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Solid lipid nanoparticles modified with chitosan oligosaccharides for the controlled release of doxorubicin

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ABSTRACT

Solid lipid nanoparticles (SLN) modified with chitosan oligosaccharide was prepared by solvent diffusion method and subsequent ionic interaction. Using doxorubicin as a model drug, the effects of amount and molecular weight of chitosan oligosaccharide, and its crosslink degree by glutaraldehyde on the physicochemical properties of nanoparticles were investigated. After modification with chitosan oligosaccharide, the zeta potential of nanoparticles changed from about −20 mV to above 30 mV, and the drug loading could be improved from 6.76% to 22.3%. *In vitro* drug release tests showed the burst release of SLN could be significantly reduced by the chitosan oligosaccharide modification, and the release rate could be controlled by changing the molecular weight of chitosan oligosaccharide and the crosslink degree. *In vitro* antitumor activity tests indicated the SLN could mediate the cellular internalization of doxorubicin, and the chitosan oligosaccharide modification and crosslink could further improve the cellular uptake and cytotoxicity of doxorubicin.

1. Introduction

Solid lipid nanoparticles (SLN) represent an alternative carrier system to traditional colloidal carriers since the early 1990s (Müller, Mäder, & Gohla, 2000). Compared with traditional carriers, SLN combine the advantages of polymeric nanoparticles and emulsions for drug delivery, such as low toxicity, good biocompatibility, and targeting effect on brain (Yang, Lu, Cai, & Zhu, 1999). SLN have been applied via parenteral, oral, dermal, ocular, pulmonary and rectal routes (Joshi & Müller, 2009; Kalam et al., 2010; Ojewole, Mackraj, Naidoo, & Govender, 2008; Pardeike, Hommoss, & Müller, 2009; Weyenberg et al., 2007), most of which have been characterized *in vitro* and *in vivo* (Bhaskar et al., 2009; Gulbake, Jain, Khare, & Jain, 2010; Wissing & Müller, 2002). However, the application of SLN was limited by the low drug loading capacity and burst release behavior. In the nanoparticles structure, the lipid forming highly crystalline state with a perfect lattice would lead to drug expulsion. In recent years, modifying SLN has attracted increasing attention for overcoming the drawbacks in application (Santander-Ortega, Lozano-Lopez, Bastos-Gonzalez, Peula-Garcia, & Ortega-Vinuesa, 2010; Wu, Tang, & Yin, 2010; Zhang, Liu, Li, Chen, & Liu, 2008; Zhang, Pan, et al., 2008).

Chitosan, a cationic polysaccharide, exists abundantly in nature and shows potential for safe use in healthcare field. It is derived from chitin by alkaline deacetylation and consists of 2-amino-2-deoxy-(1-4b)-D-glucopyranose residues (D-glucosamine units) and *N*-acetyl-D-glucosamine units (Kean, Roth, & Thanou, 2005). Chitosan is a biodegradable natural polymer with great potential for pharmaceutical applications due to its biocompatibility, high charge density, non-toxicity and mucoadhesive propertiey (Du et al., 2010; Illum, Jabbal-Gill, Hinchcliffe, Fisher, & Davis, 2001). Chitosan has been used as a drug carrier for sustained release preparations and improvement of bioavailability for hydrophobic drugs, and as a vehicle for directly compressed tablets, disintegrant, binder and granulating agent (Sinha et al., 2004). It has been shown to possess mucoadhesive properties (Kockisch, Rees, Young, Tsibouklis, & Smart, 2003) due to molecular attractive forces formed by electrostatic interaction between positively charged chitosan oligosaccharide and negatively charged mucosal surfaces. In recent years, many researchers focused on the water-soluble chitosan oligosaccharide with relatively low molecular weight, to synthesize the chitosan oligosaccharide hydrophobic derivatives for the controlled release of hydrophobic antitumor drug (Du, Wang, Yuan, Wei, & Hu, 2009; Muzzarelli, 2010a,b; Yang et al., 2010).

Doxorubicin, a potent anticancer drug, is widely used clinically in treating leukemia, lymphomas and various solid tumors of lung and breast (Young, Ozols, & Myers, 1981), however, its clinical application is limited by the severe side effects, such as

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Table 1
Recipes for preparing doxorubicin-loaded SLN, doxorubicin-loaded SLN modified with chitosan oligosaccharide and crosslinked doxorubicin-loaded SLN modified with chitosan oligosaccharide.

Formula			Chitosan				
	Doxorubicin) (mg)	MS (mg)	M_w (kDa)	Amount	Molar ratio of glutaraldehyde to CSO	DMSO (mL)	Water
1	0.125	4.875	–	–	–	1	9
2	0.25	4.75	–	–	–	1	9
3	0.375	4.625	–	–	–	1	9
4	1	2	18	5	–	1	9
5	2	2	18	5	–	1	9
6	1	2	8	5	–	1	9
7	1	2	42	5	–	1	9
8	1	2	8	1	–	1	9
9	1	2	8	2	–	1	9
10	1	2	8	5	2:5	1	9
11	1	2	8	5	6:5	1	9
12	1	2	18	5	2:5	1	9
13	1	2	18	5	6:5	1	9
14	1	2	42	5	2:5	1	9
15	1	2	42	5	6:5	1	9

MS, M_w, CSO and DMSO indicate monostearin, weight average molecular weight, chitosan oligosaccharide and dimethyl sulfoxide, respectively.

cytotoxicity and dose-dependent congestive heart failure (Singal & Iliskovic, 1998; Torti et al., 1986). To minimize its side effects, various drug delivery systems have been developed, including SLN (Subedi, Kang, & Choi, 2009; Ying, Du, Chen, Yuan, & Hu, 2008). It is important to develop an intracellular transport drug carrier system to increase the doxorubicin concentration in tumor cells and improve its efficiency.

In this study, chitosan oligosaccharide was adsorbed onto the negative charge surface of SLN through the electrostatic interaction. Using hydrophobic doxorubicin base as a model drug, the effects of molecular weight and charged amount of chitosan oligosaccharide, and the crosslink degree by glutaraldehyde on the physicochemical properties of SLN modified with chitosan oligosaccharide were investigated, including number average diameter, zeta potentials, drug encapsulation efficiency, drug loading, *in vitro* drug release behavior, and uptake ability by tumor cells and cytotoxicity.

2. Materials and methods

2.1. Materials

Chitosan oligosaccharide was obtained by enzymatic degradation of 95% deacetylated chitosan (M_w = 450 kDa), which was supplied by Yuhuan Marine Biochemistry Co., Ltd., Zhejiang, China. 50 g of chitosan (M_w = 450 kDa) was dispersed in 2 L deionized water (DI water), and 18 mL of 36.5% (w/v) hydrochloric acid was added. After the temperature of the mixture was raised up to 55 °C, 1 g chitosanase was added. The molecular weight of chitosan oligosaccharide was controlled by reaction time of hydrolysis, which was determined by gel permeation chromatography (GPC) with TSK-gel column (G3000SW, 7.5 mm I.D. × 30 cm) at 25 °C (Hu et al., 2006). Monostearin was purchased from Shanghai Chemical Reagent Co. Ltd., China. Glutaraldehyde solution 25% was supplied by Sinopharm Chemical Reagent Co., Ltd. Doxorubicin hydrochloride (Doxorubicin·HCl) was kindly donated from Zhejiang Haizheng Pharmaceutical Co. Ltd., China. Iron II, III. Dimethyl sulfoxide (DMSO) was purchased from Haishuo Biochemistry Co., Ltd.,Wuxi, China. Polyvinyl alcohol (PVA 0486) was purchased from Beijing Chemicals Co. Ltd., China. All other chemicals were analytical or chromatographic grade.

MCF-7 (human breast carcinoma cell line) was donated by the second affiliated hospital of Medicine College in Zhejiang University, Hangzhou, China. FBS (fetal bovine serum) was purchased from Sijiqing Biologic, Hangzhou, China. RPMI 1640 was purchase from Gibco BRL, USA.

2.2. Preparation of doxorubicin-loaded solid lipid nanoparticles

Doxorubicin-loaded solid lipid nanoparticles were prepared by solvent diffusion method in aqueous solvent (Hu, Yuan, Zhang, & Fang, 2002). Triethylamine was utilized to convert doxorubicin·HCl into doxorubicin base (Lee, Na, & Bae, 2005). The preparation formulas were indicated in Table 1. Doxorubicin and monostearin were completely dissolved in 1 mL DMSO at 75 °C. Then the resultant organic solution was quickly dispersed into 9 mL deionized water under mechanical agitation (DC-40, Hangzhou Electrical Engineering Instruments, China) with 400 rpm for 5–10 min at room temperature, and the nanoparticles dispersion was obtained. To purify the nanoparticles containing doxorubicin, 500 μL 1 M HCl was added into 10 mL nanoparticles dispersion. After the dispersion was frozen and melted, the precipitate of nanoparticles were collected by centrifugation (64R, Beckman, USA) at 20,000 rpm for 35 min. The precipitate of nanoparticles was washed by deionized water twice by the help of centrifugation. Then precipitate was redispersed in PBS (pH 7.4) containing 1% PVA (Hu, Hong, & Yuan, 2004).

2.3. Preparation of doxorubicin-loaded SLN modified with chitosan oligosaccharide

To prepare doxorubicin-loaded SLN modified with chitosan oligosaccharide, doxorubicin and monostearin were firstly dissolved in DMSO, and then dispersed in deionized water containing chitosan oligosaccharide. Here, the charged amount or molecular weight of chitosan oligosaccharide was varied in different formulas (see Table 1), and various doxorubicin-loaded SLN modified with chitosan oligosaccharide dispersion was obtained. For the preparation of glutaraldehyde crosslinked doxorubicin-loaded SLN modified with chitosan oligosaccharide, the glutaraldehyde (glutaraldehyde/chitosan oligosaccharide = 2/5, 6/5, mol/mol) was added into the doxorubicin-loaded SLN modified with chitosan oligosaccharide dispersion, the cross-link reaction between chitosan oligosaccharide and glutaraldehyde was conducted under stirring at room temperature for 7 h. The purification method of nanoparticles was same as mentioned in Section 2.2.

2.4. Characteristics of nanoparticles

The number average diameter and surface zeta potential of doxorubicin-loaded SLN and doxorubicin-loaded SLN modified with chitosan oligosaccharide in dispersion were determined by

a Zetasizer (Nano-zs90, Malvern Instruments, UK; Zetasizer 3000 HS Malvern Co., UK) after the nanoparticles dispersion was diluted 10 times with distilled water.

The morphological examinations of doxorubicin-loaded SLN, doxorubicin-loaded SLN modified with chitosan oligosaccharide and cross-linked doxorubicin-loaded SLN modified with chitosan oligosaccharide were performed by a transmission electronic microscopy (TEM) (JEM-1230, JEOL, Japan). The samples were placed on copper grids, and stained with 2% (w/v) phosphotungstic acid for viewing.

The doxorubicin content was measured by a fluorescence spectrophotometer (F-2500, HITACHI Co., Japan). The detection was accomplished at $\lambda_{ex}505/\lambda_{em}565$, and the slit openings were both set at 5.0 nm. To determine the drug entrapment efficiency of the doxorubicin-loaded nanoparticles, 500 μL 1 M HCl was added into 10 mL nanoparticles dispersion. After treated by vortex for 30 s, the dispersion was frozen and melted. The supernatant and precipitate of nanoparticles were collected by centrifugation (64R, Beckman, USA) at 20,000 rpm for 35 min. The 100 μL supernatant was demolished by adding 900 μL DMSO in 75 °C water bath for 15 min. This solution was cooled down to room temperature and centrifuged for 15 min at 12,000 rpm. The drug content in supernatant (W_S) was estimated by comparing to standard curve obtained from the doxorubicin DMSO aqueous solution (DMSO/H_2O = 9:1, v/v). The drug encapsulation efficiency (EE) and drug loading (DL) could be calculated from the equations below:

$$EE = \left(\frac{W_D - W_S}{W_D}\right) \times 100\% \quad (1)$$

$$DL = \left(\frac{W_D - W_S}{W_D - W_S + W_M + W_{CSO}}\right) \times 100\% \quad (2)$$

where W_D, W_M and W_{CSO} represent the charged amount of doxorubicin, monostearin and chitosan oligosaccharide, respectively.

2.5. In vitro drug release studies

The *in vitro* drug release profiles of doxorubicin-loaded nanoparticles were then carried out using PBS (pH 7.4) solution as dissolution medium. 500 μL 1 M HCl was added into prepared nanoparticles dispersion. After treated by vortex for 30 s, the dispersion was frozen and melted. The precipitates of nanoparticles were collected by centrifugation (64R, Beckman, USA) at 20,000 rpm for 35 min. The collected nanoparticles were redispersed in 1 mL PBS (pH 7.4) containing 1% PVA and treated by vortex for 90 s, and put into dialysis bag (MWCO: 7.0 kDa, Spectrum Laboratories, Laguna Hills, CA). The dialysis bag was set into plastic tube containing 20 mL PBS (pH 7.4) solution. The plastic tube was then placed in an incubator shaker (SHELLAB1227-2E, SHELLAB, USA) at 37 °C, horizontally shaken at 60 rpm. At definite time intervals, all medium outside the dialysis membrane was replaced with fresh PBS. The drug concentration was determined by a fluorescence spectrophotometer. All drug release tests were performed thrice.

2.6. Cytotoxicity tests

MCF-7 cells were maintained in RPMI 1640 supplemented with 10% (v/v) FBS (fetal bovine serum) and penicillin/streptomycin (100 U mL^{-1}, 100 U mL^{-1}) at 37 °C in a humidified atmosphere containing 5% CO_2. Cells were subcultured regularly using trypsin/EDTA.

Cytotoxicities of doxorubicin·HCl, doxorubicin-loaded nanoparticles against MCF-7 cells were evaluated with MTT methods. Briefly, 10,000 cells per well in 1 mL growth medium were plated in 96-well plates (Nalge Nunc International, Naperville, IL, USA) and grown for 24 h. Then the cells were exposed to different concentrations of free doxorubicin·HCl, doxorubicin-loaded nanoparticles at 37 °C for another 48 h. At the end of incubation time, 20 μL MTT solution with the concentration of 5 mg mL^{-1} was added and incubated for further 4 h at 37 °C. After that, 100 mL DMSO was added to each well to dissolve the MTT formazan crystals. Finally, the plates were shaken for 30 min, and the absorbance of formazan product was measured at 570 nm in a microplate reader (Bio-Rad, Model 680, USA). All the experiments were performed in trice.

2.7. Cell internalization observation

MCF-7 cells was seeded at 30,000 mL^{-1} cells/well in a 24-well plate (Nalge Nunc International, Naperville, IL, USA) and grown for 24 h, respectively. A certain amount (final drug content was 3.75 mg mL^{-1}) of doxorubicin·HCl, doxorubicin-loaded nanoparticles were added, and the cells were further incubated for 0.5 and 6 h, respectively. After cells were washed with PBS three times, the cellular uptake was observed by a fluorescence microscopy (Olympus America, Melville, NY).

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. Preparation and characteristics of doxorubicin-loaded SLN modified with chitosan oligosaccharide

Doxorubicin loaded monostearin SLN prepared by solvent diffusion method had negative surface charged. Dispersing chitosan oligosaccharide into aqueous phase in the SLN preparation, chitosan oligosaccharide could be adsorbed onto the negative charged surface of SLN through electrostatic interaction to form doxorubicin-loaded SLN modified with chitosan oligosaccharide.

The recipes and properties of prepared doxorubicin-loaded SLN, doxorubicin-loaded SLN modified with chitosan oligosaccharide and glutaraldehyde crosslinked doxorubicin-loaded SLN modified with chitosan oligosaccharide were shown in Table 1 and Table 2, respectively. From, formulas 1–3 from Table 1 and 2, it was clear that the monostearin (MS) SLN loading doxorubicin had negative charge with about −20 mV Zeta potential, and nano-ordered size. When the charged percentage of the amount of doxorubicin to doxorubicin and MS increased from 2.5% to 7.5, the size of SLN enhanced from 83.7 nm to 163.3 nm, and the drug encapsulation efficiency was also improved from 84.88% to 89.72%. The increased drug encapsulation efficiency was due to the limited drug solubility in SLN preparation solution. The enhanced size could be contributed to the low stability of the formed nanoparticles when charged drug content increased. Notice the absolute value of zeta potential of the SLN decreased from 21.8 to 18.2, when the charged percentage of doxorubicin increased from 2.5% to 7.5%. Indeed, the further increase of charged percentage of doxorubicin, the stable SLN could not be obtained. Fig. 1(a) showed the *in vitro* doxorubicin release behavior from SLN, the SLN presented burst release and fast release behavior. In the first 2 h, about 60% drugs released from the SLN, and about 80% drug released in 12 h.

From, formulas 4–5 from Tables 1 and 2, after the positive charged chitosan oligosaccharide coated with negative charged doxorubicin-loaded SLN, the zeta potential of doxorubicin-loaded SLN modified with chitosan oligosaccharide changed to positive, and the zeta potentials were above 30 mV. It was reported that the

Table 2
Characteristics of doxorubicin-loaded SLN, doxorubicin-loaded SLN modified with chitosan oligosaccharide and crosslinked doxorubicin-loaded SLN modified with chitosan oligosaccharide.

Formula	d_N (nm)	PI (–)	Zeta potential (mV)	EE (%)	DL (%)
1	83.7 ± 11.2	0.225 ± 0.014	−21.8 ± 1.0	84.88 ± 1.72	2.13 ± 0.05
2	120.3 ± 7.6	0.148 ± 0.03	−19.1 ± 2.5	87.91 ± 1.49	4.41 ± 0.09
3	163.3 ± 22.1	0.188 ± 0.025	−18.2 ± 0.7	89.72 ± 1.05	6.76 ± 0.09
4	278 ± 14.0	0.143 ± 0.027	37.6 ± 0.6	89.54 ± 1.94	11.32 ± 0.24
5	273.3 ± 21.5	0.235 ± 0.002	33.1 ± 0.4	87.1 ± 0.01	19.92 ± 0.01
6	256.7 ± 9.6	0.147 ± 0.021	33.8 ± 0.2	88.34 ± 0.75	11.27 ± 0.01
7	308.3 ± 12.2	0.17 ± 0.012	45.4 ± 0.5	79.6 ± 0.01	10.2 ± 0.01
8	169.3 ± 20.6	0.229 ± 0.051	32.2 ± 3.2	85.9 ± 0.01	22.3 ± 0.01
9	182.7 ± 10.5	0.17 ± 0.026	30.5 ± 0.5	85.7 ± 0.01	17.6 ± 0.01
10	221.7 ± 12.6	0.13 ± 0.011	34.4 ± 0.5	88.97 ± 0.01	11.28 ± 0.01
11	214.3 ± 12.2	0.131 ± 0.037	34.2 ± 0.5	88.66 ± 0.01	11.24 ± 0.01
12	251.3 ± 16.3	0.19 ± 0.019	38.7 ± 0.3	89.86 ± 0.09	11.37 ± 0.01
13	245 ± 9.4	0.22 ± 0.022	38.4 ± 0.3	89.9 ± 0.01	11.39 ± 0.01
14	293.3 ± 9.3	0.16 ± 0.017	42.4 ± 0.2	77.7 ± 0.02	9.98 ± 0.01
15	259 ± 10.0	0.17 ± 0.04	40.3 ± 0.3	77.14 ± 0.04	9.92 ± 0.01

d_N, PI, EE and DL presented number average diameter, polydispersity index of diameter, drug encapsulation efficiency and drug loading, respectively.

absolute value of zeta potentials above 30 mV indicated nanoparticles dispersion highly stable (Cunningham et al., 2006). From Table 2, it was also found that the particle size became large (about 270 nm) when the SLN was modified with chitosan oligosaccharide, however stable doxorubicin-loaded SLN modified with chitosan oligosaccharide having high drug loading (about 20%) was obtained. The physical stability of doxorubicin-loaded SLN modified with chitosan oligosaccharide redispersed in PBS (pH 7.4) containing 1% PVA, such as particle size and drug encapsulation efficiency were also investigated. After the nanoparticles redispersion stored at room temperature for two weeks, it was found that no obvious changes in particle size and drug encapsulation efficiency were observed. Fig. 1(b) indicated the doxorubicin release behavior of doxorubicin-loaded SLN and doxorubicin-loaded SLN modified with chitosan oligosaccharide. It was clear the drug release rates were slowed in the initial stage after the modification of chitosan oligosaccharide. In the initial 2 h, only 20% drug released. The drug release behavior was prolonged, only near 60% drug released from doxorubicin-loaded SLN modified with chitosan oligosaccharide. Fig. 2(a) and (b) presented the TEM images of doxorubicin-loaded SLN (formula 2) and doxorubicin-loaded SLN modified with chitosan oligosaccharide (formula 7). Doxorubicin-loaded nanoparticles had spherical morphologies, and the observed size was close to that obtained by DLS (Dynamic Light Scattering). It was also confirmed that the particle size became large when the SLN was modified with chitosan oligosaccharide.

3.2. Preparation of doxorubicin-loaded SLN modified with chitosan oligosaccharide using chitosan oligosaccharide with different molecular weight

The molecular weight is an important characteristic of chitosan oligosaccharide which influences the properties of pharmaceutical formulations based on chitosan oligosaccharide. As shown in Table 1, the doxorubicin-loaded SLN modified with chitosan oligosaccharide was prepared using chitosan oligosaccharide with different molecular weight (formulas 4, 6 and 7). From Table 2, the number average diameter and zeta potential of prepared doxorubicin-loaded SLN modified with chitosan oligosaccharide enhanced from 256.7 nm to 308.3 nm ($p < 0.05$), and 33.8 mV to 45.4 mV ($p < 0.05$), respectively, as the chitosan oligosaccharide molecular weight increased from 8 kDa to 42 kDa. While no obvious difference was found in drug encapsulation efficiency or drug loading. The smaller size using chitosan oligosaccharide with low molecular weight might be due to the stronger folding ability of chitosan oligosaccharide with low molecular weight, which led to the tight binding between chitosan oligosaccharide and SLN. The tight binding between chitosan oligosaccharide and SLN led the low surface charge obtained from the zeta potential determination. Fig. 2(b) and (d) presented the TEM images of the doxorubicin-loaded SLN modified with chitosan oligosaccharide using different molecular weight of chitosan oligosaccharide (8 kDa and 42 kDa). The particle size observed

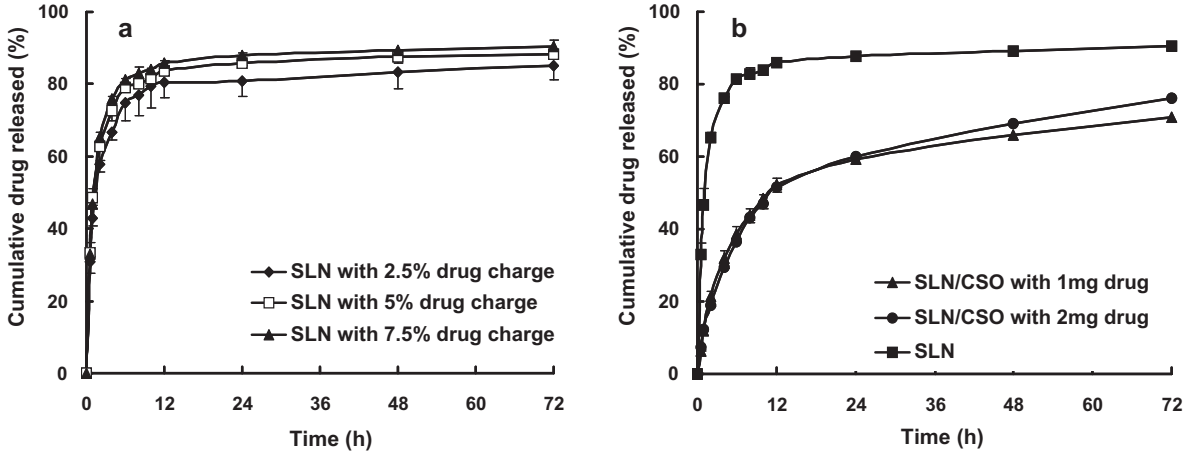

Fig. 1. *In vitro* doxorubicin release profiles of doxorubicin loaded SLN prepared using different drug charge (a), and doxorubicin loaded SLN modified with chitosan oligosaccharide (SLN/CSO) prepared using different drug content (b) (formulas 4 and 5).

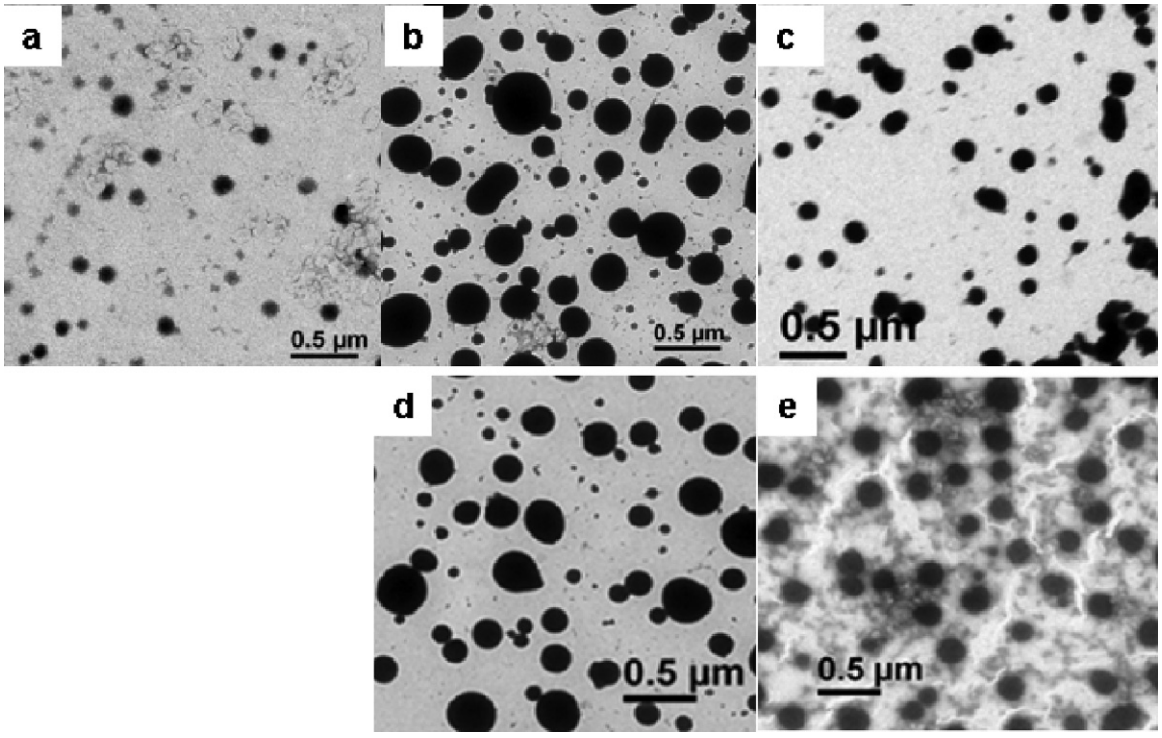

Fig. 2. TEM images of doxorubicin loaded SLN, doxorubicin loaded SLN modified with chitosan oligosaccharide and cross-linked doxorubicin loaded SLN modified with chitosan oligosaccharide. (a) TEM image of doxorubicin loaded SLN (formula 2); (b) TEM image of doxorubicin loaded SLN modified with chitosan oligosaccharide (formula 7); (c) TEM image of cross-linked doxorubicin loaded SLN modified with chitosan oligosaccharide (formula 15); (d) TEM image of doxorubicin loaded SLN modified with chitosan oligosaccharide (formula 6); (e) TEM image of doxorubicin loaded SLN modified with chitosan oligosaccharide (formula 8).

from TEM images were corresponded to that obtained by Zetasizer.

Fig. 3 shows the *in vitro* doxorubicin release behaviors from doxorubicin-loaded SLN modified with chitosan oligosaccharide with different molecular weight of chitosan oligosaccharide (8, 18 and 42 kDa). When the charged amount of doxorubicin and chitosan oligosaccharide was fixed, the drug release rate was slowed with decreasing the molecular weight of chitosan oligosaccharide in nanoparticles. It could also be explained the stronger folding ability of chitosan oligosaccharide with low molecular weight, which led to the tight binding between chitosan oligosaccharide and SLN.

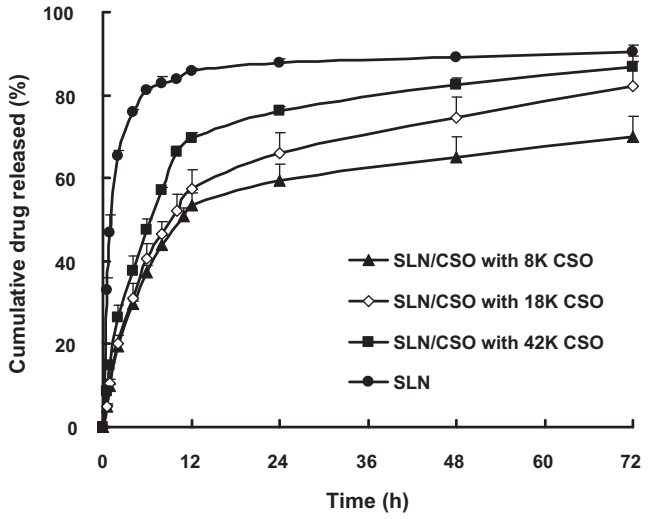

Fig. 3. *In vitro* doxorubicin release profiles of doxorubicin loaded SLN modified with chitosan oligosaccharide (SLN/CSO) prepared using different molecular weights of chitosan oligosaccharide (formulas 4, 6 and 7).

The drug diffused easily from the chitosan oligosaccharide membrane with loose structure, and reached a relative fast drug release rate.

3.3. Preparation of doxorubicin-loaded SLN modified with chitosan oligosaccharide with different charged amount of chitosan oligosaccharide

The effects of charged amount of chitosan oligosaccharide on the properties of resulted doxorubicin-loaded SLN modified with chitosan oligosaccharide were also investigated (formulas 6, 8 and 9 in Table 1). As shown in Table 2, it was found that the particle size of prepared doxorubicin-loaded SLN modified with chitosan oligosaccharide increased with increasing the amount of chitosan oligosaccharide when the amount of lipid and doxorubicin was fixed, however, no difference was found in the surface zeta potentials ($p > 0.05$). Moreover, the amount of chitosan oligosaccharide could affect the drug loading in doxorubicin-loaded SLN modified with chitosan oligosaccharide, although the drug encapsulation efficiency was not changed. The higher drug loading, as high as 22.3%, could reach when small amount of chitosan oligosaccharide was used. Fig. 2(d) and (e) presented the TEM images of the doxorubicin-loaded SLN modified with chitosan oligosaccharide using different amount of chitosan oligosaccharide (formulas 6 and 8). The particle size observed from TEM images were corresponded to that obtained by Zetasizer.

Fig. 4 shows the *in vitro* doxorubicin release profiles from doxorubicin-loaded SLN modified with chitosan oligosaccharide with different charged amounts of chitosan oligosaccharide (molecular weight is 8 kDa). It could also be seen that the doxorubicin released rate was slowed down as the SLN was modified with chitosan oligosaccharide. However, no significant difference was found among doxorubicin-loaded SLN modified with chitosan oligosaccharide using different amount of chitosan oligosaccharide.

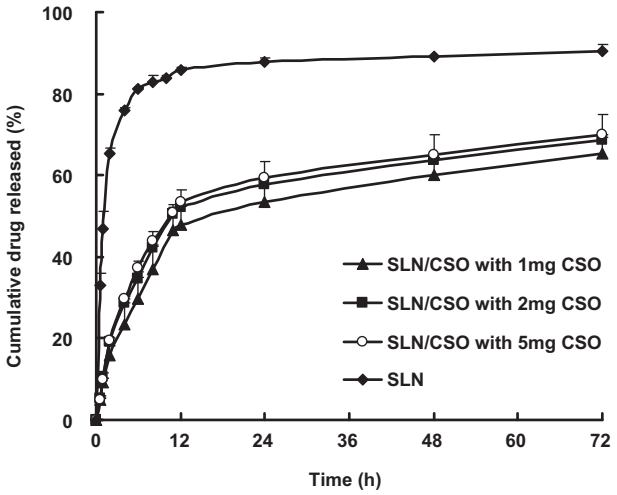

Fig. 4. *In vitro* doxorubicin release profiles of doxorubicin loaded SLN modified with chitosan oligosaccharide (SLN/CSO) prepared using different amount of chitosan oligosaccharide (formulas 6, 8 and 9).

3.4. Preparation of glutaraldehyde cross-linked doxorubicin-loaded SLN modified with chitosan oligosaccharide

Glutaraldehyde cross-linked doxorubicin-loaded SLN modified with chitosan oligosaccharide was prepared by solvent diffusion method and cross linked with glutaraldehyde through nucleophilic reaction between amino groups of chitosan oligosaccharide and aldehyde groups of glutaraldehyde. By alternating the molar ratio of glutaraldehyde to chitosan oligosaccharide, the properties of glutaraldehyde cross-linked doxorubicin-loaded SLN modified with chitosan oligosaccharide was investigated (see Tables 1 and 2). When the molecular weight of chitosan oligosaccharide was fixed, d_N was smallest when the molar ratio of glutaraldehyde to chitosan oligosaccharide was 6:5 ($p < 0.05$). The particles size confirmed by TEM images corresponded to that obtained from Zetasizer (see Fig. 2(b) and (c)). This phenomenon might be attributed to tight structure of chitosan oligosaccharide membrane when the chitosan oligosaccharide was highly crosslinked by glutaraldehyde. Besides, after doxorubicin-loaded SLN modified with chitosan oligosaccharide was cross-linked, no obvious change in the surface zeta potential was found ($p > 0.05$). The crosslink reduced the primary amine group number, while the decreased size reduced the surface area per nanoparticles.

The effects of molar ratios of glutaraldehyde to chitosan oligosaccharide on *in vitro* drug release rate of glutaraldehyde cross-linked doxorubicin-loaded SLN modified with chitosan oligosaccharide were shown in Fig. 5. As shown in Fig. 5(a)–(c), the drug release behavior of glutaraldehyde doxorubicin-SLN modified with chitosan oligosaccharide using different molecular weight chitosan oligosaccharide was different. No difference was found between glutaraldehyde doxorubicin-SLN modified with chitosan oligosaccharide using 8 kDa chitosan oligosaccharide (Fig. 5(a)). However, in cross-linked glutaraldehyde doxorubicin-SLN modified with chitosan oligosaccharide (18 or 42 kDa), the drug release rate was slowed down by increasing the molar ratio of glutaraldehyde to chitosan oligosaccharide. The crosslink of chitosan oligosaccharide by glutaraldehyde led the tight structure of chitosan oligosaccharide membrane, which highly slowed the drug release rate. However the crosslink of chitosan oligosaccharide by glutaraldehyde also reduced the particle size, which could enhance the specific surface area, and consequently enhanced the drug release rate. So, the drug release behavior of glutaraldehyde doxorubicin-SLN modified with chitosan oligosaccharide using different molecular weight chitosan oligosaccharide.

3.5. In vitro cytotoxiocity and celluar uptake of doxorubicin-loaded SLN modified with chitosan oligosaccharide

Using the MTT method, the 50% cellular growth inhibitions (IC_{50}) within 48 h were determined for cytotoxicity investigation against MCF-7 (human breast carcinoma cell line) cells. The present blank nanoparticles showed relatively low cytotoxicity against MCF-7 cells, for both monostearin and chitosan oligosaccharide had good bio-compatibility. IC_{50} value of monostearin was around 174.56 $\mu g\,mL^{-1}$, and the cellular growth inhibition rate was only 28% when the concentration of chitosan oligosaccharide (42 kDa) was 900 $\mu g\,mL^{-1}$. Compared with doxorubicin·HCl solution (IC_{50}: $0.26 \pm 0.06\ \mu g\,mL^{-1}$), All of doxorubicin-loaded nanoparticles (doxorubicin-loaded SLN (IC_{50}: $2.87 \pm 0.21\ \mu g\,mL^{-1}$), doxorubicin-loaded SLN modified with chitosan oligosaccharide (IC_{50}: $1.85 \pm 0.08\ \mu g\,mL^{-1}$) and cross-linked doxorubicin-loaded SLN modified with chitosan oligosaccharide (IC_{50}: $1.05 \pm 0.11\ \mu g\,mL^{-1}$)) showed lower cytotoxicity in MCF-7 cells, which might be due to that the doxorubicin could not release completely from the nanoparticles inside the cells within 48 h. Moreover, compared with doxorubicin-loaded SLN and doxorubicin-loaded SLN modified with chitosan oligosaccharide, cytotoxicity of cross-linked doxorubicin-loaded SLN modified with chitosan oligosaccharide was higher ($p < 0.05$), which might be due to the increasing cellular uptake ability (see Fig. 6). It was reported that nanoparticles with smaller size could be more easily internalized by cells (Hu, Xie, Tong, & Wang, 2007). After cross-linked with glutaraldehyde, the particle size decreased significantly ($p < 0.05$) from 308.3 nm to 259 nm, which was the main reason for

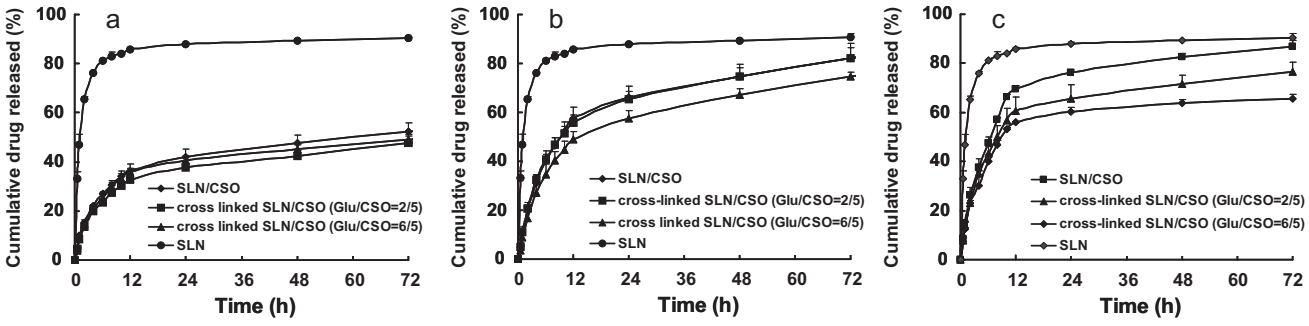

Fig. 5. *In vitro* doxorubicin release profiles of glutaradehyde cross-linked doxorubicin loaded SLN modified with chitosan oligosaccharide (SLN/CSO) prepared using different molar ratios of glutaradehyde to chitosan oligosaccharide (Glu/CSO). (a) The M_w of chitosan oligosaccharide is 8 kDa (formulas 6, 10 and 11); (b) the M_w of chitosan oligosaccharide is 18 kDa (formulas 4, 12 and 13); (c) the M_w of chitosan oligosaccharide is 42 kDa (formulas 7, 14 and 15).

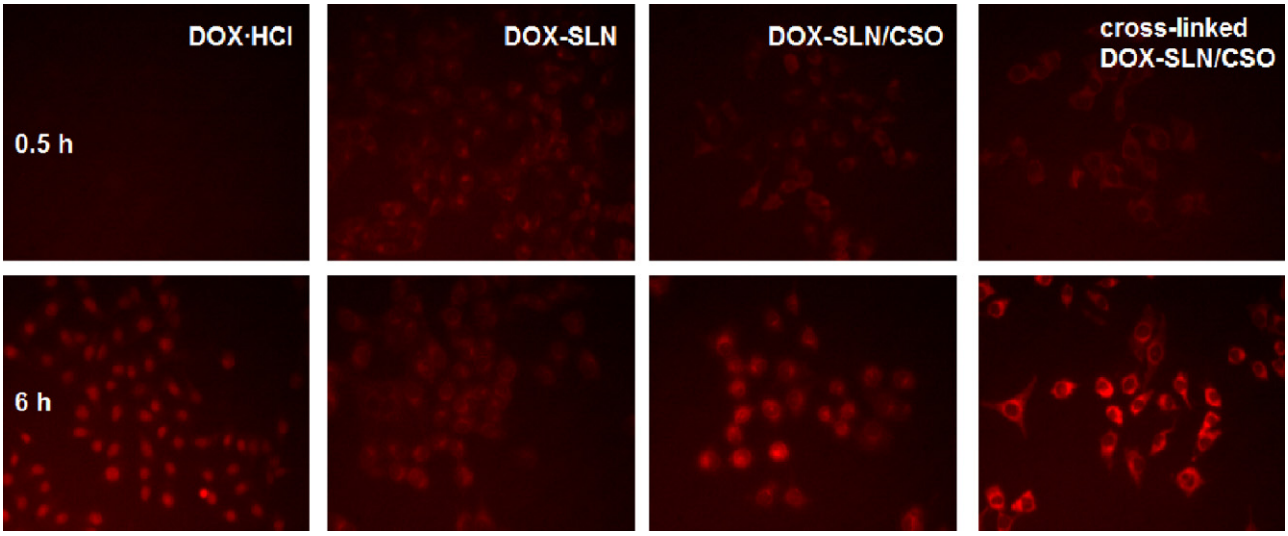

Fig. 6. Fluorescence images after the MCF-7 cells were incubated with doxorubicin·HCl solution (DOX·HCl), doxorubicin-loaded SLN (DOX-SLN), doxorubicin-loaded SLN modified with chitosan oligosaccharide (DOX-SLN/CSO) and cross-linked doxorubicin-loaded SLN modified with chitosan oligosaccharide (cross-linked DOX-SLN/CSO) solution for 0.5 h and 6 h, respectively.

the increase in cellular uptake ability, thus the cytotoxicity was also improved.

4. Conclusion

The doxorubicin-loaded SLN modified with chitosan oligosaccharide could be easily prepared by solvent diffusion method and dispersing chitosan oligosaccharide in the aqueous phase. After the modification of chitosan oligosaccharide, the drug loading of SLN could be highly improved, and the burst drug release and release rate could also be reduced significantly. The doxorubicin release from doxorubicin-loaded SLN modified with chitosan oligosaccharide could continue for 72 h, and could be adjusted by the amount and molecular weight of chitosan oligosaccharide, and the molar ratio of glutaraldehyde to chitosan oligosaccharide. Though the chemical cross-linking, the drug release of SLN/chitosan oligosaccharide could further delay. Doxorubicin-loaded SLN modified with chitosan oligosaccharide prepared in this study showed the potential as a carrier for improving drug loading content and controlling drug release rate.

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