad band from 3450 and 2850 cm^{-1}. The peak at 1381 cm^{-1} represented the —C—O stretching of primary alcoholic groups (—CH_2—OH). The other major absorption band between 1250 and 1085 cm^{-1} represented the free —NH_2 groups at the C2 position. In the IR spectra of the DEAE–chitosan [Figure 1(b)], the peak at 1386 cm^{-1}, which represented the —C—O stretching of primary alcoholic groups (—CH_2—OH) became weaker, and the absorption at 1000–1150 cm^{-1} due to C—O—C stretching became stronger; this supported the occurrence of the substitution in the C6 position of chitosan.[43] For DEAE–CMC [Figure 1(c)], the absorption peak (1250 cm^{-1}) of the free —NH_2 groups disappeared. Also, the strong peaks at 1592 cm^{-1} could be assigned to the asymmetric stretching vibration of COO^-.[19] The results indicate that carboxymethyl group substitution occurred in the chitosan backbone.

^{1}H-NMR Analysis

Figure 2(a) shows the ^{1}H-NMR spectrum of chitosan. The proton signals of the chitosan backbone were observed at 3.0 ppm for H2, 3.4–4.0 ppm for H3–H6, and 4.7 ppm for H1 (overlapped with the D_2O proton). Figure 2(b) shows the ^{1}H-NMR spectrum of DEAE–chitosan. The new signals at 1.15 and 2.65 ppm were for methyl and methylene protons of the diethylaminoethyl groups, respectively. We estimated that C-6 hydroxyl

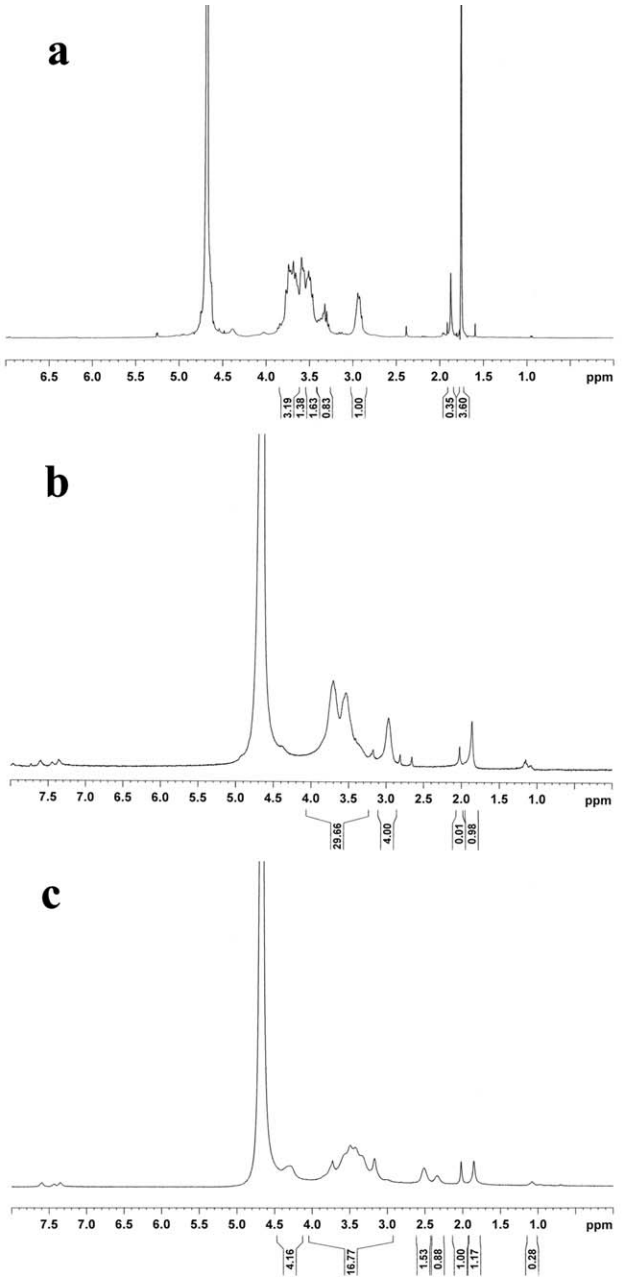


Figure 2. ^{1}H-NMR spectra of (a) chitosan, (b) DEAE–chitosan, and (c) DEAE–CMC.

groups were possibly replaced by diethylaminoethyl groups, which was consistent with data previously reported.[16] In the ^{1}H-NMR spectrum of DEAE–CMC [Figure 2(c)], the chemical shift of 4.3 ppm was assigned to the protons of $-CH_2-COO^-$ at the C2 position of DEAE–CMC. This indicated that carboxymethyl group substitution was observed on some of the primary amino sites of the modified chitosan structure.

XRD Results

The XRD patterns of chitosan, DEAE–chitosan, and DEAE–CMC are shown in Figure 3. There were two strong peaks in the diffractograms of chitosan at 2θ values of 10.6 and 21.0°, this indicated the high degree of crystallinity of chitosan, with

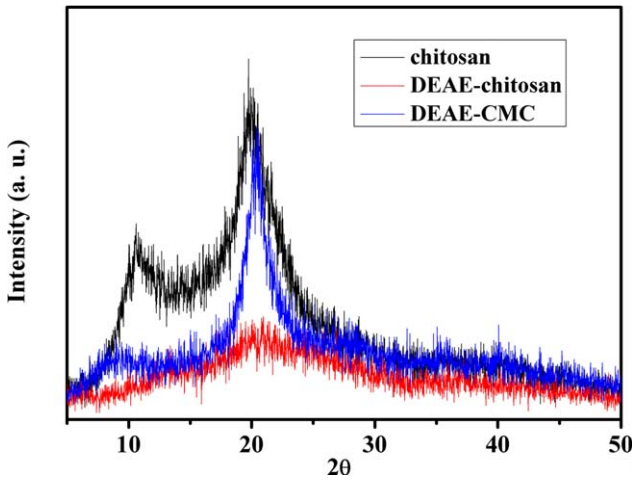


Figure 3. XRD patterns of chitosan, DEAE–chitosan, and DEAE–CMC. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

their crystal lattice constants corresponding to 8.470 and 4.075.[44] For DEAE–chitosan, the original characteristic peak at 10.6° totally disappeared, and another original crystalline peak around 21.0° was greatly decreased. These result revealed that greater disarray in the chain alignment occurred in DEAE–chitosan, and the original crystalline structure of chitosan was seriously destroyed. Conversely, the two strong peaks at 2θ values of 9.1 and 20.4° appeared again in the diffractogram of DEAE–CMC. It showed that the crystallinity was significantly improved after carboxymethyl groups were introduced.

Thermal Degradation Studies

It is essential to study the thermal properties of a material for its applications, and they are directly related to the composition of a material. The TGA and derivative thermogravimetry (DTG) curves of chitosan and its derivatives are shown in Figure 4(A,B). All of the copolymers had three decomposition stages and two maximum decomposition temperatures. All of the data for the samples, with regard to the onset temperature of the thermal degradation, defined as the temperature corresponding to a weight loss of 5% (T_{onset}); the temperature at the maximum degradation rate (T_{max}) determined by the DTG curves; and the weight loss at the maximum degradation rate (WL_{max}), are summarized in Table I.

The T_{max} values of chitosan, DEAE–chitosan, and DEAE–CMC were 312, 257, and 265°C, respectively. The thermal stability of chitosan was better than that of its derivatives. We concluded that the introduction of functional groups had a significant influence on its thermal stability. Generally, it is known that strong polar groups, rigid groups, and more chain alignment between polymer chains contribute to increases in the degradation temperature.[45] The hydrogen bond of chitosan was partially destroyed by the introduction of diethylaminoethyl groups onto the backbone of chitosan. The introduction of strong polar groups, such as carboxymethyl groups, resulted in another increase in the degradation temperature.

Figure 4(C) shows the heating scans of chitosan and its derivatives. All of the relevant enthalpies (ΔH_m), T_g values, and T_m

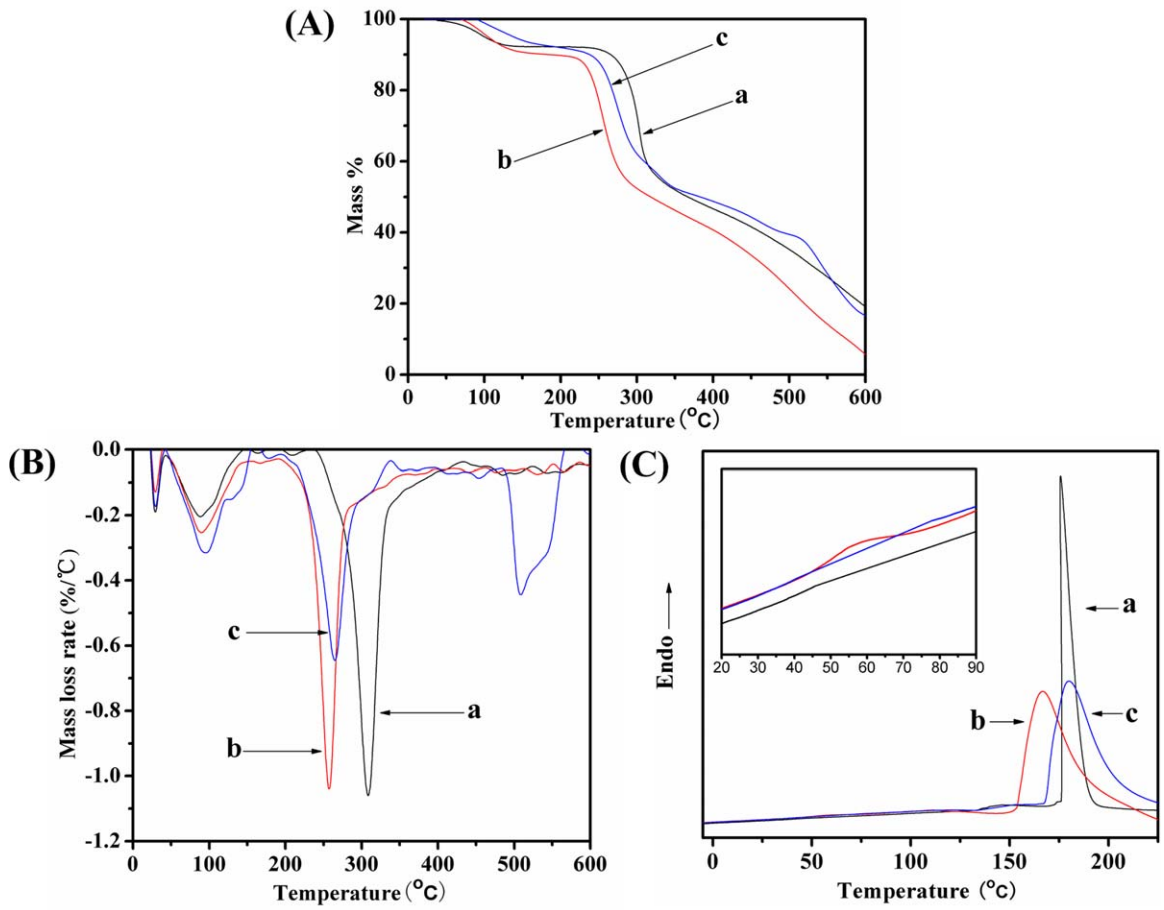


Figure 4. (A) TGA, (B) DTG, and (C) DSC curves of (a) chitosan, (b) DEAE–chitosan, and (c) DEAE–CMC. (Inset showing the DSC curves at initial temperature period). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

values determined from Figure 4(C) are listed in Table II. The DSC thermograms revealed that all of the samples exhibited endothermic peaks in the vicinities of 175.8, 166.5, and 180.2°C; these resulted from the melting of the chitosan backbone.

As a rule, the crystallinity of a polymer is calculated from the ratio of ΔH_m measured at T_m for the polymer sample. This behavior is frequently detected in many polysaccharides, such as cellulose and chitosan derivatives. The ΔH_m values of the samples were 229.5, 173.6, and 196.0 J/g, respectively. No obvious T_g was observed in the DSC thermograms obtained for chitosan; this observation was ascribed to the structure and properties of chitosan. It is known that chitosan is a semicrystalline polymer because of its strong intramolecular hydrogen bonds on the backbone, and it also had a rigid amorphous phase because of its heterocyclic units; as a result, when it was heated within a certain range of temperature below its decomposition temperature, the variations in heat capacity corresponding to the change in specific volume near T_g was probably too small to be detected by the DSC technique.[46,47] DEAE–chitosan had poor crystallinity, which was proven from its XRD diffractograms (Figure 3), and it showed a broad jump at 45–65°C; this was recognized as its T_g reflecting an increase in the molecular movement because of the dissociation of hydrogen bonds and the beginning of molecular chain scissions.[48] After the introduction of carboxymethyl groups into the DEAE–chitosan

Table I. Data Collected from the TG and DTG Thermograms

Sample	T_{onset} (°C)	T_{max} (°C)	WL_{max} (°C)
Chitosan	100	312	47%
DEAE-chitosan	104	257	30%
DEAE-CMC	138	265	18%

The values in the table are the average values from three specimens for each sample.

Table II. Relevant Transitions and Enthalpies Determined by DSC

Sample	T_m (°C)	T_g (°C)	ΔH_m (J/g)
Chitosan	175.8	—	229.5
DEAE-chitosan	166.5	50.6	173.6
DEAE-CMC	180.2	—	196.0

The values in the table are the average values from three specimens for each sample.

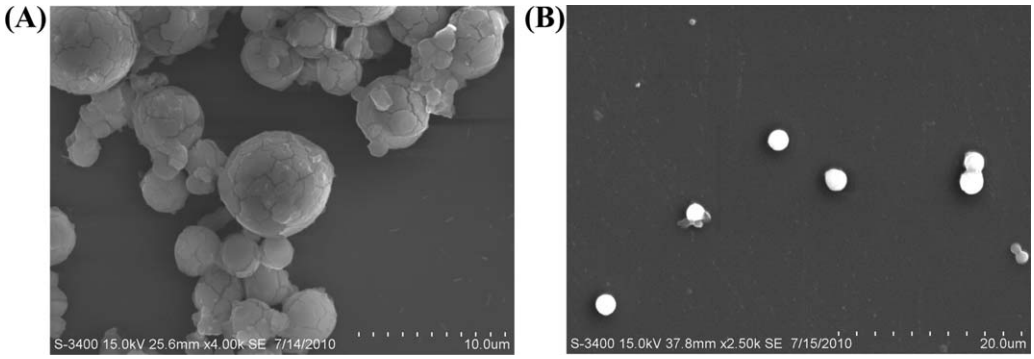


Figure 5. SEM image of the DEAE–CMC microspheres and VB_{12}-loaded DEAE–CMC microspheres.

backbone, its crystallinity was significantly improved compared to DEAE–chitosan (Figure 3). Moreover, there was no T_g in the DSC curves of DEAE–CMC.

Morphology and Particle Size Distribution Studies

The particle size distributions of the DEAE–CMC microspheres and VB_{12}-loaded DEAE–CMC microspheres are shown in Figures S1 and S2 (see the Supporting Information online). The mean particle size distributions were 14.01 ± 2.56 and 4.52 ± 1.21 μm, respectively. The size and morphology of the DEAE–CMC microspheres and VB_{12}-loaded DEAE–CMC microspheres were also confirmed by SEM images (Figure 5). The DEAE–CMC microspheres had an open and cystoids structure, and their agglomerate phenomenon were obvious. However, the incorporation of VB_{12} into the DEAE–CMC microspheres altered their surface morphology. The whole size of VB_{12} loaded DEAE–CMC microspheres was dispersed in 0.2–20 μm. The microspheres became smaller with good dispersion, and had uniform size and less agglomeration. These results reveal that incorporation of drug imparted a high degree of surface smoothness to the microspheres.

Blood Compatibility Studies

At low concentration (<4 mg/mL), the liquid solution formulation of the polymer was nonhemolytic (Figure 6), and we hypothesized that although the polymer was a soluble amphiphile, the higher molecular weight of the polymer prevented the effective partitioning into and the solubilization of the erythrocyte membranes. Hemolysis was observed when concentrated solutions (>8 mg/mL, gel-like) were applied to the erythrocytes; this was presumably due to the high osmotic stress caused by the gel. Even at a high concentration, the DEAE–CMC also exhibited fewer hemolysis phenomena. This shows that DEAE–CMC possesses very good blood compatibility.

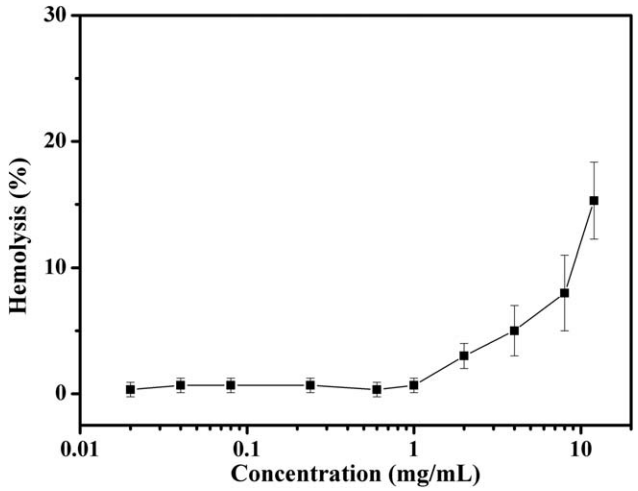


Figure 6. Blood compatibility of DEAE–CMC relative to Triton X-100.

Cytotoxicity Assay

One of the major requirements for cationic polymers used in drug-delivery systems is noncytotoxicity. The issue of cytotoxicity of chitosan and its derivatives has to be addressed. The results of MTT cytotoxicity with human SiHa cells are shown in Table III. These cells were selected for this assay as they were substrates-dependent, nonspecific cell lines. The results in Table III indicate that there was no statistical difference in the cytotoxicities among DEAE–chitosan and DEAE–CMC (91 ± 2 and 90 ± 3%, respectively; $p > 0.05$) as compared to chitosan (96 ± 3%). The slightly lower cell viability of DEAE–chitosan and DEAE–CMC could possibly have been due to the presence of quaternary ammonium moieties in the chitosan backbone. We found that the synthetic polymer (DEAE–CMC) was demonstrated as a safe material because of its high cell viability. It should also be noted that the results obtained with chitosan were not significantly different from those of the nontoxic control (growth culture medium).

In Vitro Drug-Release Behavior

The release of VB_{12} from drug-loaded microspheres was performed by the immersion of the microspheres in pH 7.4, 6.3, and 1.2 solutions, respectively. The drug-loading efficiencies for the chitosan and DEAE–CMC microspheres were 81 and 86%, respectively. Figure 7 depicts the release profiles of VB_{12} from

Table III. Cytotoxicity Assay of Human SiHa Cells on the Chitosan and Its Derivatives

Sample	Cell viability of the control (%)
Growth medium	100 ± 2
Chitosan	96 ± 3
DEAE-chitosan	91 ± 2
DEAE-CMC	90 ± 3
Triton X-100 (1 wt %)	1 ± 0.4

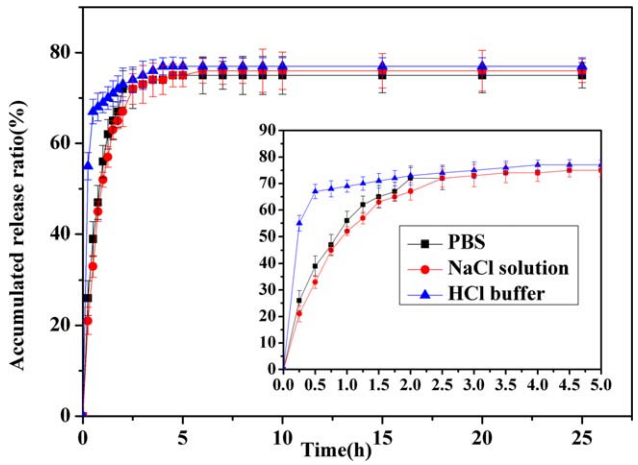


Figure 7. Release of VB_{12} from chitosan microspheres in phosphate buffer (pH 7.4), NaCl (0.9% w/w) aqueous solution (pH 6.3), and HCl buffer (pH 1.2). (The inset shows the release data at the initial time period). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

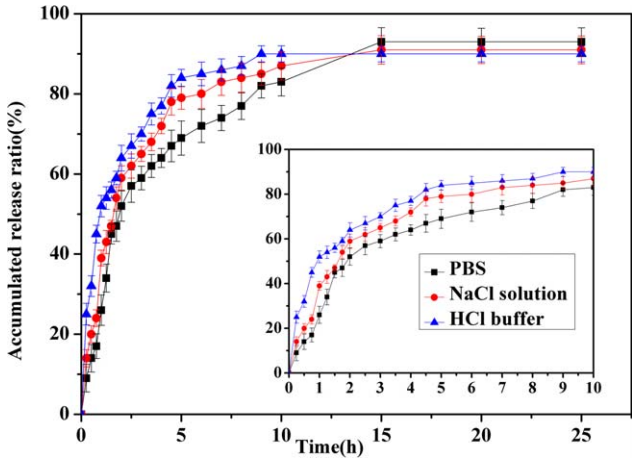


Figure 8. Release of VB_{12} from the DEAE–CMC microspheres in phosphate buffer (pH 7.4), NaCl (0.9% w/w) aqueous solution (pH 6.3), and HCl buffer (pH 1.2). (The inset shows the release data at the initial time period). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

chitosan microspheres. The control chitosan microspheres, which had less interaction with the drug molecules, showed a faster release. About 60–70% of the drug was released in about 1.5 h in these three release media.

For VB_{12}-loaded DEAE–CMC microspheres (Figure 8), a biphasic release pattern was observed with all of the samples. Within the first 2 h, 48, 56, and 64% of the loaded VB_{12} were released from respective DEAE–CMC microspheres. This was considered to be a burst effect, and it was attributed to the unbound excess VB_{12} on the surface of the polymer.[18] Then, all of the microspheres gave a slow drug release up to 15 h after the initial burst effect. After 10 h, VB_{12} steadily released from all of microspheres, and cumulative drug releases of 93, 91, and 90% were realized. The release behavior showed no obvious changes between the PBS and NaCl solution. However, when the microspheres were transferred to the low-pH solution (pH 1.2), the diethylaminoethyl groups were positively charged. Thus, the microspheres swelled rapidly because of osmotic pressure and electrostatic repulsion between the DEAE–CMC chains.

Basically, the DEAE–CMC chain skeleton consisted of hydrophilic and hydrophobic moieties; therefore, microspheres could form with a hydrophobic inner core and a hydrophilic outer shell. The water-soluble nature of VB_{12}, ascribed to its hydrophilicity, might have governed its release pattern and was accompanied by physical interaction with DEAE–CMC, such as van der Waal's forces, hydrogen bonding, hydration forces, static hindrance effects, and hydrophobic interaction.[49,50] We concluded that the hydrophobic inner core and hydrophilic outer shell surrounded VB_{12} and played an important role in the controlled release. First, because of the physical interaction between DEAE–CMC molecules and the hydrophilic drug molecules, the hydrophobic core served as a reservoir for the hydrophilic drug molecules. At the same time, the hydrophilic shell affected their pharmacokinetic behavior.[19] Second, the slow release may have also been due to a hydrophobic barrier that limited the access of water and the dissolution of the drug. The previous reasons all led to the controlled release of VB_{12} from the DEAE–CMC microspheres.

CONCLUSIONS

Chemically modified chitosan has the potential to be used in controlled drug-delivery systems. In this study, DEAE–CMC was synthesized through a protection–graft–deprotection procedure to produce a system possessing amphiphilic properties. The polymer was nonhemolytic when present as a liquid solution and was relatively noncytotoxic. Because of this character, DEAE–CMC could be used as a matrix for controlled drug delivery. The preliminary results for VB_{12} as a model drug, including loading and release experiments, indicate that this system seemed to be a very promising vehicle. The amphiphilic properties originating from the chain structure of the DEAE–CMC molecule could have been the main reason for the slow and steady release of the drug from DEAE–CMC microspheres.

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