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Core-modified chitosan-based polymeric micelles for controlled release of doxorubicin

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Abstract

Amphiphilic stearic acid-grafted chitosan oligosaccharide (CSO-SA) micelles have been shown a good drug delivery system by incorporating hydrophobic drugs into the core of the micelles. One of the problems associated with the use of CSO-SA micelles is disassociation or the initial burst drug release during the dilution of drug delivery system by body fluid. Herein, the core of CSO-SA micelles was modified by the physical solubilization of stearic acid (SA) to reduce the burst drug release and enhance the physical stability of CSO-SA micelles. The CSO-SA micelles had 27.4 ± 2.4 nm number average diameter, and indicated pH-sensitive properties. The micelle size and drug release rate from micelles increased with the decrease of pH value. After the incorporation of SA into CSO-SA micelles, the micelle size was increased, and the zeta potential was decreased. The extents of the increase in micelle size and the decrease of zeta potential related with the incorporated amount of SA. The *in vitro* drug release tests displayed the incorporation of SA into CSO-SA micelles could reduce the drug release from the micelles due to the enhanced hydrophobic interaction among SA, hydrophobic drug and hydrophobic segments of CSO-SA.

Keywords: Chitosan oligosaccharide; Stearic acid; Micelles; Doxorubicin; Core modification; Controlled drug release

1. Introduction

Polymeric micelles, self-assemblies of amphiphilic graft or block copolymers, are currently recognized as one of the most promising formulations of anti-tumor drug deliveries, and several formulations have been intensively studied in clinical trials (Kataoka et al., 1992, 2001; Kwon and Okano, 1996; Aliabadi and Lavasanifar, 2006). It is well known that polymer micelles have unique core–shell architecture that composed of hydrophobic segments as internal core and hydrophilic segments as surrounding corona in aqueous medium. The hydrophobic core provides a loading space for water-insoluble drugs, whereas the modification of hydrophilic shell affects pharmacokinetic behavior (Huh et al., 2005; Van Nostrum, 2004; Hruby et al., 2005). Additionally, the nano-scaled polymer micelles exhibit many advantages for the use of drug delivery carriers, such as prolonged circulation; tumor localization by enhanced permeability and retention (EPR) effect (Maeda et al., 2000; Maeda, 2001); and the controlled drug release by using stimuli-sensitive copolymers (Liu et al., 2004; Yoo and Park, 2004; Li et al., 2006; Lee et al., 2005).

Polymeric micelles have been used as delivery carriers for extensive variety of therapy and diagnoses drugs (Rapoport, 2004; Nakanishi et al., 2001; Lee et al., 2005; Hruby et al., 2005; Kabanov et al., 2002; Gillies and Frechet, 2005; Huh et al., 2005; Nishiyama et al., 2001). For anti-cancer drugs such as doxorubicin (DOX), polymeric micelles had been extensively utilized for passive targeting to solid tumors. Those DOX formulations exhibited a number of attractive features and advantages over the other types of drug carriers. Some DOX-loaded micelles exhibited prolonged systemic circulation time and much lower cardiotoxicity than free DOX (Kabanov et al., 2002). The folate-targeted DOX-loaded micelles showed superior cytotoxitity in

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cultured folate receptor (+) tumor cells *in vitro* (Yoo and Park, 2004).

In most cases, anti-cancer drugs such as DOX are incorporated into the hydrophobic core of micelles by hydrophobic interaction (Yokoyama et al., 1996; Nishiyama et al., 2001) and/or electrostatic interaction (Kabanov et al., 1996). These interactions are weak between core-forming blocks and incorporated drugs. Because intravenous injections of micellar solution are associated with extreme dilutions by blood, polymer micelles are easily deformable and disassemble which result in the leakage of loaded drugs (Borovinskii and Khokhlov, 1998; Sens et al., 1996). There are evidences that polymeric micelles will release drugs fleetly in a period of hours (Kang et al., 2002; Yu et al., 1998). It is one limitation of micelles as drug delivery carriers.

A number of strategies are used to overcome the drug leakage limitation of polymer micelles. In order to control the drug release rate and reduce the burst release, drug had been chemically conjugated to polymer chains to improve the interaction between the drug and polymer (Kataoka et al., 2001). The shell of micelles had also been cross-linked to reduce the drug leakage in our previous research (Hu et al., 2006b). However, the former strategy was complicate and the drug was pharmacological inactive until the conjugate bond was hydrolyzed. The latter one would change the surfaces structure and properties of micelles, such as the quality of primary amine on the micelle surface, the zeta potential and the ability of cellular uptake. In our early studies, chitosan oligosaccharide (CSO, the low-molecular weight chitosan) was chosen as a main material to prepare drug delivery carrier, because it is a biodegradable, biocompatible, and low-toxic material (Zhang et al., 2003; Vishu Kumar et al., 2004; Liu and Yao, 2002; Andres and Martina, 1998; Janes et al., 2001). The amphiphilic stearic acid-grafted chitosan oligosaccharide (CSO-SA) polymer was synthesized. The CSO-SA could self-aggregate to form polymer micelles in aqueous solution, which was applied to the loading of bovine serum albumin, DNA and paclitaxel (Hu et al., 2006a,b,c). In order to reduce the leakage of drug in future *in vivo* transport process, the core modification of CSO-SA micelle was developed as an alternative approach. Herein, SA was physical solubilized into the core of CSO-SA micelles to get the core-modified micelles. The effect of incorporation of SA on the properties of CSO-SA micelles was investigated. DOX was then used as a model drug to incorporate into the micelles. The effect of incorporation of SA on the properties of drug-loaded CSO-SA micelles, such as micelle size, zeta potential, surface morphology, drug entrapment efficiency and the *in vitro* drug release behaviors, were further investigated in detail.

2. Materials and methods

2.1. Materials

Chitosan (95% deacetylation degree, $M_w = 450$ kDa) was supplied by Yuhuan Marine biochemistry Co. Ltd. (Zhejiang, China). Stearic acid was purchased from Shanghai Chemical Reagent Co. Ltd. (China). 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and 2,4,6-trinitrobenzene sulfonic acid (TNBS) were purchased from Sigma (St. Louis, MO, USA). Master sample of polysaccharide (Part Number: 2090-0100) was purchased from Polymer Labortories Co. Ltd. (USA) and pyrene was purchased from Aldrich Chemical Co. Ltd. Doxorubicin was kindly donated from Zhejiang Hisun Pharmaceutical Co. Ltd. (China). Other chemicals were analytical or chromatographic grade.

2.2. Synthesis of CSO-SA-grafted copolymer

Chitosan oligosaccharide (CSO) was prepared by enzymatic degradation of chitosan as described in our previous reports (Hu et al., 2006a,b,c). The molecular weight of CSO used in this paper was 18.7 kDa (weight average of molecular weight), which was determined by gel permeation chromatography (GPC) with TSK-gel column (G3000SW, 7.5 mm i.d. × 30 cm) at 25 °C.

CSO-SA polymer was synthesized *via* the reaction of carboxyl groups of SA with amine groups of CSO in the presence of EDC as reported in previous research (Hu et al., 2006b). Briefly, CSO (0.8 g) was dissolved in 30-ml distilled water. Stearic acid (0.2 g) and EDC (1.33 g) were dissolved in 20-ml ethanol by sonicate treatment (Sonic Purger CQ250, Academy of Shanghai Shipping Electric Instrument) in water bath at room temperature for 30 s. The mixture was added to the CSO solution, under mechanic stirring with 400 rpm at 80 °C. The reaction mixture was stirred under 80 °C for 5 h, then the reaction mixture was cooled to room temperature and the reaction was continued for further 24 h under stirring. After the reaction, the reaction mixture was extensively dialyzed (molecular weight cut-off 7 kDa, Spectrum Laboratories, Laguna Hills, CA) against distilled water for 48 h with successive exchange of fresh distilled water in order to remove by-product. The dialyzed product was lyophilized. The lyophilized product was washed twice with 20 ml ethanol to remove unreacted SA.

^{1}H NMR spectrum was used to analysis of synthesized CSO-SA. The sample was measured at 298 K with about 0.05 wt.% D_2O solution using a NMR spectrometer (AC-80, Bruker Biospin. Germany).

2.3. Determination of substitution degree of CSO-SA

Substitution degree (SD) of CSO-SA, defined as the number of SA groups per 100 amino groups of CSO, was determined by TNBS method (Andres and Martina, 1998). 4 mg CSO-SA were suspended in 1 ml distilled water and incubated with 2 ml of 4% $NaHCO_3$ and 2 ml of 0.1% TNBS under 40 °C for 1.5 h. Then, 2 ml of 2N HCl was added. The ultra-violet (UV) absorbance of sample at 344 nm was measured by UV spectroscopy (TU-1800PC, Beijing Purkinje General Instrument Co. Ltd., China). The substitution degree of CSO-SA was calculated using a calibration curve obtained by the amino-group determination of a series of solutions with different concentrations.

2.4. Measurement of critical aggregation concentration for CSO-SA-grafted copolymer

The critical aggregation concentration (CAC) of CSO-SA-grafted copolymer was determined by fluorescence measurement using pyrene as a probe (Lee et al., 1998). The concentration of pyrene was controlled at 6×10^{-7} M. Pyrene was firstly dissolved in acetone for quantitation. After the acetone was evaporaed under 50 °C, 10 ml of CSO-SA solution with different concentrations were added into pyrene. The fluorescence spectrum was obtained with a fluorometer (F-2500, Hitachi High-Technologies Co., Tokyo, Japan). The excitation wavelength (λ_{ex}) was 336 nm and the slit openings were set at 10 nm (excitation) and 2.5 nm (emission). Then the intensity ratio of the first (374 nm, I_1) and the third (386 nm, I_3) highest energy bands in the pyrene emission spectra was calculated.

2.5. Preparation of CSO-SA micelles

CSO-SA micelles were prepared by dispersing CSO-SA (20 mg) in 20 ml of distilled water with probe-type ultrasonic treatment for 10 times (active every 1 s for a 6 s duration) in ice-bath (400 W, JY92-II, Ningbo Xinzhi Scientific Instrument Institute, Zhejiang, China).

DOX-loaded CSO-SA micelles were prepared by dialysis method. A certain amount of doxorubicin was dissolved in dimethyl sulphoxide (DMSO). Then the doxorubicin solution was added into CSO-SA solution. The DMSO was removed by dialyzing with dialysis bag (molecular weight cutoff 7 kDa) against distilled water.

The SA-modified micelles were prepared by physical solubilizing SA into the core of micelles. A suitable amount of SA ethanol solution was added drop wise to the CSO-SA micelles solution under stirring at 70 °C. The beaker was opened to air for 2 h, allowing slow evaporation of ethanol. The residual ethanol was completely removed by vacuum distillation with a rotary evaporator. The solution was filtered with a syringe filter (pore size: 0.45 μm) to eliminate the unsolubilized SA. The weight ratios of SA to CSO-SA micelles (w_{SA}:$w_{CSO\text{-}SA}$) performed at 0.5–10%.

The mean size and zeta potential of the micelles were measured by dynamic light scattering using a Zetasizer (3000HS, Malvern Instruments Ltd., UK). An atomic force microscopy (AFM, SPA3800N, SEIKO, Japan) was employed to observe the morphology of CSO-SA micelles. The sample was prepared by casting a dilute micelle solution on freshly cleaved mica surface for 15 min for imaging. The imaging was performed in tapping mode with a gold-coated tip operating with 3.2 N/m force constant.

2.6. Determination of drug entrapment efficiency and drug loading of CSO-SA

The content DOX was measured by fluorescence spectrophotometer. The DOX-loaded micelle solution (0.2 ml) was placed in ultrafilter tube with molecular weight cutoff of 10k (Microcon YM-10, Millipore Co., Bedford, USA) and centrifuged at 14,000 × *g* for 5 min (3K30, SIGMA Labrorzentrifugen GmbH, Germany). The DOX content in filtrate (C_f) was measured. 3.8 ml DMSO (90%) was added into 0.2 ml DOX-loaded micelle solution to dissolve the micelles, and the drug content (C_t) was also determined. The drug content incorporated into micelles was obtained from $C_t - C_f$. The drug entrapment efficiency (EE) and drug loading (DL) were then calculated by the following equations:

$$\mathrm{EE}\,(\%) = \frac{\text{drug content in micelles}}{\text{the initial added drug amount}} \times 100 \quad (1)$$

$$\mathrm{DL}\,(\%) = \frac{C_t - C_f}{C_t - C_f + C_m} \times 100 \quad (2)$$

where C_m represent the amount of CSO-SA in 0.2 ml drug-loaded micelle solution.

2.7. In vitro drug release studies from DOX-loaded micelles

In vitro DOX releases from drug-loaded micelles were investigated using phosphate buffer solution with different pH (2.7, 5, 6.5, 7.2) as dissolution medium. 1 ml DOX-loaded micelles solution with 1 mg/ml CSO-SA concentration was dispersed into 12.5 ml phosphate buffer solution with different pH in a beaker. The beaker was then placed in a 37 °C water bath and stirred at 100 rpm. At certain time intervals, 0.2 ml medium was withdrawn and centrifuged by using ultrafilter tube with a molecular weight cutoff of 10k for 5 min at 14,000 × *g*. The DOX content in filtrate was measured by fluorescence spectrophotometer.

2.8. Statistical analysis

Data were expressed as means of three separate experiments, and were compared by analysis of variance (ANOVA). A p-value <0.05 was considered statistically significant in all cases.

3. Results and discussion

3.1. Synthesis of CSO-SA

CSO-SA was synthesized *via* the reaction of carboxyl group of SA with amine group of CSO in the presence of EDC. ^{1}H NMR spectrum was used to conform the binding between SA and CSO. As shown in Fig. 1, it is clear the proton peaks of CH_3 (chemical shift 0.9 ppm, Fig. 1a) and CH_2 (chemical shift at

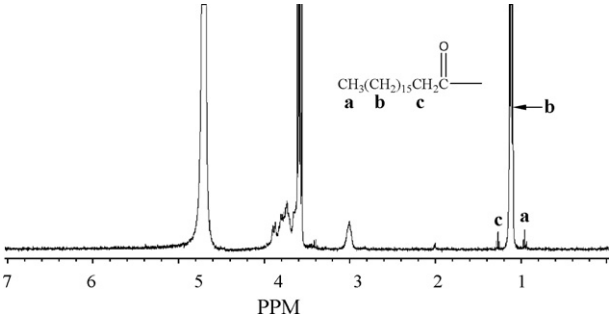

Fig. 1. ^{1}H NMR spectrum of synthesized CSO-SA.

about 1.1 ppm and 1.3 ppm, Fig. 1b and c) for SA were observed in ^{1}H NMR spectrum of CSO-SA, while no such peaks were observed in the same chemical shifts of ^{1}H NMR spectrum for CSO. By controlling reaction time or the charged amount of CSO, SA and EDC, CSO-SA copolymer with different SD could be obtained. The SD of CSO-SA copolymer in present study was about 25.5%, which was determined by TNBS method.

The dye solubilization method using pyrene as a probe molecule was used to examine the CAC value of CSO-SA copolymer (Lee et al., 1998). The intensity ratio of the first energy band (374 nm, I_1) to the third energy band (386 nm, I_3) of the pyrene emission spectra was used as an indicator of the polarity of the pyrene environment. Upon micellization of CSO-SA, pyrene molecules are preferably partitioned into the less polar hydrophobic core, thereby resulting in the change of I_1/I_3 ratios. Fig. 2 shows I_1/I_3 of pyrene emission spectra as a function of the concentration of CSO-SA copolymer. The CAC value of CSO-SA is 0.022 mg/ml, which is significantly lower than the critical micelle concentration (CMC) of low-molecular weight surfactants in water (Lee et al., 1998). The CAC value is one of the important characteristics for polymeric micelles as a drug delivery carrier. The low CAC value is desired to avoid the disassociation of micelles during the dilution of drug delivery systems by body fluid. It means that the present CSO-SA-grafted copolymer can form micelle under highly diluted condition.

3.2. Preparation of CSO-SA micelles

CSO-SA micelles were prepared by dispersing CSO-SA-grafted copolymer in distilled water with probe-type ultrasonic treatment, and the DOX-loaded CSO-SA micelles were obtained by dialysis method. Table 1 summarizes the average micelle size and zeta potential of micelles and drug-loaded micelles. At 1 mg/ml of CSO-SA concentration, the number average diameter and zeta potential of micelles was 27.4 ± 2.4 nm and 51.7 ± 3.0 mV, respectively. After the loading of drug, the size of drug-loaded micelles was 20.4 ± 1.1 nm. It behaved

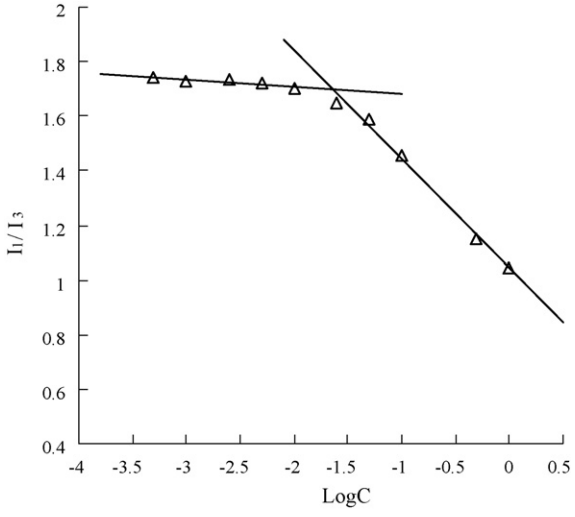

Fig. 2. Fluorescence intensity ratio variation of I_1/I_3 for pyrene emission against the concentration of CSO-SA.

Table 1
Mean sizes and zeta potentials of CSO-SA micelle and DOX-loaded CSO-SA micelle ($n = 3$)

Micelle	Micelle size		Zeta potential (mV)
	d_n (nm)	PI	
CSO-SA micelle	27.4 ± 2.4	0.54 ± 0.06	51.7 ± 3.0
Drug-loaded micelle	20.4 ± 1.1	0.55 ± 0.05	53.1 ± 14.4

d_n and PI indicate the number average diameter and polydispersity index, respectively.

a relative smaller size comparing with the drug-free micelle ($p < 0.05$, $n = 3$), which may attribute to van der Waals interaction between drug and CSO-SA molecule. The zeta potential (53.1 ± 14.4 mV) of the drug-loaded micelles had no obvious change.

The morphologies of CSO-SA micelles were observed by AFM. Fig. 3 shows the AFM images of CSO-SA micelles and core-modified CSO-SA micelles with 0.5% SA. The spherical morphologies of micelles were confirmed in Fig. 3A and B. The observed micelles size was approximately 50–70 nm, which was larger than the hydrodynamic diameter obtained from the DLS experiment. This observation was due to the collapse of micelles during drying process of AFM sample.

3.3. Effect of the modification of SA on the properties on the CSO-SA micelles

The cores of CSO-SA micelles and DOX-loaded CSO-SA micelles were further solubilized with different amount of SA. The effects of SA amount on the size and zeta potential of CSO-

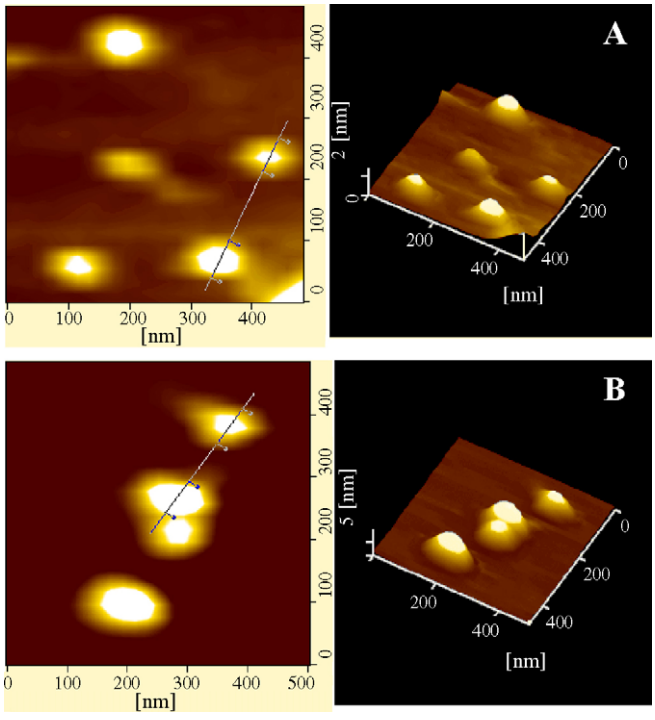

Fig. 3. Atomic force microscopy image of CSO-SA micelles (A) and CSO-SA micelles modified with 0.5% SA (B).

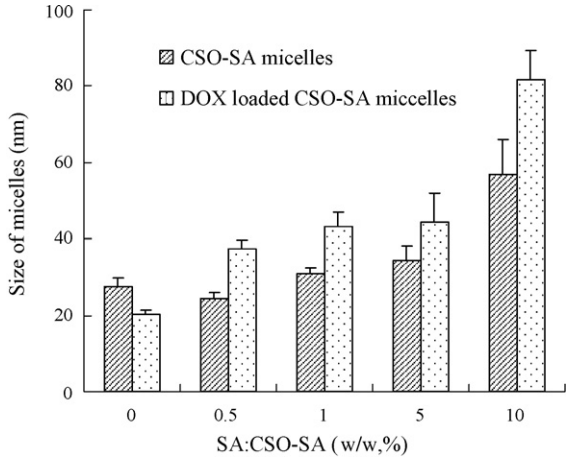

Fig. 4. Effects of amount of SA on the size of CSO-SA micelles and DOX-loaded CSO-SA micelles.

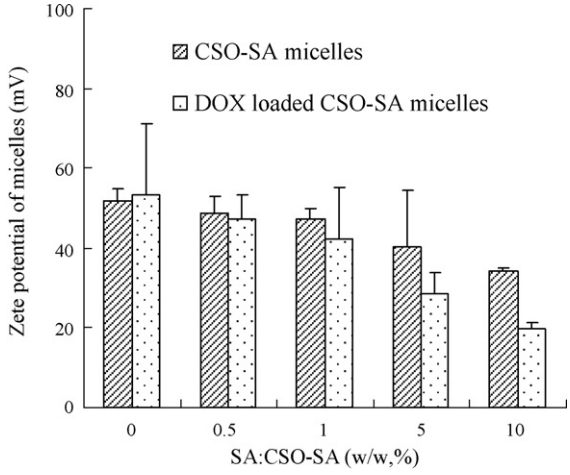

Fig. 5. Effects of amount of SA on the zeta potential of CSO-SA micelles and DOX-loaded CSO-SA micelles.

SA micelles and DOX-loaded CSO-SA micelles are shown in Figs. 4 and 5. It can be found that the particle size of core-modified CSO-SA micelles with 0.5% SA was smaller than that of unmodified CSO-SA micelles, which may be due to the van der Waals interactions between the SA molecule and the SA segments of CSO-SA. The particle size of core-modified CSO-SA micelles was then increased with increasing the incorporated amount of SA. According to the statistics analysis, the particle size of CSO-SA micelle with 10% SA was significantly larger than that of CSO-SA micelle ($p < 0.05$). On the other hand, the core-modified CSO-SA micelles loading DOX were higher than that of core-modified CSO-SA micelles with the same amount of SA ($p < 0.05$). The zeta potentials of core-modified CSO-SA micelles and DOX-loaded CSO-SA micelles decreases as the increasing of added SA amount (Fig. 5, $p < 0.05$). The reduction of zeta potential of CSO-SA micelles and DOX-loaded CSO-SA micelles could be contributed to the deprotonation of carboxyl group of SA incorporated into the micelles.

The influences of pH value of PBS medium on DOX-loaded CSO-SA micelles modified by different amount of SA are shown in Fig. 6. It can be seen that the mean diameter of DOX-loaded CSO-SA micelles increased from about 30 to 250 nm when the pH value of medium from decreased from 7.2 to 2.7. The size change of the CSO-SA micelles may depend on the protonation extent of remained primary amino groups of CSO-SA molecule under different pH value of environment. At low pH, the amino groups will be partially or completely ionized. Consequently, the electrostatic repulsive forces of inter-/intra-molecules increase, and lead to the chain stretch CSO-SA. Thus, the CSO-SA micelles under lower pH value would result in an increase in the mean size of micelles. The pH effects on size were also observed in the cases of drug-loaded CSO-SA micelles modified by SA.

On the other hand, the zeta potential of DOX-loaded CSO-SA micelles modified by different amounts of SA have positive charges in different pH PBS and with the value of about 20–60 mV. With the pH value decreased, the zeta potential of micelles were increased, which also caused by the higher protonation degree of the primary amine of CSO segments at lower pH value.

The effect of the modification of SA on the EE and DL of DOX-loaded CSO-SA micelles modified by different amounts of SA are listed in Table 2. It can be seen that all formulations indicated almost the same EE (about 50%) and DL (5%). This means the modification of DOX-loaded CSO-SA micelles by physical incorporation of SA would not affect the EE and DL of DOX-loaded CSO-SA micelles.

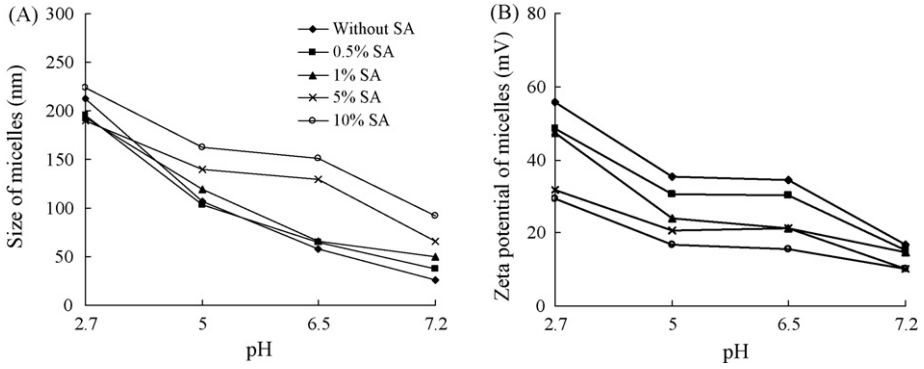

Fig. 6. Effects of the pH value of dispersion medium on the micelle size (A) and zeta potential (B) of drug-loaded CSO-SA micelles modified by different amount of SA.

Table 2
Drug entrapment efficiency and drug loading of DOX-loaded CSO-SA micelles modified by different amount of SA ($n = 3$)

w_{SA}:$w_{CSO\text{-}SA}$ (%)	EE (%)	DL (%)
0	55.09 ± 0.11 a	5.51 ± 0.01 b
0.5	53.21 ± 1.24 a	5.29 ± 0.12 b
1	50.92 ± 1.64 a	5.04 ± 0.16 b
5	51.07 ± 2.21 a	4.86 ± 0.21 b
10	56.21 ± 7.01 a	5.11 ± 0.63 b

a and b: present $p > 0.05$.

3.4. The effect of the modification of SA on the in vitro drug release of DOX-loaded CSO-SA micelles

Figs. 7 and 8 show the drug release profiles of DOX-loaded CSO-SA micelles modified by different amount of SA using dissolution medium with different pH value. As shown in Fig. 7A, the drug release profiles of DOX-loaded CSO-SA micelles without SA modification shown a fast release rate. About 80% drug released in 24 h. The result was similar as our early report (Hu et al., 2006b). It was found that the pH value of dissolution medium affect the drug release rate of DOX-loaded CSO-SA micelles. The drug release rate from CSO-SA micelles reduced with the increasing of the pH value of dissolution medium (significant difference of drug release in 24 h was found in the runs under pH 2.7 and 7.4 media, $p < 0.05$). The fastest drug release was found when the pH value of dissolution medium was 2.7, in which the drug release reached up to 70% in 8 h. The slowest drug release was found when the pH value of dissolution medium was 7.2, in which the drug release only reached up to 30% in 8 h. The faster drug release rate in lower pH medium could be contributed to two factors: the one is the loose micelle structure, which caused by the stronger protonation of amino groups of CSO in lower pH; the other is the higher solubility of DOX in lower pH. The pH-sensitive drug release behaviors were also observed in the cases of DOX-loaded CSO-SA micelles modified by SA (Fig. 7B–D, significant difference of drug release in 24 h was found in the runs modified by SA under pH 2.7 and 7.4 media, $p < 0.05$). All of the drug release profiles did not fit the Peppas model or Higuchi model. The Fickian diffusion and polymer relaxation were the release mechanisms for the present case.

Furthermore, as shown in Fig. 8, it was obvious the drug release rates were delayed with increasing the SA concentration. Significant differences of drug release in 24 h were found in the runs with different SA modification under different pH media ($p < 0.05$). The DOX-loaded CSO-SA micelles unmodified by SA presented the faster drug release rate under dissolution medium with different pH, and the DOX-loaded CSO-SA micelles modified by 5% SA indicated the slower drug release rate under dissolution medium with different pH. The slowest drug release was achieved when the DOX-loaded CSO-SA micelle was modified by 5% SA using dissolution medium with pH 7.2. Only about 20% drug was released in 24 h. The increase amount of SA in CSO-SA micelles resulted in the enhanced hydrophobic interaction between SA and SA segments in CSO-SA to form a tightly packed hydrophobic

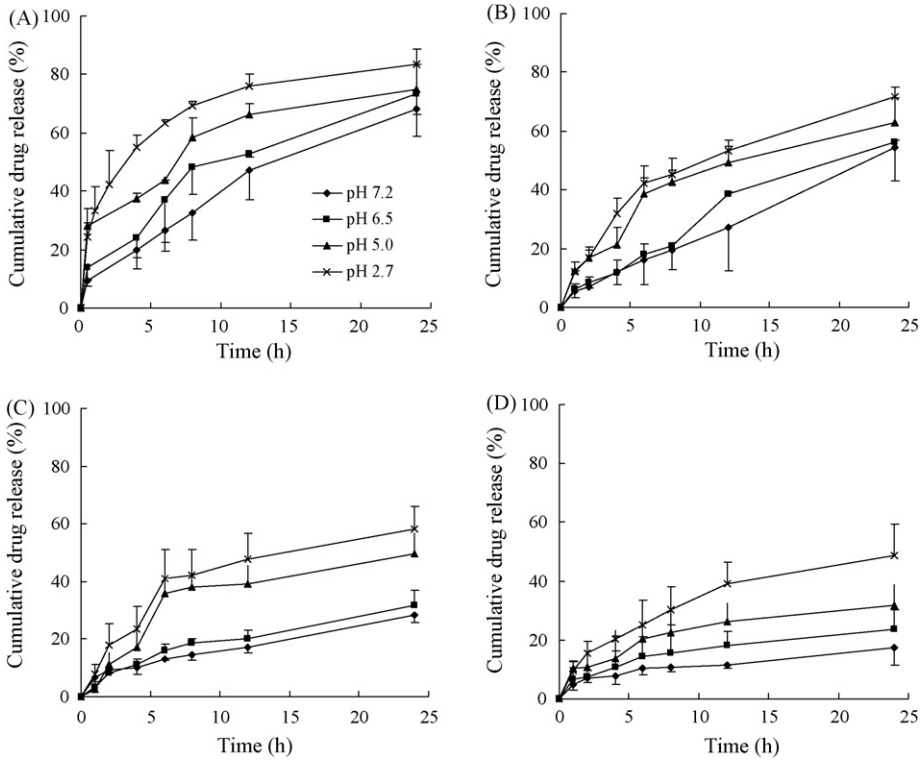

Fig. 7. *In vitro* drug release profiles of DOX-loaded CSO-SA micelles modified by different amount of SA (A: without SA; B: 0.5% SA; C: 1% SA; D: 5% SA) using dissolution medium with different pH value ($n = 3$).

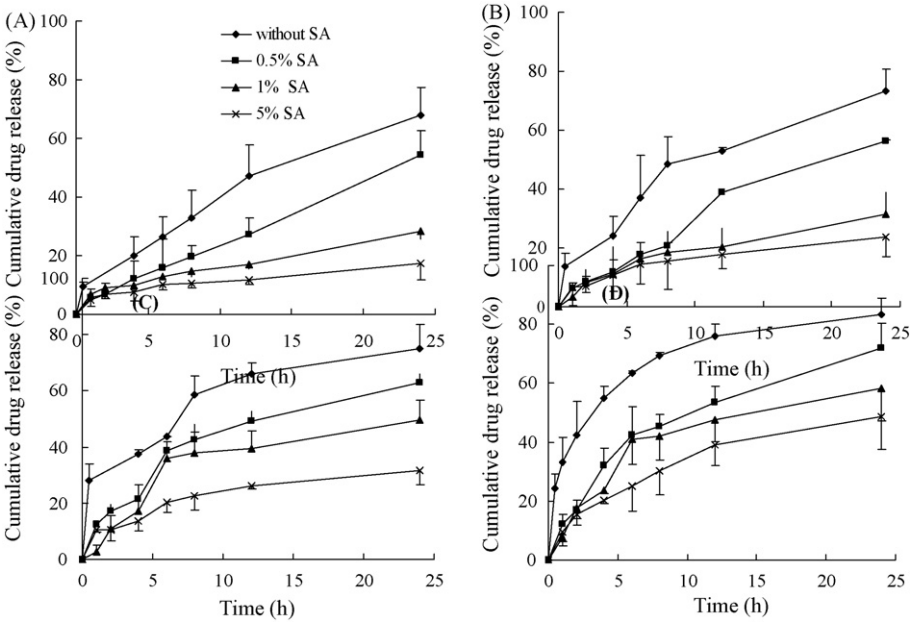

Fig. 8. *In vitro* drug release profiles of DOX-loaded CSO-SA micelles modified by different amount of SA using dissolution medium with different pH values (A: pH 7.2; B: pH 6.5; C: pH 5.0; D: pH 2.7) ($n = 3$).

core, and the ionic interaction between SA and DOX. Due to these reasons, the drug release was delayed by the modification of SA.

4. Conclusions

The CSO-SA micelles with small size and low CAC value presented an excellent candidate for drug delivery carrier. To solve the initial burst dug release during the dilution of drug delivery system by body fluid, the CSO-SA micelles was modified by physical solubilization of SA. Although the incorporation of SA could cause the change of the size and zeta potential, but it did not affect the EE and DL of CSO-SA micelles. DOX-loaded CSO-SA micelles showed pH-sensitive properties on the size, zeta potential and drug release. The incorporation of SA into DOX-loaded CSO-SA micelles could significantly reduce the drug release rate. We believe the present modification method of micelles can be widely used for the controlled release of drug-loaded polymeric micelle systems.

Acknowledgements

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