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Synthesis of Chitosan-*graft*-β-Cyclodextrin for Improving the Loading and Release of Doxorubicin in the Nanopaticles

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ABSTRACT: Chitosan-*graft*-β-cyclodextrin (CS-*g*-β-CD) copolymer was synthesized by conjugating β-cyclodextrins to chitosan molecules through click chemistry. The copolymer structure was characterized by Fourier transform infrared spectroscopy (FTIR) and nuclear magnetic resonance (NMR). CS-*g*-β-CD/CMC nanoparticles were prepared by a polyelectrolyte complexation process in aqueous solution between CS-*g*-β-CD copolymer and carboxymethyl chitosan (CMC), which was used to load anticancer drug (Doxorubicin hydrochloride, DOX·HCl) with hydrophobic group. The particle size, surface charge, zeta potential, and morphology of the nanoparticles were characterized with dynamic light scattering. The drug loading efficiency and *in vitro* release of DOX·HCl of the nanoparticles were measured by ultraviolet spectrophotometer. The results demonstrated that the size, surface charge and drug loading efficiency of the nanoparticles could be modulated by the fabrication conditions. The drug loading efficiency of CS-*g*-β-CD/CMC nanoparticles was improved from 52.7% to 88.1% because of the presence of β-CD moieties with hydrophobic cavities, which can form inclusion complexes with the drug molecules. The *in vitro* release results showed that the CS-*g*-β-CD/CMC nanoparticles released DOX·HCl in a controlled manner, importantly overcoming the initial burst effect. These nanoparticles possess much potential to be developed as anticancer drug delivery systems, especially those drugs with hydrophobic group. © 2014 Wiley Periodicals, Inc. J. Appl. Polym. Sci. **2014**, *131*, 41034.

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INTRODUCTION

Polymeric drug delivery devices have many advantages over conventional systems, because they allow releasing bioactive substances for longer periods of time with a constant concentration in blood plasma. For this purpose, chitosan (CS), a deacetylated derivative from chitin, is one of the most promising polymers with good biocompatibility and biodegradability, which has been widely used in drug/gene delivery and tissue engineering.[1–3] However, the application of CS as hydrophobic drug carriers is significantly limited because of its water solubility. Several methods have been employed to overcome this matter, including hydrophobic modification of CS to from core-shell micelle structure,[4] host-guest complex formation,[5] development of novel techniques like electrospray ionization,[4] etc.

Cyclodextrins (CD) are versatile malt oligosaccharides that have been applied in diverse fields,[6] and have shown particular success in their ability to the delivery of drugs and nucleic acids.[7–9] The unique hydrophobic cup-like structure within these molecules can be readily functionalized to improve the solubility of hydrophobic drugs in water and protect drug molecules from degradation. β-CD has been widely used in pharmaceutical applications and supermolecular chemistry research among different types of CDs. β-CD has ready availability and cavity size which is suitable for widest range of drugs or guest molecules.[10] To improve the safe delivery, bioavailabilitym, and absorption of drugs, various attempts have been made to bind drugs with polymers.[11] Nevertheless, the mixture of β-CD and polymer as drug carriers is not practical because of limited drug absorption and undesirable stability.[12,13]

The water solubility of CS can be improved by grafting β-CD to CS and the load amount of hydrophobic drug can be increased due to the hydrophobic cavities of β-CD. Recently, CS-grafted-CD copolymers have been prepared by many groups for various studies and applications.[5,14–17] The recent invention of click chemistry by Sharpless[18] is of particular interest, which is insensitive to water and oxygen and has broad applications in quantitative synthesis reactions. In this study, the click chemistry is used to graft β-CD to CS in order to develop suitable pharmaceutics for anticancer drug. Doxorubicin hydrochloride (DOX·HCl), a water soluble anticancer drug, shows a broad spectrum of antitumor efficacy against hematological and solid cancers,[19] which was used as model drug to be encapsulated in

the nanoparticles in this study. Because of the inclusion complexes formation between the drug molecules and the β-CD moieties[20] and the hydrophobic interaction between the hydrophobic cavities of β-CD and the hydrophobic group of the drug, DOX·HCl could be easily loaded in the nanoparticles and the drug release property could be effectively sustained.

In this work, amphipathic chitosan-*graft*-β-cyclodextrin (CS-*g*-β-CD) copolymer was synthesized by conjugating β-CD to CS molecules through click chemistry to combining the beneficial qualities of CS and β-CD. The nanoparticles were prepared through a self-assembly process of CS-*g*-β-CD copolymer and CMC by polyelectrolyte complexation in aqueous solutions at room temperature. The physiochemical properties of the nanoparticles were characterized and the DOX·HCl loading and *in vitro* release behavior of the nanoparticles was measured to evaluate the nanoparticles pharmaceutics for hydrophobic drug.

MATERIALS AND METHODS

Materials

The CS ($M_w = 50$ kDa, deacetylation degree = 95%) was purchased from Haidebei Marine Bioengineering (Jinan, China). β-CD was purchased from Boao Biotechnology (Beijing, China) and recrystallized from water before use. Low MW carboxymethyl chitosan (CMC) (MW ~ 6.1×10^4, the degree of substitution of carboxymethyl group (DS) ~0.66) was purchased from Aoxing Biotechnology (Zhejiang Province, China). 1-(3-dimethylaminopropyl)−3-ethylcarbodiimide hydrochloride (EDC), *N*-hydroxysuccinimide (NHS), propiolic acid, doxorubicin hydrochloride (DOX·HCl), CuCl, sodium azide and other reagents were obtained from Sijia Biotechnology (Guangzhou, China).

Synthesis of Mono-6-(p-tolylsulfonyl)-β-cyclodextrin (6-OTs-β-CD)

The 6-OTs-β-CD was synthesized by referring to previously reported procedure.[21] Briefly, β-CD (200 g, 176.2 mmol) was suspended in 1500 mL of water, and then NaOH (21.6 g, 54 mmol) in 70 mL of water was dropwise added over 30 min under stirring. The suspension became homogeneous and slightly yellow after the addition of NaOH solution. P-tolunesulfonyl chloride (33.6 g, 176.2 mmol) in 100 mL of acetonitrile was dropwise added over 60 min, resulting in white precipitate formation immediately. After stirring for 4 h at 23°C, the precipitate was removed by filtration. The filtrate was adjusted to about pH = 6 with diluted HCl solution (8 mmol) to recover white precipitate by filtration. The pure white solid of 6-OTs-β-CD (23.9 g) with 11% yield was afforded after drying for 12 h.

Synthesis of Mono-6-(azido)-β-cyclodextrin (N_3-β-CD)

The N_3-β-CD was prepared via the reaction of 6-OTs-β-CD with sodium azide.[22] Briefly, 6-OTs-β-CD (19.32 g, 15 mmol) was suspended in water (150 mL) at 80°C, and sodium azide (9.75 g, 150 mmol) was then added. After stirring for 6 h at 80°C, the mixture was cooled to 21°C. The resulting solution was precipitated with acetone (800 mL) and the resulting white powder was recovered by filtration. Pure N_3-β-CD was yielded with 81.0% yield by recrystallization of this crude product in hot water.

Synthesis of Alkynyl-Chitosan (alkynyl-CS)

The precursor for a click reaction, alkynyl-CS, was prepared by the amidation of CS with propiolic acid in the presence of EDC/NHS. In this case, propiolic acid (0.35 g, 5 mmol) was dissolved in CH_2Cl_2 (20 mL), into which NHS (0.64 g, 5.5 mmol) and EDC (1.06 g, 5.5 mmol) were then added. The reaction proceeded overnight at room temperature in dark. After vacuum distillation to remove CH_2Cl_2, acetonitrile (40 mL) was added to dissolve the intermediate product. The acetonitrile solution was dropwise added to CS solution (2 g in 100 mL of 1% acetic acid and 6 g in 300 mL of 1% acetic acid, respectively). The reaction conducted overnight at room temperature under stirring. The reaction liquid was then precipitated with ethanol for three times to obtain the pure product of alkynyl-CS with yield of 85.6% and 83.9%, respectively.

Synthesis of CS-g-β-CD

The CS-*g*-β-CD was synthesized by the click chemistry of N_3-β-CD and alkynyl-CS with the catalyst of Cu (I). Briefly, alkynyl-CS (0.5 g, 0.2 mmol) and N_3-β-CD (0.35 g, 0.3 mmol) were dissolved in 150 mL of 1% acetic acid solution. Under a nitrogen atmosphere, $CuSO_4 \cdot 5H_2O$ (0.05 g, 0.2 mmol, in 2 mL of water) and sodium L-ascorbate (0.12 g, 0.6 mmol, in 3 mL of water) were added successively. Then, the pH value of the solution was adjusted to about 6.0 with NaOH solution. The yellow mixture was stirred for 24 h at room temperature in dark. The product was dialyzed against EDTA for 2 days and against distilled water for 3 days at room temperature, respectively, and then being lyophilized into dry powder of CS-g-β-CD with 82.5% yield.

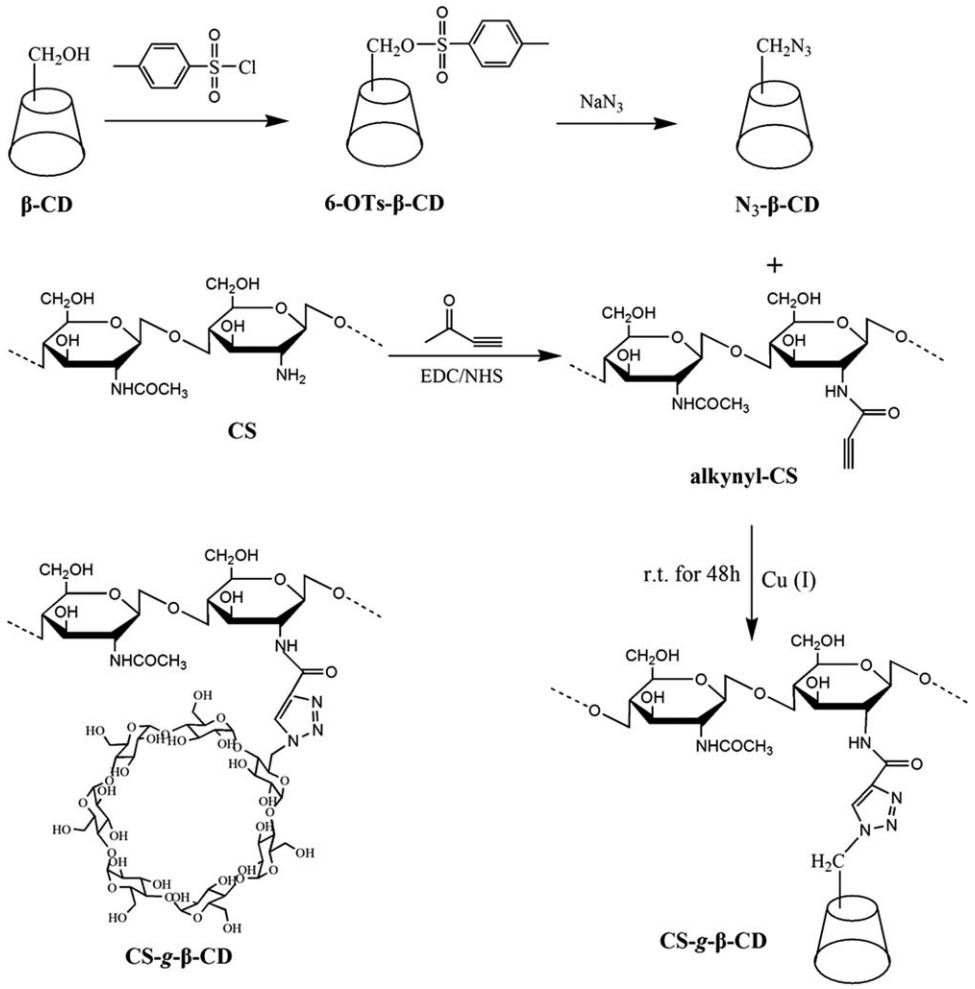
Preparation of CMC/CS-g-β-CD or CMC/CS Nanoparticles for DOX Loading

CS and CS-g-β-CD solution were prepared by dissolving 1 g CS in 1% (w/v) 100 mL acetic acid solution under stirring overnight at room temperature. The CS and CS-*g*-β-CD solution were diluted with deionized water (refer to water thereafter) to produce different concentrations. CMC/CS-*g*-β-CD and CMC/CS nanoparticles were prepared according to the polyelectrolytes self-assembly. In brief, CMC aqueous solution at different concentrations was a dropwise dded to the CS-*g*-β-CD or CS solution and stirred (600 rpm) for 30 min at room temperature to obtain blank nanoparticles. For preparation of DOX-loaded chitosan-based nanoparticles, the DOX solution with concentration of 50 μg/mL was added slowly to the CS-*g*-β-CD or CS solution with mild stirring (600 rpm) for 30 min at room temperature, and then CMC solution was dropwise added to the mixture with mild stirring (600 rpm) for another 30 min. The CMC/CS-*g*-β-CD and CMC/CS volume ratio used throughout this study was 2 : 5.

The drug-loading efficiency, which was calculated as the ratio of the weight of load drug with that of initial added drug, was measured by means of ultraviolet (UV) spectrophotometer.

$$\text{Loading efficiency (\%)} = \frac{\text{Amount of capsulated drug}}{\text{Total amount of drug}} \times 100$$

To study drug release behaviors, DOX-loaded nanoparticles were dissolved in 10 mL of Tris–HCl buffer (pH = 7.4). The incubation was conducted at 37°C with shaking speed of 90 rpm. The concentration of DOX in the release buffer was



Scheme 1. Synthesis route of CS-g-β-CD.

then determined at 481.5 nm by an UV–visspectrophotometer (SHIMADZU UV-2550).

Characterizations

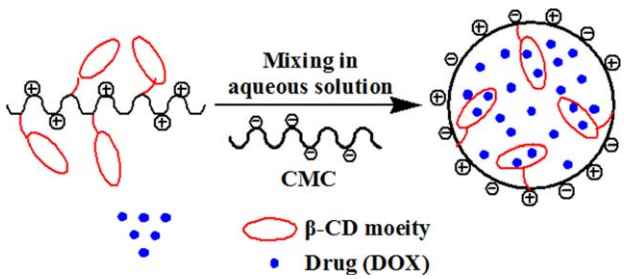
Fourier transform infrared spectroscopy (FTIR) was performed with EQUINOX 55 equipment (EQUINOX 55, Bruker, Germany). The FTIR spectra were recorded from 400 $\sim$ 4000 cm^{-1} using KBr pellets. The ^{1}HNMR spectra were recorded using a Bruker AVANCE AV 400 spectrometer with DMSO or D_2O/CDF_3 as the solvent. The particle size and Zeta potential was measured by Zeta potential instrument (Zetasize Nano, Malvern, Britain).The particle morphology was determined with TEM picture by TECNAI 10 transmission Electronic Microscope (TECNAI 10, Philips, Holland).

RESULTS AND DISCUSSION

Chemical Characterizations of CS-g-β-CD

CS-g-β-CD was synthesized as vectors for drug delivery (Scheme 1). The first step involves the formation of azide group onto β-CD. Second, carboxyl groups of propiolic acid were conjugated with the amino groups of chitosan using EDC and NHS to obtain alkynyl-CS. Then, CS-g-β-CD was synthesized by the click chemistry of N_3-β-CD and alkynyl-CS with the catalyst system of Cu (I). The grafting of β-CD onto chitosan was confirmed by FTIR and ^{1}HNMR.

Azidation of β-CD: Figure 1 shows the FTIR spectra of N_3-β-CD in comparison with its precursory β-CD and 6-OTs-β-CD. In the spectrum of 6-OTs-β-CD [Figure 1(a)], the peaks at 1592 and 1179 cm^{-1} were assigned to the skeletal vibration of



Scheme 2. Schematic representation of the nanoparticles preparation. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

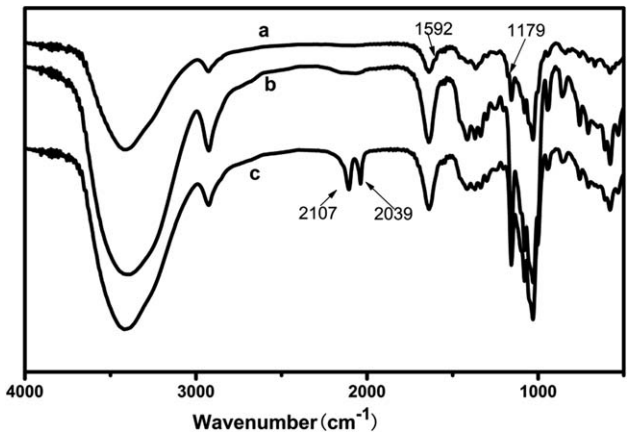


Figure 1. FTIR spectra of (a) 6-OTs-β-CD, (b) β-CD, and (c) N_3-β-CD.

benzene ring. In Figure 1(c), the characteristic signals of azide group of N_3-β-CD appeared at 2107 and 2039 cm^{-1}.

The ^{1}HNMR spectra of the samples are shown in Figure 2, and all samples exhibited the characteristic peaks of β-CD. In Figure 2(b), two new peaks appeared at 7.75 and 7.42 ppm, corresponding to the protons of p-tolylsulfonyl. The spectrum of N_3-β-CD [Figure 2(c)] has more resemblance to that of β-CD and the peaks assigned to 6-OTs-β-CD disappeared. In the spectra, hydroxy of C-6 at 4.4∼4.5 ppm and H(1) proton of β-CD moiety at 4.8 ppm were observed. From the area ratio of these signals, the grafting degree of azide group to β-CD was 100 mol %.

Alkynyl-CS: Figure 3 shows the FTIR spectra of CS and alkynyl-CS. Owing to its weak signal, the vibration peak of alkynyl

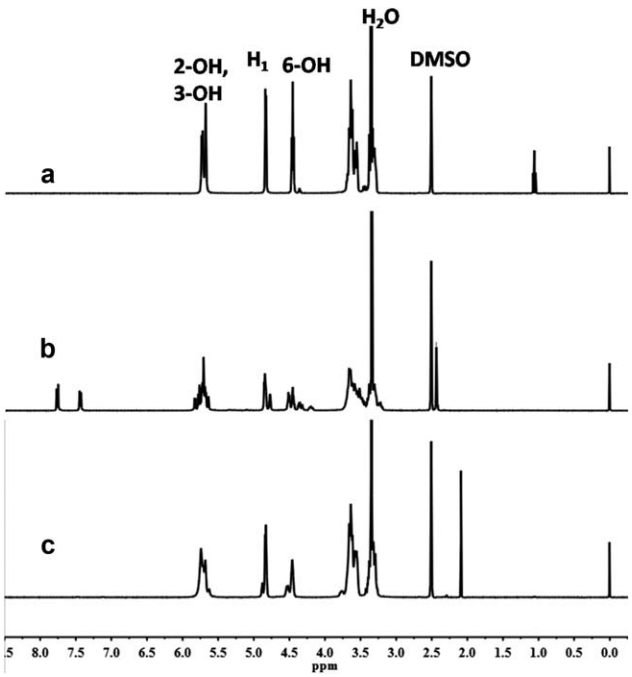


Figure 2. ^{1} HNMR spectra (400 MHz, 6 mg/mL in DMSO) of (a) β-CD, (b) 6-Ots-β-CD, and (c) N_3-β-CD.

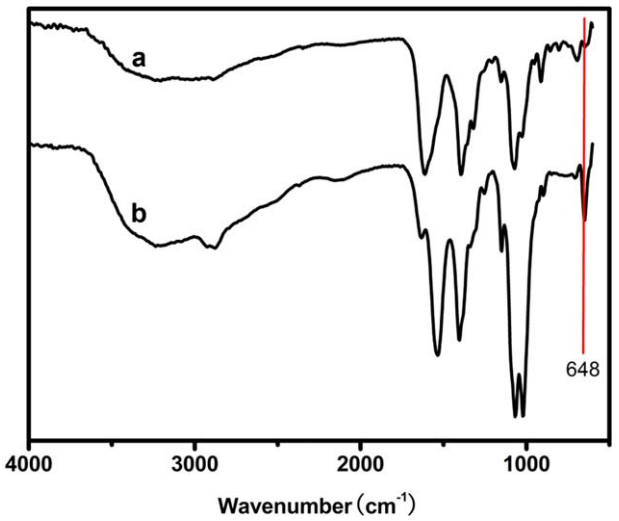


Figure 3. FTIR spectra of (a) CS and (b) alkynyl-CS. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

group (C≡C) cannot be observed. The introduction of alkynyl group was observed with the sharp intensity increase of the absorption band near 648 cm^{-1} which is associated with the bending vibration sharp peak of C≡C—H.

CS-*g*-β-CD: In the final step, the synthesis of CS-*g*-β-CD was accomplished by the click reaction of alkynyl-CS and N_3-β-CD under mild conditions. To guarantee the grafting efficiency, an excess of N_3-β-CD was used to ensure the complete consumption of alkynyl moieties, and the removal of excess N_3-β-CD was achieved by dialysis. The CD content was tunable by changing the degree of substitution of the alkynyl-CS.

The FTIR spectrum of the purified product of CS-*g*-β-CD is shown in Figure 4, which clearly reveals the characteristic

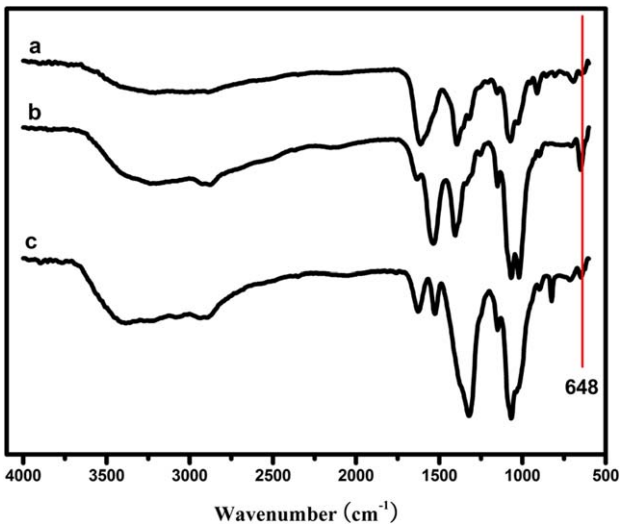


Figure 4. FTIR spectra of (a) CS, (b) alkynyl-CS, and (c) CS-*g*-β-CD. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

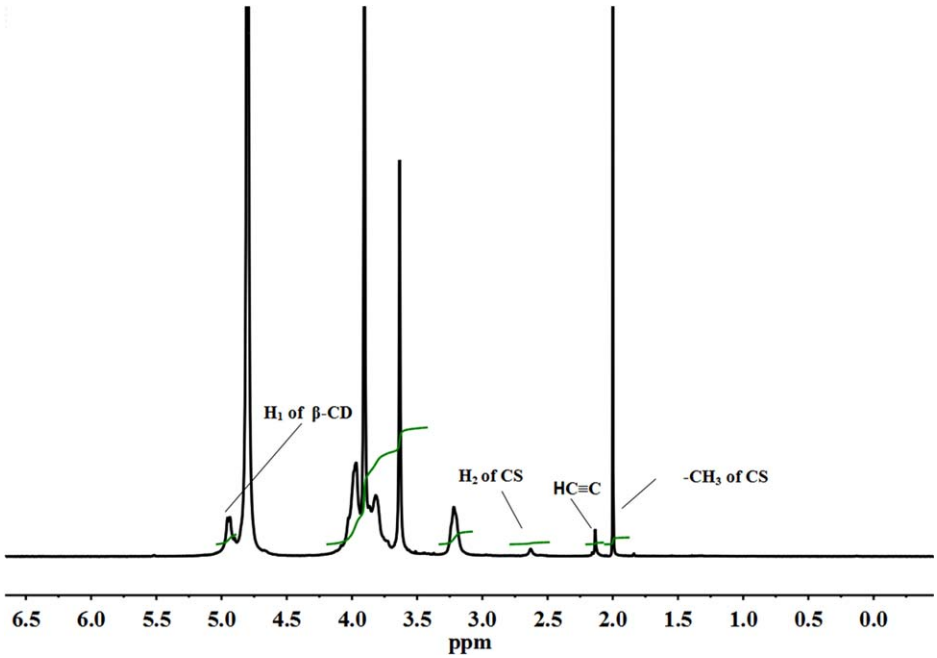


Figure 5. ^{1}HNMR spectrum (400 MHz, 6 mg/mL in D_2O/CDF_3) of CS-*g*-*β*-CD. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

absorbance peaks of both CS and *β*-CD. In Figure 5, a typical ^{1}HNMR spectrum of CS-*g*-*β*-CD shows the characteristics peaks of both CS at 3.7–4.0 ppm and *β*-CD at 3.5–3.6 ppm, respectively. The signals at 2.20–2.50 ppm corresponding to methane proton in alkynyl group indicate the incomplete consumption of alkynyl moieties. However, anomeric protons of α-D-glucopyranosyl residues in the *β*-CD moiety at 4.9–5.0 ppm and those of 2-amino-2-deoxy-*β*-D-glucopyranosyl residues in the CS skeleton at 4.80 ppm were observed. From these results, it is clear that *β*-CD was grafted onto CS backbone. The substitution degree of *β*-CD onto CS (*β*-CD/glucopyranose, mol%) was calculated according to the integrated area ratio of the signals of these anomeric protons, referring to the previous report.[23] When the feed ration (mol %, molar ratio between *β*-CD and glucopyranose unit) was fixed to 1 : 15 and 1 : 5, the prepared CS-*g*-*β*-CD products with two substitution degrees were of 5.81% and 9.62% determined from ^{1}HNMR date, as termed as CS-*g*-*β*-CD-5.81% and CS-*g*-*β*-CD-9.62%, respectively.

Table I. Particle Size and Zeta Potential of the Nanoparticles

Formula	Particle size (nm)	Polydispersity index	Zeta potential (mV)
A1[a]	128.5 ± 19.3	0.236 ± 0.020	23.5 ± 1.3
A2[b]	165.2 ± 28.7	0.364 ± 0.028	20.8 ± 1.1

[a] A1 : 1 mg/mL of CMC+2 mg/mL of CS-*g*-*β*-CD-5.81%, volume ratio = 2 : 5.
[b] A2 : 1 mg/mL of CMC+1 mg/mL of CS-*g*-*β*-CD-5.81%, volume ratio = 2 : 5.

Characterizations of the Nanoparticles

In aqueous solution, CS-*g*-*β*-CD and CMC self-assembled to polyelectrolyte nanoparticles mediated by the electrostatic attraction of positively charged CS-*g*-*β*-CD and negatively charged CMC. The reduced sizes of the nanoparticles formulations are very interesting in view of their potential application in drug delivery. Indeed, it is well known that particles in nanoscale are more efficiently transported through biological barriers;[24] therefore, the reduction in particle size could result in more efficient drug delivery. The physicochemical properties of the nanoparticles are tunable through the change of the volume ratio and the concentration of CS-*g*-*β*-CD and CMC. The prepared nanoparticles ranged in diameter from 128.5 to 165.2 nm

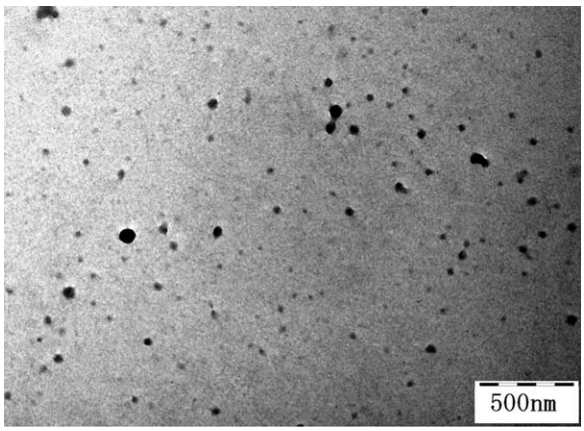


Figure 6. Transmission electron microscopy (TEM) photographs of CM-CS/CS-*g*-*β*-CD nanoparticles, the nanoparticle samples with CS-*g*-*β*-CD concentration of 2 mg/mL, while CM-CS concentration was fixed at 1 mg/mL.

Table II. Effects of β-CD Substitution Degree on DOX Loading Efficiency in the Nanoparticles

Nanoparticles (1 mg/mL/1 mg/mL)	Particle size (nm)	Polydispersity index	Zeta potential (mV)	Loading efficiency (%)
CMC/CS	343.1 ± 20.3	0.370 ± 0.028	45.2 ± 3.7	52.7
CMC/CS-*g*-β-CD-5.81%	109.3 ± 0.5	0.169 ± 0.019	35.6 ± 2.1	65.4
CMC/CS-*g*-β-CD-9.62%	104.4 ± 1.9	0.166 ± 0.003	20.8 ± 1.1	88.1

with a polydispersity index between 0.236 and 0.364 (as listed in Table I).

The effect of different formations on particle size and zeta potential of nanoparticles was examined. The nanoparticles appear to be dependent on the formation. Increasing the concentration of CS-*g*-β-CD-5.81% from 2 mg/mL (Formula A1) to 1 mg/mL (Formula A2) at a constant CMC concentration (1 mg/mL) showed a significant increase in the particle diameter from 128.5 to 165.2 nm. The increased particles diameters were attributed to the increase of CS viscosity[25] and the higher availability of protonated amine groups for ionic interaction with increasing CS-*g*-β-CD concentration.[26] Our research has supported the assumption that through adjusting the concentration of CS-*g*-β-CD or CMC, nanoparticles with appropriate zeta potential and particle size can be obtained according to the properties required.

To complement the studies above, the morphologies of the nanoparticles on different formations was examined via TEM, as shown in Figure 6. It was found that CMC/CS-*g*-β-CD nanoparticles were irregularly spherical in shape and the size was within 100 nm.

From Table II, it can be seen that the increase of hydrophobic cavities by introduction of β-CD reduced the zeta potential of the nanoparticles. The reason might due to the lower degree of substitute and the higher availability of protonated amine groups. The particles size was dramatically reduced with the hydrophobic cavities while it seemed scarcely influenced by the DS value. In addition, the effect of β-CD substitution degree on DOX loading efficiency is also demonstrated in Table II. With the increase of β-CD substitution degree from 0 to 9.62%, the DOX loading efficiency increased from 52.7 to 88.1 mol%. This result was expected, because CS-*g*-β-CD was armed with the unique hydrophobic cup-like structure through the conjugation of β-CD. Thus, the water solubility of CS was improved and the amount of loaded drug DOX increased owing to the formation of inclusion complexes between DOX and the β-CD moieties and hydrophobic interaction between the hydrophobic cavities of β-CD and the hydrophobic group of the drug DOX. Especially, the loading efficiency of DOX reached 88.1%, which is a remarkable increase. In previous studies, the DOX loading efficiency was ~80% at most.[27]

Drug Release *In Vitro*

Figure 7 presents the *in vitro* release profiles of DOX from the nanoparticles. The result shows a rapid initial burst (0–1 h), followed by a sustained release in all samples. Compared with the CMC/CS nanoparticles, the CMC/CS-*g*-β-CD nanoparticles released DOX slower, which could be attributed to host-guest complex formation between DOX and β-CD. In addition, in the case of the CMC/CS-*g*-β-CD nanoparticles, along with the substitution of the amine groups by β-CD, a decrease in the amount of protonated amine groups may lead to shorter intermolecular distances, which would decrease the drug release rate. Furthermore, the initial burst effect seemed to be slightly reduced with the addition of β-CD. For example, the cumulative DOX release amounts at 210 min from the CMC/CS-*g*-β-CD-(0–9.62%) nanoparticles were 53.4%, 36.1%, and 32.9%, respectively. This result indicates that the more hydrophobic

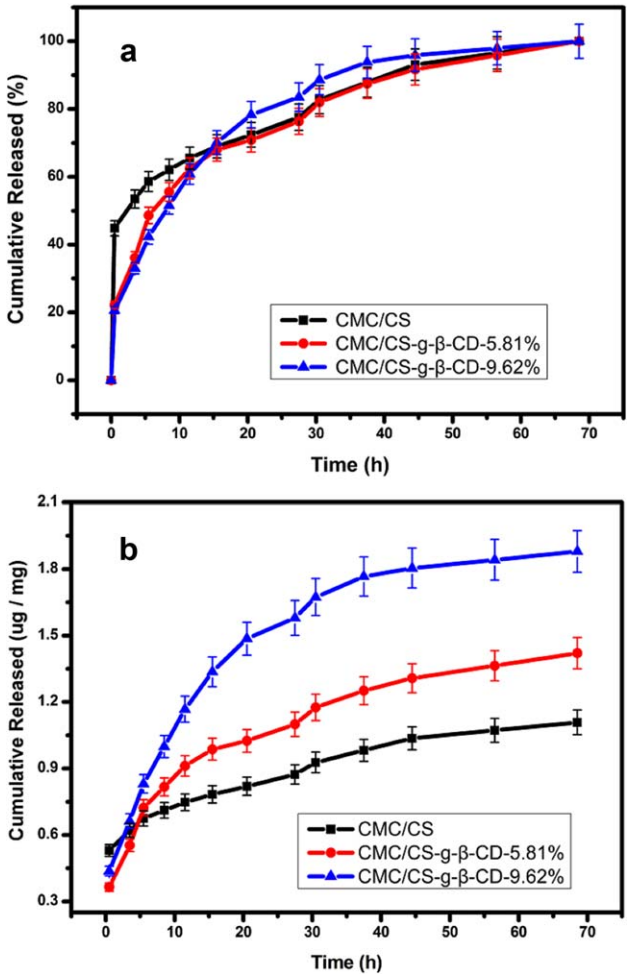


Figure 7. Release profile of DOX from the nanoparticles. a, the ratio of the weight of released drug with the weight of nanoparticles; b, the ratio of the weight of released drug with the weight of loaded drug. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

cavities of β-CD in the nanoparticles result in the lower release rate, which presents a sustained release behavior.

The mechanisms of drug release from polymeric systems have been discussed previously.[15] Three most common mechanisms are erosion, diffusion, and swelling followed by diffusion. Erosion may take place via hydration or hydrolysis of the bulk, the polymer being slowly degraded starting at the periphery of the particle. Diffusion can occur through the no hydrated polymer matrix but will generally be facilitated as the polymer gradually swells in contact with the body fluids. In the case of CMC/CS-*g*-β-CD nanoparticles, the drug release is faster at the initial period of time, followed by a constant and much slower release rate. It seems that the release obeys a swelling-controlled release mechanism, especially at this initial period of release. After the initial period, the release was most probably followed by a diffusion-controlled mechanism. In this diffusion step, the drug release from the CMC/CS-*g*-β-CD nanoparticles was slower than that from CMC/CS nanoparticles due to the higher interaction between the drug and β-CD.

CONCLUSIONS

Amphiphilic CS-*g*-β-CD can be synthesized via click chemistry. The CMC/CS-*g*-β-CD nanoparticles with tunable physicochemical properties can be successfully prepared under mild conditions via ionic gelation method. With increasing the grafting degree of β-CD, the loading efficiency of DOX in the nanoparticles increased dramatically and the *in vitro* release was controlled. The CMC/CS-*g*-β-CD nanoparticles possess a great potential for anticancer drugs delivery, especially those with hydrophobic group.

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