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Self-assembly and characterization of paclitaxel-loaded *N*-octyl-*O*-sulfate chitosan micellar system

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Abstract

N-octyl-*O*-sulfate chitosan micellar system loaded paclitaxel was prepared by using dialysis method. The critical micelle concentration (CMC) of the modified chitosan was found to be 0.45 mg/ml. Compared with the amount of *N*-octyl-*O*-sulfate chitosan, the paclitaxel loading amount in the system was up to 25% (w/w), depending on both of the solvents used in dialysis and the feed weight ratio of paclitaxel to the derivative. The polymeric micelles forming and loading occurred simultaneously in the dialysis process when ethanol and water were utilized as the solvents for paclitaxel and the polymer, respectively. Paclitaxel-loaded micellar system of *N*-octyl-*O*-sulfate chitosan was characterized by DSC, WXRD and TEM. TEM photograph revealed that paclitaxel existed as the colloid particulates in ethanol before loading and in the cores of the spherical polymeric micelles after loading. The results of DSC and WXRD indicated that paclitaxel was transferred from the crystalline state to amorphous state after loading. The lyophilized powder of micellar system (25% (w/w) loading) could be reconstituted easily in aqueous media even after 2 months storage at 4 °C without the change of paclitaxel entrapment and micelle size. The reconstituted solution (2.1 mg paclitaxel/ml) also showed good stability. The dilution with saline may decrease the loading and physical stability based on the dilution times which was related with CMC of the polymer. In vitro tests showed that paclitaxel was slowly released from micellar solution and the release lasted up to 220 h by means of the dialysis method.

Keywords: *N*-octyl-*O*-sulfate chitosan; Self-assembly; Polymeric micelle; Solubilization; Paclitaxel

1. Introduction

Paclitaxel has been successfully used in the clinical treatment of several solid tumor malignancies such as breast cancer, non-small cell lung cancer and epithelial ovarian cancer [1–4]. Due to its very low water solubility of approximately 1 μg/ml [5], paclitaxel is currently solubilized in a 50:50 mixture of cremophore EL (polyoxylated castor oil) and dehydrated ethanol as clinical formulation [6]. However, a few of studies reported that cremophore EL induced serious side effects such as hypersensitivity, neurotoxicity, nephrotoxicity, and the extraction of plasticizer from the i.v. infusion tubing [7–9]. A number of alternative formulations were investigated for the solubilization of paclitaxel, including liposomes, lipid emulsions, mixed micelles, cyclodextrin complexes and paclitaxel conjugates [10–19].

The polymer micelles are effective vehicles for the solubilization of hydrophobic drugs [20]. The drugs can be physically incorporated within the hydrophobic cores of polymeric micelles or covalently coupled with polymers to form micellar structures [21]. Polymer micelles are conventional passive targeting carriers of anticancer drugs since they are structurally strong and not captured by the reticuloendothelial cell system (RES) due to their small particle size [22,23]. The efforts were made to solubilize paclitaxel by preparing the micelles of chitosan derivative or amphiphilic diblock copolymer [24,25]. The polymers used for micelle forming should be non-toxic, biodegradable and metabolized or excreted in the body. Chitosan is a polysaccharide derived

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from chitin by alkaline deacetylation and consists of 2-amino-2-deoxy-(1-4β)-D-glucopyranose residues (D-glucosamine units) with little or without *N*-acetyl-D-glucosamine units. It is generally regarded as non-toxic, biocompatible and biodegradable [26–28].

Chitosan is soluble in aqueous acidic solutions, but cannot form micelles in water. Miwa et al. synthesized *N*-lauryl-carboxymethyl-chitosan and prepared its micelles of paclitaxel [24]. The micelles encapsulated paclitaxel were thereby water-soluble and in addition lack of common side effects associated with cremophor vehicle. In our previous report, we synthesized a series of novel water-soluble chitosan derivative carrying long chain alkyl groups ($n = 8$, 10, 12) as hydrophobic moieties and sulfated groups as hydrophilic moieties in order to solubilize paclitaxel [29]. The primary results showed that the *N*-octyl-*O*-sulfate chitosan micellar solution had the high solubilization capacity for paclitaxel. The solubility of paclitaxel in the micellar system was up to 2.6 mg/ml, 1000 times higher than that of the saturated concentration of the drug in water.

In this study, we aimed to develop the polymeric micellar system of *N*-octyl-*O*-sulfate chitosan for paclitaxel delivery. The micelles preparation, paclitaxel solubilization and micelles properties were investigated by the size measurement, HPLC assay, TEM, DSC and WXRD in the micellar solution or in the state of lyophilized powder. The critical micelle concentration (CMC) of *N*-octyl-*O*-sulfate chitosan was measured by using the standard fluorescence substance of pyrene. Paclitaxel release in vitro from the micelles was performed based on the dialysis method. The stability of paclitaxel-loaded micellar system was also evaluated.

2. Materials and methods

2.1. Materials

Chitosan was provided by the Nantong Suanglin Biochemical Co. Ltd., China with deacetylation degrees of 97% and viscosity average molecular weight of 65 000 D. Paclitaxel was obtained by Taihua Natural Plant Pharmaceutical Co. Ltd., China. Pyrene was purchased from Fluka Company (>99%). All commercially available solvents and reagents were used without further purification.

2.2. Synthesis of N-octyl-O-sulfate chitosan

The modified chitosan (Fig. 1) was prepared following a procedure reported by our group [29]. Briefly, chitosan (1.0 g) was suspended in 50 ml methanol with stirring at room temperature, and then octaldehyde (1.02 g) was added. After reaction of 24 h, KBH_4 (0.5 g) dissolved in 5 ml water was slowly added to the solution. After a further 24 h continuous stirring, the reaction solution was neutralized with 2N hydrochloric acid and the product was precipitated with methanol. The precipitate was filtered and repeatedly washed with methanol and water. The product, *N*-octyl chitosan was dried under vacuum at 60 °C overnight.

N-octyl chitosan (1.05 g) suspended in *N*,*N*-dimethylformamide (DMF) (40 ml) was magnetically stirred overnight. Chlorosulfonic acid (20 ml) was added dropwise into DMF (40 ml) with stirring at 0 °C under N_2 atmosphere. After completely dripped, the solution was kept in agitation for 1 h, and then the suspension of *N*-octyl-chitosan and DMF was added to the above solution. The mixture was reacted at 10 °C under N_2 atmosphere for 24 h. The reaction solution was neutralized with 20% NaOH until pH = 7, and the filtered solution was dialyzed (MWCO 10 000) against distilled water, then lyophilized and the *N*-octyl-sulfate chitosan powder was obtained.

The chemical structure and substitution degree of the derivative were determined by FT-IR (Nicolet 2000), NMR spectroscopy (Bruker AVACEAV-500) and the elemental analysis (Element Vario EL III analyzer).

2.3. Preparation of paclitaxel-loaded micelles

Paclitaxel-loaded micelles were prepared by dialysis method [30]. A certain amount of *N*-octyl-sulfate chitosan

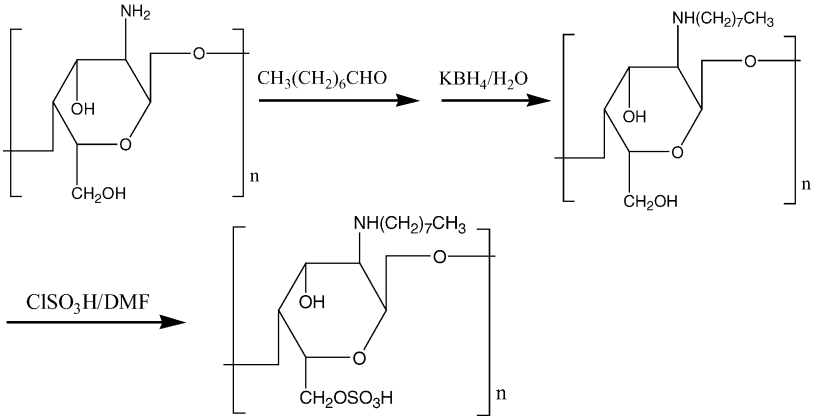

Fig. 1. Synthetic scheme of the modified chitosan.

Table 1
The entrapment efficiency and paclitaxel loading in the modified chitosan micelles (feed weight ratio of paclitaxel to modified chitosan = 1:1.7)

Solvent		Entrapment efficiency (%)	Loading (% (w/w))	*d* (nm)
For modified chitosan	For paclitaxel			
Water	Ethanol	59.11	25.25	240
Water	DMF	6.2	16	369
Water	DMSO	1.33	1.2	287
10% ethanol	Ethanol	0.30	0.13	–
10% DMF	DMF	0.39	0.17	–
10% DMSO	DMSO	0.08	0.08	–
10% Tween 80	Ethanol	1.7	1.9	–

was dissolved in 2 ml of water or other aqueous solutions, such as ethanol solution (10% (w/w)), Tween 80 solution (10% (w/w)), DMF solution (10% (w/w)) and dimethyl sulfoxide (DMSO) solution (10% (w/w)), respectively. Paclitaxel (6.8 mg) was dissolved in 0.22 ml of ethanol, DMF and DMSO, respectively. Then the selected polymer solution and paclitaxel solution were mixed (Table 1), sonicated for 30 min at room temperature (JY 92-II ultrasonic processor, China) and followed by dialysis against distilled water overnight at room temperature, in which the dialysis membrane with 10 000 molecular weight cut-off was used. The micellar solution was centrifuged at 3000 rpm for 5 min, and filtered with a 0.45 μm pore-sized filtration membrane. A suitable amount of mannitol was added to adjust the similarity and then freeze-dried. The lyophilized powder was kept in refrigerator at 4 °C until use. The total content of paclitaxel in the micellar systems was measured by HPLC after resolving the lyophilized powder in acetonitrile. The entrapment efficiency in the micelles was calculated as the ratio of the content to the feeding drug amount (6.8 mg). The loading of paclitaxel was calculated based on the content and the feeding amount of the polymer.

2.4. HPLC analysis

Paclitaxel concentrations were measured by HPLC (LC-6A, Shimadzu, Japan). The mobile phase was a mixture of methanol, acetonitrile and water (30:40:32 (v/v)). The column was Diamohsil^{TM} C18 (250 × 4.6 mm) with 5 μm particles. The flow rate was 1.0 ml/min, the detection wavelength was 227 nm (SPD-10A, UV detector, Shimadzu, Japan), and the column temperature was maintained at 30 °C. Injected volume of the sample was 20 μl.

2.5. Characterization of paclitaxel-loaded micellar system

The diameter and polydispersity (V) of the polymeric micelles were measured by Zetasizer 3000HS instrument (Malvern Instruments, Malvern, UK) with 633 nm He–Ne lasers at 25 °C while the lyophilized powder was reconstituted with double distilled water. DSC was carried out for the powder samples by using NETZSCH DSC 204 equipment, the temperature range was 50–500 °C and heating rate was 20 °C/min. X-ray diffraction was performed with a XD-3A powder diffraction meter with Cu Kα radiation in the range of 5–40° (2θ) at 40 kV and 30 mA. Transmission electron microscopy (TEM) observation was carried out at 75 kV with H-7000 (Hitachi, Japan) in the micellar solution, which was negatively stained with 0.01% phosphotungstic acid and placed on a copper grid coated with film.

2.6. Measurement of critical micelle concentration

Critical micelle concentration (CMC) of the modified chitosan was determined by using pyrene (Fluka, >99%) as a hydrophobic probe in fluorescence spectroscopy (Shimadzu RF-5301 PC, Japan) [31–34]. Briefly, a known amount of pyrene in acetone was added to each of a series of 10 ml vials and the acetone was evaporated, then 6 ml of various concentrations of *N*-octyl-sulfate chitosan solutions (1×10^{-6} to 2 mg/ml) were added to each vial (the final concentration of pyrene was 6×10^{-7} M), sonicated for 30 min at room temperature. The sample solutions were heated at 65 °C for 3 h to equilibrate pyrene and the micelles, and then left to cool overnight at room temperature. The solutions were filtered with a 0.22 μm pore-sized filtration membrane. Fluorescence emission spectra were measured at excitation wavelength of 339 nm, emission wavelength was 350 to 450 nm for excitation spectra. Both excitation and emission bandwidths were 3 nm, respectively.

2.7. In vitro drug release studies

The lyophilized powder of paclitaxel-loaded micellar system (containing 2 mg paclitaxel) and 2 ml phosphate buffer solution (PBS, 0.1 M, pH 7.4) were put into a dialysis tube (MWCO 10 000). Then the tube was introduced into a stainless steel basket immerged in 250 ml PBS (0.1 M, pH 7.4) containing 0.1% (w/v) Tween 80 with stirring at 100 rpm and 37 °C. At predetermined time intervals, the whole medium was taken for HPLC analysis and replaced with fresh PBS. In addition, in vitro drug release test was compared with that of cremophore EL-based paclitaxel solution, in which 2 mg of paclitaxel was dissolved in 1 ml of the blend of cremophor EL and ethanol (1:1) and diluted with 5 ml dextrose solution (5%).

Table 2
Relationship between entrapment efficiency and feed weight ratio of paclitaxel to the modified chitosan (paclitaxel and modified chitosan was dissolved in ethanol and water, respectively)

Feed weight ratio paclitaxel:polymer	Entrapment efficiency (% (w/w))	Loading (% (w/w))
1:1	37.6	27.3
1:1.5	55.4	27.0
1:1.7	59.1	25.1
1:2	35.7	12.2
1:2.5	40.6	11.8
1:3	38.4	9.7
1:4	44.5	9.1
1:5	45.3	8.3

2.8. Stability of paclitaxel-loaded micellar system

The lyophilized powder (25% (w/w) paclitaxel loading) and the polymeric micellar solution (2.1 mg paclitaxel/ml) were stored at 4 °C, the entrapment efficiency, content of paclitaxel and the size of the micellar system were measured by using dialysis and HPLC methods as described above. The loading amount of paclitaxel in the polymer was calculated according to the content measurement. In addition, the effect of dilution on the stability of the micellar system was observed by dissolving same amount of lyophilized powder (equal to 2.1 mg paclitaxel) in various volumes of saline.

3. Results and discussion

3.1. Micelles formation

The paclitaxel loading into micelles occurred simultaneously with self-assembly of amphiphilic *N*-octyl-sulfate chitosan micelles during dialysis process. It was found that once the micellar structure was formed completely, the drugs could not be incorporated into the micelle. The solvents used to dissolve the polymer or paclitaxel and the feed weight ratio of paclitaxel to the polymer significantly affected the micelles forming and entrapment efficiency. The results were summarized in Tables 1 and 2. It indicated that much higher entrapment efficiency was obtained only when dehydrated ethanol and water were used as the solvent for paclitaxel and the polymer, respectively. It was interesting that there was no self-assembly of micelles existed and almost no drug was loaded in these systems when 10% ethanol, 10% DMF, 10% DMSO and 10% Tween 80 were used to dissolve the polymer, respectively, while DMSO and DMF were used to dissolve the drug, even some of these solvents could dissolve them easily. It suggested that all the solvent systems except dehydrated ethanol and water might not be applied for the micellar system preparation and drug loading while the dialysis procedure was applied. It was possible that paclitaxel was entrapped by the forming micelles in self-assembly process as the colloid particulates rather than the molecules. The interaction between paclitaxel molecule and the hydrophobic groups of the polymer was not strong enough to overcome the intra-molecular force of paclitaxel and the inter-molecular force between the drug molecule and the solvent molecule except dehydrated ethanol. These solvents may also hinder the formation of the micelles due to their solubilization ability.

It was unexpected that the entrapment efficiency was decreased with the increasing the amount of the polymer utilized in the dialysis process. It was observed that the loading amount of paclitaxel in the micelles decreased as the amount of the polymer was increased. The fact suggested that the micelle numbers or concentration of the modified chitosan in the dialysis system may not only the determinative factor for the solubilization of paclitaxel. The highest entrapment efficiency (55–59% (w/w)) was observed when the feed weight ratio of paclitaxel to the polymer was 1:1.5–1:1.7 (Table 2) in ethanol–water system. As the feed ratio was increased to 1:1, the precipitation of paclitaxel in aqueous phase in the dialysis tube simultaneously occurred with the incorporation of paclitaxel into the micelles. The precipitation of paclitaxel consequently resulted in the lower loading rate.

The blank and drug loaded polymeric micelles were observed by TEM as approximately spherical shapes without aggregation (Fig. 2a). The micelle sizes were about 250 nm, which were measured by dynamic light scattering and consistent with those visualized by TEM. It was interesting that TEM monographs showed that paclitaxel was homogenously dispersed in ethanol as the colloid particulates before mixing with the polymer solution (Fig. 2c), and the colloid particular state of paclitaxel was steadily kept in the hydrophobic core of the forming micelles although ethanol was removed in the dialysis process (Fig. 2b). In contrast, the drug dissolved in DMF and DMSO and formed a transparent solution, but no micelle forming was observed by TEM.

The self-assembly of the polymeric micelles was gradually completed about 5 h dialysis procedure while water and ethanol were used as the solvent system. The results suggested that ethanol-water system was more favorable for paclitaxel-loaded micelles of the modified chitosan. However, the mechanism of micelle forming and drug loading in the solvent system has not been understood.

3.2. Fluorescence spectroscopy measurement

The CMC of self-assembled micelle formation was determined by fluorescence probe techniques. The fluorescence intensity was increased with increasing the concentration of the modified chitosan when pyrene was selected as the probe. Pyrene molecules have a strong hydrophobic character with very low solubility in water and preferentially solubilize into the hydrophobic core of micelles. The fluorescence intensity of pyrene in the micellar solution was obviously affected by *N*-octyl-*O*-sulfated-chitosan concentration (Fig. 3). Below the CMC, there were no micelles present in the polymer solution, so the fluorescence intensity was very low. The curve of fluorescence intensity versus modified chitosan concentration shows a sharp increase at the CMC, which

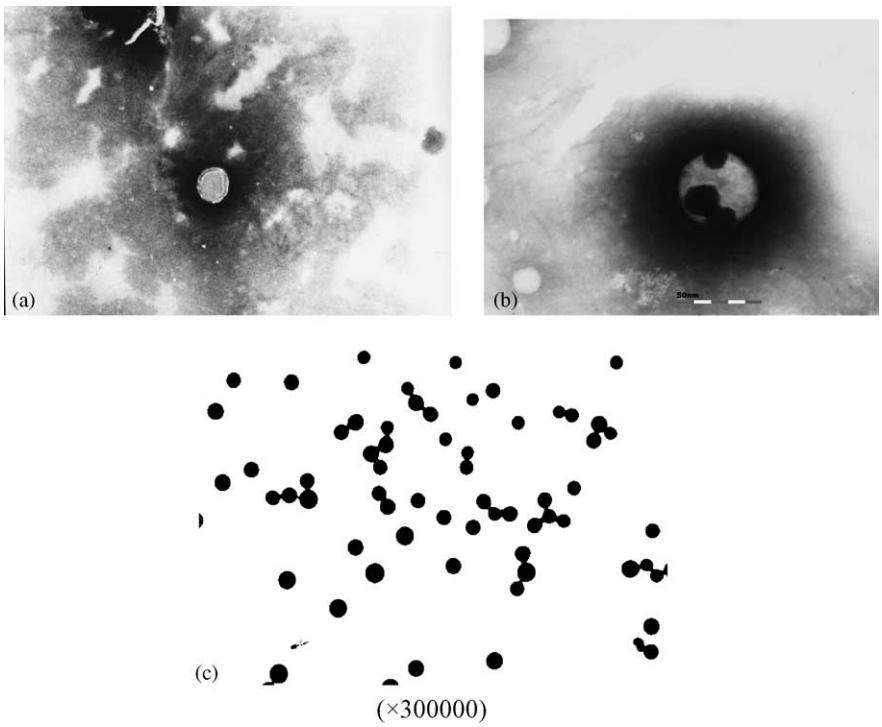

Fig. 2. Transmission electron micrograph: (a) blank micelle (×200 000), (b) paclitaxel-loaded micelle (×500 000) and (c) paclitaxel colloid particle in ethanol (×300 000).

N-octyl-*O*-sulfated-chitosan concentration was calculated to be 0.45 mg/ml.

3.3. Stabilities of micelles and drug release from the micellar system

The physical stabilities of both the lyophilized powder and micellar solution of paclitaxel were evaluated. It was found that the lyophilized powder (25% (w/w) paclitaxel loading) and micellar solution (2.1 mg paclitaxel/ml) could be stored at 4 °C for at least 2 months without the change of paclitaxel content and micelle size. The stability results of the reconstituted solution of lyophilized samples (Table 3) showed that the same amount of powder samples was dissolved in a series of volumetric saline at room temperature and the stating time of opalescent appearance was recorded. The results indicated that the stability of the reconstituted micellar solution was dependent on the diluted concentration of the polymer. The lower concentration of the polymer existed in the system, the more unstable property of the micelles appeared. For example, as the micellar solution was reconstituted to paclitaxel concentration of 2.1 mg/ml with 1 ml of saline for the lyophilized powder (25% (w/w) paclitaxel loading), the haziness occurred after more than 10 days. However, same amount of the lyophilized powder was dissolved with 5–9 times volume of saline, the cloudy phenomenon was found in 3 days to 1.5 h. Moreover, immediate haziness appeared in

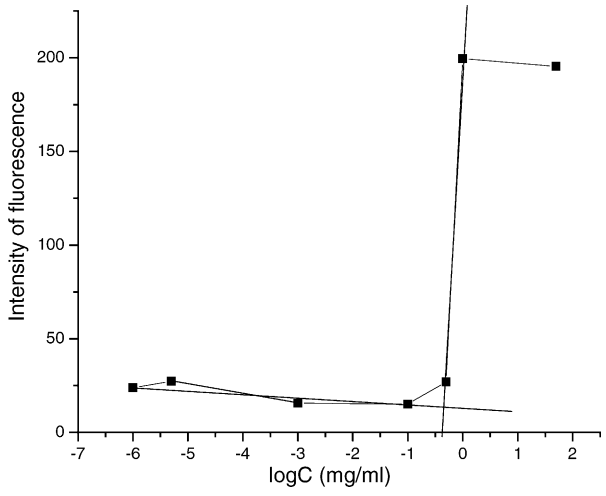

Fig. 3. Plots of the fluorescence intensity of pyrene vs. log *C* for modified chitosan.

Table 3
Solution stability of paclitaxel-loaded micelle in solution (25 °C)

Diluted volume (ml)	Micelle concentration (mg/ml)	Time first appearance of haziness
1	6	>10 days
2	3	10 days
3	2	7 days
4	1.5	7 days
5	1.2	3 days
6	1	3 days
7	0.86	2 days
8	0.75	1 days
9	0.67	1.5 h
10	0.6	Immediately

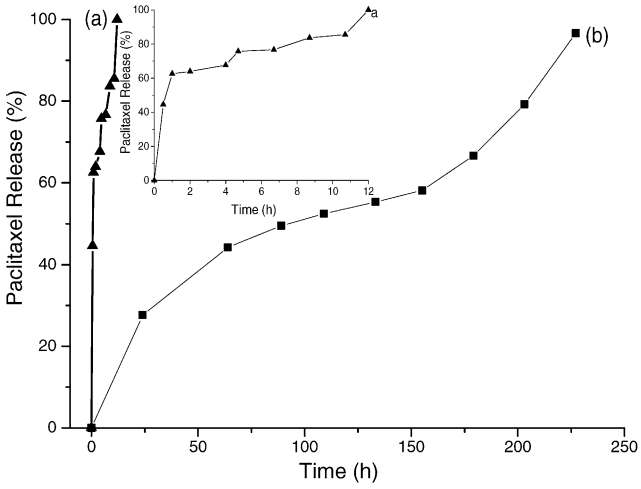

Fig. 4. The in vitro release of paclitaxel from: (a) (▲) cremophore EL-based formulation and (b) (■) modified chitosan micelle in PBS (0.1 M, pH 7.4) at 37 °C ($n = 3$).

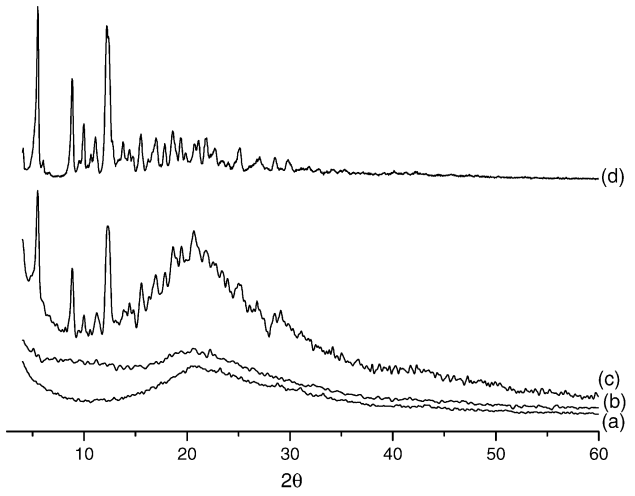

Fig. 5. Powder X-ray diffraction patterns for the lyophilized powder: (a) the blank micellar system; (b) paclitaxel micellar system (25% (w/w) loading); (c) physical mixture of blank micellar system and paclitaxel (3:1 (w/w)), (d) paclitaxel.

the solution while reconstituted in 10 times volume of saline, in which the micelle concentration (0.6 mg/ml) was close to CMC (0.45 mg/ml) of the polymer.

In vitro release test of paclitaxel from the micellar system showed that 40% of paclitaxel was released within 60 h at 37 °C, afterwards, the release rate was slow down and it was released completely after 220 h (Fig. 4b). In comparison, 60% of paclitaxel was released rapidly from cremophore EL-based formulation within 4 h and almost completed within 22 h (Fig. 4a).

3.4. Physicochemical properties of the lyophilized micellar system

The X-ray diffraction patterns of paclitaxel and the lyophilized powders of the micellar systems were shown in Fig. 5. The graphs showed that three typical crystal peaks at 2θ of 6, 9 and 12.5° and numerous small peaks between 15 and 25° for paclitaxel. The lyophilized blank micellar system gave one broad peak at 2θ of 21° contributed to the polymer. When the two samples were physically mixed according to the ratio of 3:1 (lyophilized blank micellar system to paclitaxel, e.g., equal to 25% (w/w) loading), three crystals peaks of paclitaxel and one broad peak of modified chitosan were still observed. However, the lyophilized drug loaded micellar system had no paclitaxel peaks and had a broad peak at 2θ of 21° similarly with the lyophilized blank micellar system. It indicated that paclitaxel was either in molecular state or in an amorphous state dispersed in the polymer after the lyophilization. It should be mentioned that the colloid particulates of paclitaxel were found again in the reconstituted micellar solution of the lyophilized powder.

DSC thermograms revealed one endothermic peak at 221 °C and one exothermic peak at 240 °C for paclitaxel (Fig. 6). The former was the melting temperature and the latter was assigned to decomposition. The exothermic peak at 242 °C for lyophilized blank micellar system was attributed to the decomposed temperature of the modified polymer. The weak endothermic peak (221 °C) and sharp exothermic peak (240 °C) of the physical mixture of lyophilized blank micellar system with paclitaxel (25% (w/w)) were associated with the melting temperature and the decomposing temperature of paclitaxel, respectively, but the later may also related with the polymer, because the decomposing temperatures of the two components was very close to each another.

The lyophilized drug loaded micellar system showed no decomposing peak in DSC thermograms but a new peak at 230 °C, which suggested that a solid dispersion of paclitaxel and the polymer was formed when the micellar solution was freeze-dried.

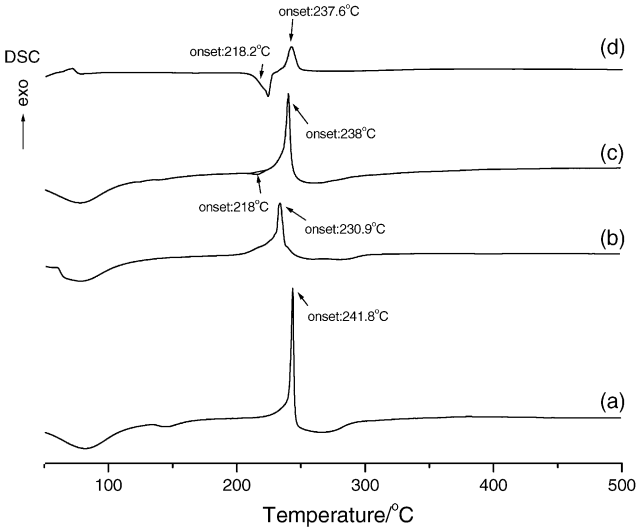

Fig. 6. DSC thermograms of: (a) blank micelle (b) 25% paclitaxel micellar system (25% (w/w)); (c) physical mixture of blank micellar system and paclitaxel (3:1 (w/w)); (d) paclitaxel.

4. Conclusions

As a novel carrier material for paclitaxel, the micelle-forming chitosan derivative, *N*-octyl-*O*-sulfate chitosan, was synthesized. The polymeric micellar system has a comparative solubilization capacity for paclitaxel, a hydrophobic with very slight soluble drug in water. The reasonable solvent system is important for the micelle formation and paclitaxel loading into the micelles. The self-assembly of the micellar system and paclitaxel loading were completed simultaneously in a simple dialysis process only when the solvent system of ethanol–water was selected. Although the solubilization mechanism would be elucidated further, the experiments revealed an interesting evidences that paclitaxel dispersed in both of dehydrated ethanol and the hydrophobic core of the polymeric micelles as the colloid particulates rather than the molecular state. Furthermore, some solvents that could dissolve the drug and form a clear solution, but they were failure to constitute the drug loading micellar system. The loading in micelles was dependent on the feed weight ratio of paclitaxel to the modified chitosan in ethanol–water system. The highest entrapment efficiency was reached in the range of 1:1.5–1:1.7.

Taking the advantage of the average diameter of 250 nm and high paclitaxel loading, the micellar system of the modified chitosan seems a promising nano-carrier for some of insoluble drugs. In addition, the lyophilized powder of paclitaxel-loaded micellar system and its solution could be generally recognized as the stable system when stored at 4 °C, even the solution was diluted into a certain extent. At last, the slow release rate in vitro of paclitaxel micellar system may be beneficial to the long circulation and targeting of the drug.

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