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Polymeric micelle systems of hydroxycamptothecin based on amphiphilic *N*-alkyl-*N*-trimethyl chitosan derivatives

Can Zhang [a,b], Ya Ding [a], Liangli (Lucy) Yu [b], Qineng Ping [a,*]

[a] *College of Pharmacy, China Pharmaceutical University, Nanjing 210009, PR China*
[b] *Department of Nutrition and Food Science, University of Maryland, College Park, MD 20742, United States*

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Abstract

This research investigated the possible utilization of amphiphilic *N*-octyl-*N*-trimethyl chitosan (OTMCS) derivatives in solublization and controlled release of 10-hydroxycamptothecin (10-HCPT), a hydrophobic anticancer drug. The release behavior of the 10-HCPT-OTMCS micelles was measured and compared to that of a commercial 10-HCPT lyophilized powder *in vitro* and *in vivo*. This research also examined the effects of chemical structure of the chitosan derivatives and the micellar preparation conditions on the encapsulation efficiency, drug loading content, and particle size of the polymeric micelles. The results showed that these chitosan derivatives were able to self-assemble and form spherical shape polymeric micelles with an average particle size range of 24–280 nm and a drug loading content of 4.1–32.5%, depending on the modified structures and loading procedures. The solubility of 10-HCPT in aqueous fluid was increased about 80,000-fold from 2 ng/ml in water to 1.9 mg/ml in OTMCS micellar (degree of octyl and trimethyl substitution is 8% and 54%, respectively) solution. In addition, OTMCS was able to modulate the *in vitro* release of 10-HCPT and improve its pharmacokinetic properties and lactone ring stability *in vivo*. These data suggested the possible utilization of the amphiphilic micellar chitosan derivatives as carriers for hydrophobic drugs for improving their delivery and release properties.

Keywords: Chitosan; Amphiphilic; Polymeric micelles; 10-HCPT; Solubilization

1. Introduction

Camptothecin is a water-insoluble natural alkaloid that was originally extracted from Chinese tree *Camptotheca acuminata* (Nyssaceae) [1]. Growing evidence indicates that camptothecin and its derivatives such as 10-hydroxycamptothecin (10-HCPT), 9-nitro-camptothecin, topotecan and irinotecan are a group of novel chemotherapy agents with a broad spectrum of antitumor activity against breast, colon, lung, and ovarian cancers [2–6]. These camptothecins are capable of targeting the nuclear enzyme topoisomerase I and inhibiting the relegation of the cleaved DNA strand, and consequently suppressing cell proliferation and leading to the death of cancer cells [6,7]. Camptothecins may improve the tissue selectivity and reduce the side effects because the level of topoisomerase I is higher in malignancy than that in the normal cells [6]. Camptothecins share a α-hydroxy δ-lactone moiety in their structures (Fig. 1). The integrity of this δ-lactone ring is required for both passive diffusion of these antitumor agents into cancer cells and suppressing topoisomerase I activity [8,9]. This δ-lactone ring is stable at pH 7 or lower, but it will be opened rapidly and changed into carboxylate form completely under alkaline condition [3,4]. At physiological pH, camptothecins coexist in both lactone (the ring-closing structure) and the carboxylate (the ring-opening structure) forms. In the plasma of mice at pH 7.4, the two structures had a ratio of 50–55% [10]. The carboxylate form of camptothecins has no antitumor activity, but has potential toxic effects [8,9]. This led to the research effort for improving the lactone stability through esterification of the α-hydroxyl group in the lactone ring because of its role in opening the ring structure [7]. The esterification further decreases the water solubility while improving the lactone ring stability. Decreasing water solubility may further lower the erratic bioavailability of these camptothecins, which is known to be consistent with the dissolution rate-limiting absorption characteristics associated with the highly hydrophobic agents [6]. Additional approaches such as

* Corresponding author. Tel.: +86 25 8327 1098; fax: +86 25 8533 3041.
E-mail address: pingqn@cpu.edu.cn (Q. Ping).

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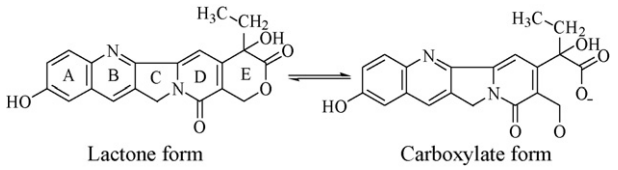

Fig. 1. Chemical structure of lactone and carboxylate forms of 10-HCPT.

nanocrystalline suspension, the suspension in Tween 80-saline or lipid media, liposomes and polymeric nanomicellar systems have been investigated for their potential for both solving the water insolubility and the poor lactone stability problems associated with camptothecins [3,4,6,7,11,12]. Among these tested approaches, the polymeric nanomicellar system is considered as one of the most promising methods because of its characteristics in drug loading, release, delivery, and stability [13].

The polymeric micelles with a hydrophobic core and a hydrophilic shell were reported to be effective vehicles for solubilizing hydrophobic drugs [14]. The drugs could be physically incorporated into the hydrophobic cores of the polymeric micelles or covalently coupled with the macromolecules to form micellar structures [15]. The polymeric micelles generally have strong mechanical strength and are conventional passive targeting carriers of anticancer drugs since they are not captured by the reticuloendothelial cell system (RES) due to their small particle size [14,16]. In general, the micelle-forming materials should be biodegradable and non-toxic, and could be metabolized in or excreted from the human body. Natural biomaterials are preferred because of their safety and better consumer preference, and have become a very active research area [17,18].

Chitosan is a polysaccharide derived from chitin by alkaline deacetylation and consists of 2-amino-2-deoxy-β (1 → 4)-D-glucopyranose or D-glucosamine units with few or without *N*-acetyl-D-glucosamine units. It is generally regarded as a non-toxic, biocompatible, and biodegradable material [19–21]. Chitosan has a primary amino, and a primary and a secondary free hydroxyl groups on C_2, C_6 and C_3, respectively. These amino and hydroxyl groups are chemically reactive and can be further chemically modified. Amphiphilic chitosan derivatives have been chemically synthesized. These chitosan derivatives may self-assemble in aqueous environment and form polymeric micelles, which are capable of entrapping hydrophobic drugs such as paclitaxel and enhance their water solubility [22,23]. For instance, *N*-octyl-*O*-sulfate chitosan with a negatively charged sulfate group and a non-polar octyl tail is an amphiphilic molecule, which could form polymeric micelles with hydrophobic paclitaxel and improve its water solubility [23,24]. These previous studies indicate that amphiphilic chitosan derivatives may be prepared by introducing both charged groups and non-polar linear hydrocarbon branches into chitosan molecules. These amphiphilic chitosan derivatives may entrap hydrophobic drugs and enhance their water solubility.

The present research investigated the potential of *N*-octyl-*N*-trimethyl chitosan (OTMCS) in entrapping 10-HCPT, a more potent highly lipophilic camptothecin derivative, in the polymeric micelles and enhancing its solubility in aqueous fluids. Also investigated was the release behavior of the 10-HCPT loaded OTMCS micelles *in vitro* and in rabbits by bonus intravenous injection. In addition, the storage stability of the 10-HCPT-OTMCS micelles was determined. This study may lead to a novel delivery system for 10-HCPT, and improve the efficacy and safety of its utilization in chemotherapy.

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 25 kDa. 10-Hydroxycamptothecin (10-HCPT, 98% purity) was obtained by Nanjing Fajing technological CO. Ltd. (China). Pyrene (purity > 99%) was purchased from Fluka Company (USA). All commercially available solvents and reagents were used without further purification.

2.2. Synthesis of amphiphilic chitosan derivatives

N-Octyl-*N*-trimethyl chitosan derivatives (OTMCS) were prepared by introducing the octyl group to NH_2 on C_2 of glucosamine unit in chitosan followed by *N*-methylation as illustrated in Fig. 2. To prepare *N*-octyl chitosan, 1 g octaldehyde was added in the suspension of 1.0 g of chitosan in 50 ml of methanol, while stirring at ambient temperature. After 24 h of reaction, 5 ml of KBH_4 solution with a concentration of 0.1 g/ml was added to the reaction mixture. The resulting mixture was stirred at ambient temperature for another 24 h and neutralized using 2 M hydrochloric acid. After precipitation with methanol, *N*-octyl chitosan was collected by filtration, repeatedly washed with methanol and water, and dried at 60 °C overnight under a reduced pressure.

N-octyl-*N*-trimethyl chitosan (OTMCS) derivatives were prepared from the corresponding *N*-octyl chitosan by methylation reaction following a previously reported procedure [25–27]. Briefly, a mixture of 0.96 g *N*-octyl-chitosan, 2.4 g of sodium iodide, 5 ml of 15% (w/v) sodium hydroxide solution, and 15 ml iodomethane in 15 ml *N*-methylpyrrolidone was mixed

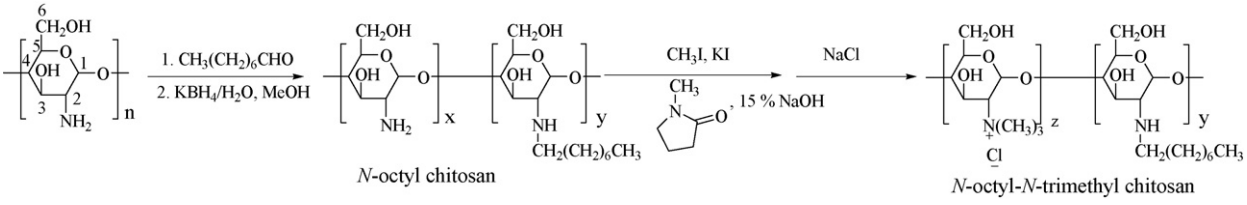

Fig. 2. Preparation of *N*-octyl-*N*-trimethyl chitosan derivatives.

and reacted at 60 °C for 1 h with stirring. The resulting OTMCS1 was precipitated using ethanol and collected by centrifugation. High degree of trimethyl substitution for *N*-octyl-*N*-trimethyl chitosan was further methylated by reacting with 3.2 ml of iodomethane in 15 ml of *N*-methylpyrrolidone containing 2.4 g of sodium iodide and 5 ml of 15% sodium hydroxide. After reacting at 60 °C for 30 min, additional 1 ml iodomethane and 0.3 g NaOH pellets were added to the mixture and stirred for another 1 h to complete the methylation reaction. OTMCS2 was precipitated with ethanol, collected by centrifugation, and purified following the same procedures described for OTMCS1.

Other amphiphilic chitosan derivatives, *N*-decanal-*N*-trimethyl (DTMCS) and *N*-lauryl-*N*-trimethyl (LTMCS) chitosan derivatives were obtained according to the same procedure.

2.3. Preparation of 10-HPCT polymeric micelle systems

10-HPCT polymeric micelle systems were prepared using the dialysis method [22]. Briefly, 11.8 mg of OTMCS with octyl substitution degrees of 8%, 20%, 36%, 47.5% or 58%, was dissolved in 2 ml of distilled water, while 6.8 mg of 10-HCPT was dissolved in 0.2 ml of DMSO. The resulting OTMCS and 10-HPCT solutions were mixed and sonicated for 30 min at ambient temperature using a JY 92-II ultrasonic processor (Tianjing, China), followed by dialysis against distilled water overnight using a membrane with a molecular weight cut-off of 10,000. The micellar solution was centrifuged at 3000 rpm for 10 min, and filtered through a 0.22 μm pore-sized membrane to collect the polymeric micelles with 10-HPCT loaded. The 10-HPCT-OTMCS micelles were lyophilized and kept at 4 °C until further evaluations.

2.4. HPLC analysis

Micellar 10-HPCT concentrations were measured by HPLC (Agilent 1100, Agilent, Germany) using a Diamohsil™ C18 column (250× 4.6 mm, 10 μm particle size). The mobile phase contained methanol and water at a ratio of 60:40 (v/v). The HPLC was performed with a flow rate of 1.0 ml/min, a column temperature of 30°, and a detection wavelength of 266 nm (Agilent 8453, UV detector, Agilent, Japan). The injected volume was 20 μl for all samples tested. The retention times of the carboxylate form and the lactone form of 10-HCPT were 3.2 and 5.2 min, respectively, under the analytical conditions. Linear calibration curves with a concentration range of 0.2–100 ng/ml were prepared for both 10-HCPT-lactone and 10-HCPT-carboxylate. All samples were analyzed in triplicate.

The total content of 10-HCPT in the resulting micellar systems was measured after resolving the lyophilized powder in double distilled water and diluting with mobile phase. The entrapment efficiency in the micelles was calculated as the percent total fed (6.8 mg) 10-HCPT entrapped in the micelles. The drug loading was calculated as the amount (mg) of 10-HCPT entrapped in the micelles every 100 mg of the chitosan derivative micelles used

$$\text{drug loading content (\%)} = \frac{\text{weight of the drug in micells}}{\text{weight of micelles}} \times 100$$

$$\text{encapsulation efficiency (\%)} = \frac{\text{weight of the drug in micelles}}{\text{weight of the feeding drugs}} \times 100$$

2.5. Characterization of 10-HCPT-polymeric micelle systems

The size of the polymeric micelles was measured using a Zetasizer 3000HS instrument (Malvern Instruments, Malvern, UK) with 633 nm He–Ne lasers at 25 °C. The lyophilized powder was reconstituted with double distilled water. Wide-angle X-ray diffraction (WXRD) was performed using a XD-3A powder diffraction meter with a Cu Kα radiation range of 5–40° (2θ) at 40 kV and 30 mA. Transmission electron microscopy (TEM) was carried out at 75 kV with H-7000 (Hitachi, Japan), for the micellar solution, which was negatively stained with 0.01% phosphotungstic acid and placed on a copper grid coated with framer film.

2.6. In vitro drug release studies

Lyophilized 10-HPCT-OTMCS micelle power (degree of octyl and trimethyl substitution is 8% and 54%, respectively, 32.5% (w/w) drug loading) containing 2 mg of 10-HPCT was dissolved in 2 ml of 0.1 M pH 7.4 phosphate buffer solution (PBS). The resulting solution was placed in a dialysis tube (MWCO 10,000), and the tube was introduced into a stainless steel basket and immerged in 400 ml PBS (0.1 M, pH 7.4) containing 0.02% (w/v) Tween 80. The PBS system was stirred with a speed of 100 rpm at 37 °C. The aliquots were taken from the PBS system at the predetermined time intervals for HPLC analysis and fresh PBS was added into the system to keep the total volume of 2 ml during the assay. In addition, the *in vitro* release behavior of the lyophilized 10-HCPT-OTMCS micelles was measured and compared with that of a commercial 10-HCPT lyophilized powder, which was obtained by dissolving 2 mg 10-HCPT in saline containing 8 mg amino acetic acid, dripping with 20% NaOH to about pH 9 and then lyophilization. All assays were performed in duplicate.

2.7. Stability of 10-HCPT-ODMCS micelles

The lyophilized powder (degree of octyl and trimethyl substitution is 8% and 54%, respectively, 32.5% (w/w) drug loading) was kept in a sealed flask at 4 °C for 3 month. The micelles were taken at the end of 3-month storage, reconstituted with distilled water, and examined for their encapsulation efficiency and the 10-HCPT loading content, using the dialysis and HPLC methods described above (Section 2.4). The micellar size was also measured using a Zetasizer 3000HS instrument following the protocol described above in Section 2.5.

2.8. In vivo drug release of 10-HCPT-OTMCS micelles

In vivo drug release property of the 10-HCPT-OTMCS micelles (degree of octyl and trimethyl substitution is 8% and 54%, respectively, 32.5% (w/w) drug loading) was examined

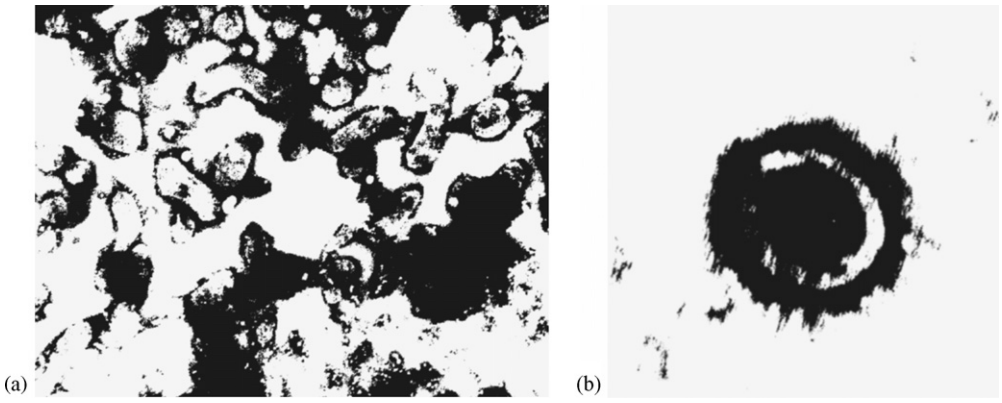

Fig. 3. TEM micrographs of (a) 10-HCPT-OTMCS micelle (degree of octyl and trimethyl substitution is 8% and 54%, respectively, 32.5% (w/w) drug loading (2,000,000×) and blank OTMCS micelle (degree of octyl and trimethyl substitution is 8% and 54%, respectively) (1,000,000×).

using New Zealand White rabbits. All animal procedures were approved by the China Pharmaceutical University Animal Care and Use Committee. Male New Zealand White rabbits were obtained at 1500–1700 g body weight from the Central Animal Laboratory of China Pharmaceutical University (CALCPU). Animals were kept on the rodent diet provided by CALCPU for 3 days, and were randomly divided into two groups. After 24 h fasting, 10-HCPT-OTMCS was given to each rabbit via marginal ear vein injection using indwelling catheter. The dose of 10-HPCT-ODMCS micelles was equivalent to 1.7 mg 10-HCPT/kg body weight. Blood samples were taken at different time intervals after drug administration from the marginal ear vein, and the plasma was immediately separated by centrifugation.

Plasma concentrations of 10-HCPT were determined by the reverse-phase HPLC procedure described above. Briefly, 500 μl of acetonitrile containing phosphorous acid at a final concentration of 25 ng/ml was vortexed with 500 μl of the plasma sample. The resulting mixture was centrifuged at 15,000 rpm for 5 min, and 100 μl of the supernatant was transferred to another tube. The supernatant was mixed with 100 μl mobile phase and centrifuged at 15,000 rpm for 10 min to obtain clear supernatant. The clear supernatant was subjected to HPLC analysis of 10-HCPT concentration using standard curves prepared with the 10-HCPT lactone and carboxylate. To prepare the standard solution, 10-HCPT was dissolved in dimethylsulfoxide (DMSO), and then diluted with the mixture of methanol and 0.01 M PBS (50:50, v/v) at pH 3 and pH 9 to obtain the stock solution of 10-HCPT lactone or carboxylate form, respectively. The detection limit was 100 ng/ml under the experimental conditions. The recovery of 10-HCPT in plasma samples was between 98% and 109%, and both intraday and interday variabilities were less then 5%.

3. Results and discussion

3.1. Preparation and characterization of 10-HCPT-polymeric micelle system

3.1.1. Particle morphology and size

N-Octyl-*N*-trimethyl chitosan was able to form polymeric nanomicelles and entrap 10-HCPT in its hydrophobic core (Figs. 3 and 4). The 10-HCPT-OTMCS micelles (degree of octyl and trimethyl substitution is 8% and 54%, respectively, 32.5% (w/w) drug loading) had no diffraction peak of 10-HCPT and exhibited a similar WXRD pattern similar to that of blank micelle, but not that of pure 10-HCPT (Fig. 4). It is widely accepted that polymeric micelles with a hydrophobic core and a hydrophilic shell may entrap lipophilic drug in the core and enhance their solubility in aqueous fluid. These WXRD data indicated that 10-HCPT was entrapped in the hydrophobic core of the OTMCS micelles, but not simply associated with non-micellar OTMCS or bound on the hydrophilic surface of the micelles.

Formation of 10-HCPT-OTMCS nanomicelles (degree of octyl and trimethyl substitution is 8% and 54%, respectively) was also examined using TEM. TEM micrographs of blank and 10-HCPT loaded OTMCS micelles are presented in Fig. 3, showing that OTMCS was capable of forming polymeric nanomicelles with (Fig. 3a) or without 10-HCPT (Fig. 3b). Furthermore, these polymeric micelles appeared in a spherical like shape with a small degree of deformation and aggregation. In agreement to the WXRD result from the present study, no

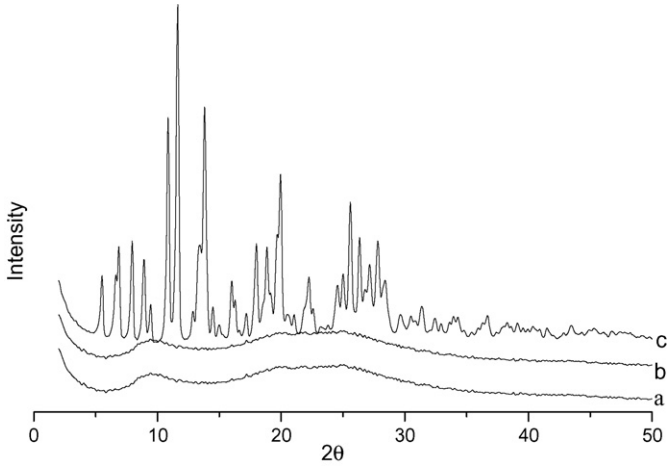

Fig. 4. WXRD patterns of (a) drug-free OTMCS micelle (degree of octyl and trimethyl substitution is 8% and 54%, respectively), (b) 10-HCPT-OTMCS micelle (the degree of octyl and trimethyl substitution is 8% and 54%, respectively, 32.5% (w/w) drug loading), and (c) pure 10-HCPT.

Table 1
Influence of different substitute degree of octyl group in OTMCS on micelle size,entrapment efficiency (%) and drug loading (%)[a]

Octyl substitute degree (%)	8	20	36	48	58
Entrapment efficiency (%)	56.5	12.1	14.4	7.1	15.0
Drug loading (%)	32.5	6.9	8.3	4.1	8.6
Size (nm)	52.5	26.0	273.1	28.7	119.7

[a] OTMCS represents *N*-octyl-*N*-trimethyl. Size is the average diameter for micelles repared from each chitosan derivatives. Data are means ($n = 3$).

Table 2
Influence of the length of hydrophobic group in dimethyl chitosan on micelle size and 10-HCPT concentrations in micellar solution[a]

	OTMCS	DTMCS	LTMCS
C (mg/ml)	1.92	0.07	1.90
Size (nm)	23.5	277.2	20.8

[a] OTMCS, DTMCS, and LTMCS represent *N*-octyl-*N*-trimethyl, *N*-decanyl-*N*-trimethyl, and *N*-lauryl-*N*-trimethyl chitosan derivatives. They were prepared following the scheme showing in Fig. 1. The degree of alkyl and trimethyl substitution is about 8% and 54%, respectively. *C* stands for the concentration of 10-HCPT in the micellar solution, and size is the average diameter for micelles repared from each chitosan derivatives. Data are means ($n = 3$).

10-HCPT crystal was visible on the surface of the 10-HCPT-OTMCS micelles under the testing conditions, supporting the conclusion that OTMCS may form polymicelles and entrap lipophilic compounds in their hydrophobic cores [28]. Both TEM and WXRD data suggestted the possible utilization of OTMCS in improving the water solubility of camptothecins including 10-HCPT and other lipophilic drugs.

Size of polymeric micelles is a crucial factor controlling the *in vivo* fate of the selected drug delivery system [24,29]. Nanoparticles with an average particle diameter range of 10–200 nm may have reduced reticuloendothelial system (RES) uptake and different membrane permeability, and consequently have different duration in the circulation system [27]. It is believed that both the mean micellar size and the polydispersity may affect the passive targeting and delivery of the entrapped drug [29]. Controlling particle size is one of the most important approaches for developing a drug carrier system. It was observed in the present study that OTMCS may form polymeric nanomicelles with 10-HCPT and enhance their water solubility (Table 1). The degree of octyl substitution altered the size of 10-HCPT-OTMCS micelles and the ability of the resulting OTMCS to improve the water solubility of 10-HCPT. Interestingly, the OTMCS with 8% octyl substitution had the greatest solubilizing capacity with a final drug loading of 32.5%, but it is not the largest polymeric micellar size (Table 1). The largest particle size of 273 nm was detected for the OTMCS with 36% octyl substitution, followed by that with 58% octyl substitution (Table 1). This larger particular size may be explained by the aggregation of micelles. In addition, the particle size does not necessarily correlate to the solubility of 10-HCPT in the micellar solution. For instance, OTMCS with 20% and 48% octyl substitution had similar micelle sizes with a diameter of 26.0 and 28.7 nm, and similar drug loadings of 6.9% and 4.1%, respectively (Table 1).

N-Octyl-*N*-trimethyl (OTMCS), *N*-decanyl-*N*-trimethyl (DTMCS), and *N*-lauryl-*N*-trimethyl (LTMCS) chitosan derivatives, with same degree of alkyl substitution and trimethylation, were prepared and used to investigate the potential effects of hydrophobic tail length on micelle size and 10-HCPT solubilizing capacity. The length of alkyl chain altered both micellar size and 10-HCPT solubilizing capacity (Table 2). OTMCS and LTMCS were able to form 10-HCPT loaded nanomicelles with similar particle size and solubility enhancing capacity (Table 2). In contrast, DTMCS was not able to form integrated polymeric micelles, which was evidenced by its very low micellar 10-HCPT concentration of 0.07 mg/ml (Table 2), suggesting the significant impact of hyhdopholic tail length on micellar forming properties of *N*-alkyl-*N*-trimethyl chitosan derivatives.

3.1.2. Encapsulation efficiency and drug loading content

Encapsulation efficiency and drug loading content are two critical characteristics for evaluating the capacity of a selected polymer to entrap and carry a selected drug. Encapsulation efficiency and drug loading content may be altered by several factors such as the chemical structure of micelle forming polymers, chemical structure of the drug, feeding ratio of the drug to polymer, and ratio of water to organic phase. In the present study, OTMCS structure and micellar preparation conditions were investigated for their possible influence on encapsulation efficiency and drug loading content using 10-HCPT. OTMCS preparations with an octyl substitution degree range of 8–58% had an encapsulation efficiency ranging from 7.1% to 56.1% and a drug loading content of 4.1–32.5% (Table 1). The encapsulation efficiency of these OTMCS preparations was positively correlated to their drug loading content with a R^2 value of 0.9996. The highest encapsulation efficiency and the greatest drug loading content were observed for the OTMCS with 48% octyl substitution, whereas the OTMCS exhibited the lowest encapsulation efficiency and drug loading content, suggesting that the overall compatibility between 10-HCPT and the core-forming block plays an important role in determining the encapsulation efficiency and drug loading content of 10-HCPT-OTMCS nanomicelles [24]. At the drug loading content of 32.5%, the solubility of 10-HCPT in the aqueous nanomicellar solution is about 80,000-folds of that in pH 7.4 PBS buffer at 37 °C, showing the potential of utilization of OTMCS in enhancing the solubility of hydrophobic camptothecins.

The feeding ratio of the drug to polymer altered 10-HCPT encapsulation efficiency (Table 3). Increasing the ratio of 10-HCPT and OTMCS from 1:4 to 1:2.4, the encapsulation efficiency was increased from 19% to 42.0% (Table 3). Further increasing the drug feeding ratio to 1:1.7 and 1:1.5 slightly decreased the encapsulation efficiency to 40.0% and 37.5%, respectively. This may be explained by the fact that the higher 10-HCPT concentration in DMSO might lead to its elevated diffusion from organic phase into water, and the higher percentage of 10-HCPT might move in water before the formation of nanomicelles and thus could not be entrapped [24]. Also noted was that the encapsulation efficiency and drug loading content differed in their responses to drug feeding ratio changes. The

Table 3
Influence feeding ratio of the drug to polymer on the entrapment efficiency and drug loading of 10-HCPT-OTMCS micelle[a]

Ratio of HCPT and OTMCS (mg/mg)	Encapsulation efficiency (%)	Drug loading content (%)
1/4	19	4.8
1/3	37.3	12.6
1/2.4	42.0	17.8
1/1.7	40.0	23.7
1/1.5	37.5	25.4

[a] OTMCS represents *N*-octyl-*N*-trimethyl and the degree of octyl and trimethyl substitution is 8% and 54%, respectively. Data are means ($n = 3$).

Table 4
Influence the volume ratio of DMSO and water (v/v) on the entrapment efficiency and drug loading of 10-HCPT-OTMCS micelle[a]

Ratio of DMSO and water (ml/ml)	Encapsulation efficiency (%)	Drug loading content (%)
0.1	40.0	23.7
0.2	14.0	8.1
0.3	6.3	3.6
0.4	2.8	1.6

[a] OTMCS represents *N*-octyl-*N*-trimethyl and the degree of octyl and trimethyl substitution is 8% and 54%, respectively. Data are means ($n = 3$).

highest encapsulation efficiency of 42% was obtained when the ratio of 10-HCPT and OTMCS was 1:2.4, but the maximum drug loading content of 25.4% was achieved at the drug-feeding ratio of 1:1.5. Therefore, the ratio of 10-HCPT and OTMCS at 1:1.5 (w/w) was selected to prepare 10-HCPT-OTMCS micelles for further drug release studies *in vitro* and *in vivo*.

It has been observed that solvent used to dissolve drug molecules may significantly affect micelle formation and encapsulation efficiency [24]. The effect of relative amount of DMSO and water on encapsulation efficiency and drug loading content was investigated in the present study. Increasing relative DMSO level decreased both encapsulation efficiency and drug loading content of the micelles (Table 4). This may be due to the interference of DMSO on micelle formation and its ability to reduce 10-HCPT diffusion into the hydrophobic core of the OTMCS micelles. Therefore, it is recommended that amount of organic solvent should be minimized for dissolving the drug, and their residues should be removed completely after the drug is loaded.

3.2. *In vitro* release of 10-HCPT loaded OTMCS micelles

The *in vitro* drug release behavior of the 10-HCPT-OTMCS micelles (degree of octyl and trimethyl substitution is 8% and 54%, respectively) was evaluated and compared to that of the commercial 10-HCPT lyophilized powder. There was no obvious burst effect of 10-HCPT releasing from the OTMCS micelles, and it took about 110 h to release 50% of the drug loaded, and took about 620 h to reach the maximum release of 10-HCPT (Fig. 5). In comparison, 70% of 10-HCPT was released rapidly from lyophilized powder formulation within 11 h and the release was almost completed within 34.5 h (Fig. 5). These data suggested the potential utilization of OTMCS and other *N*-alkyl-*N*-trimethyl chitosan derivatives in controlling the release of hydrophobic camptothecins and improve the safety, efficacy, stability, pharmacokenitic properties, and convenience of camptothecins. The significant sustained-release behavior may be attributed to the very slow diffusion of 10-HCPT from the OTMCS micelles but not simple penetration of drug molecules through the dialysis membrane.

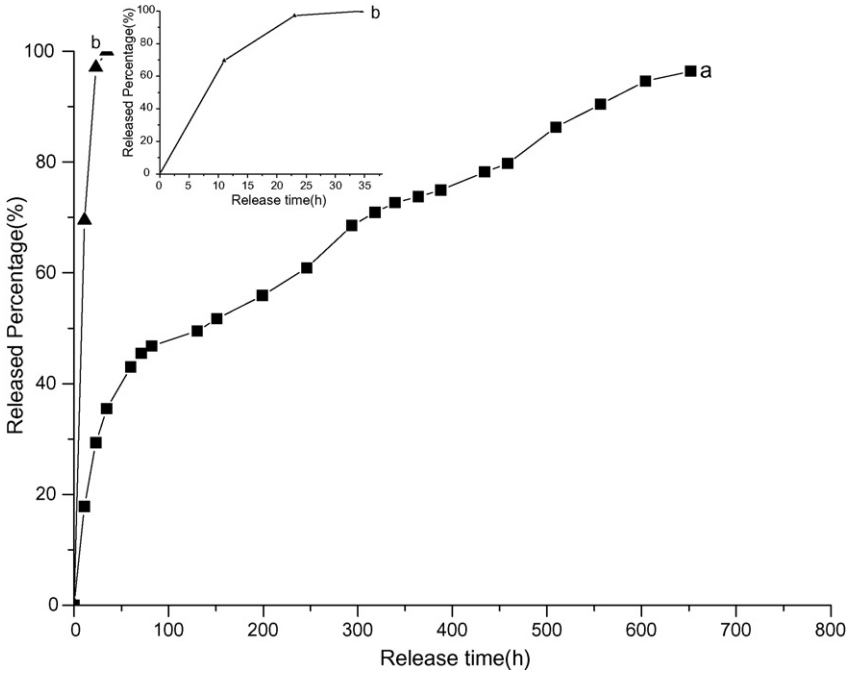

Fig. 5. *In vitro* release of 10-HPCT from (a) OTMCS micelle (the degree of octyl and trimethyl substitution is 8% and 54%, respectively, 32.5% (w/w) loading) (■), (b) commercial 10-HCPT lyophilized powder (▲) in PBS (0.1 M, pH 7.4), at 37 °C ($n = 3$).

Table 5
Stability of 10-HCPT-OTMCS micelles during storage ($n = 3$)[a]

Storage time	C (mg/ml)	Entrapment efficiency (%)	Drug loading content (%)	Micelle size (nm)
0 day	1.93 ± 3.34	56.67 ± 3.33	32.66 ± 3.35	23.5 (92.8%)
3 months	1.90 ± 1.03	55.75 ± 1.03	32.13 ± 1.03	36.4 (98.9%)

[a] OTMCS represents *N*-octyl-*N*-trimethyl and the degree of octyl and trimethyl substitution is 8% and 54%, respectively. The 10-HCPT-OTMCS micelles were kept at 4 °C. The micelle size is reported in the average diameter of the 10-HCPT-OTMCS micelles. Data are means ($n = 3$).

Table 6
Pharmacokinetic parameters of 10-HCPT-OTMCS micelle (32.5% (w/w) drug loading) and 10-HCPT solution in rabbit after i.v. administration[a]

Sample	Dose (mg/kg)	C_{max}[b] (μg/ml)	$t_{1/2\beta}$[c] (h)	$AUC_{0\rightarrow\infty}$[d] (μg h/ml)	CL[e] (ml/(h kg))	V_d[f] (l/kg)
Micelle	1.7	7.99	1.64	18.93	0.09	0.21
Solution	5	28.2	0.09	3.65	1.38	0.18

[a] OTMCS represents *N*-octyl-*N*-trimethyl and the degree of octyl and trimethyl substitution is 8% and 54%, respectively. Data are means ($n = 3$).
[b] C_{max}: peak plasma concentration.
[c] $t_{1/2\beta}$: elimination half life.
[d] $AUC_{0\rightarrow\infty}$: area under the plasma concentration-time curve.
[e] CL: total body clearance.
[f] V_d: volume of distribution at steady state.

3.3. Stability of drug loaded micellar system

The freeze-dried 10-HCPT-OTMCS micelles were able to dissolve in water easily, sterilization saline, or osmotic glucose solution after 3-month storage at 4 °C. Their drug entrapping efficiency or drug loading efficiency had no significant change, although both showed slight decrease from 56.67% and 32.66% to 55.75% and 32.13%, respectively, showing the stability of 10-HCPT-OTMCS micelles (Table 5). The average diameter of the 10-HCPT-OTMCS micelles increased from 23.5 to 36.4 nm.

3.4. Plasma concentration of 10-HCPT

The *in vivo* release behavior of 10-HCPT-OTMCS micelles was also investigated using rabbits (Fig. 6). After i.v. administration of 10-HCPT-OTMCS micelles at a dose of 1.7 mg 10-HCPT/kg body weight, the pharmacokinetic properties were determined and summarized in Table 6. The maximum plasma concentration (C) of 10-HCPT was 7.99 μg/ml, and the elimination half life (t) and the area under the plasma concentration–time curve (AUC_{0-240}) were 1.64 h and 18.93 μg/ml h, respectively. The results indicated that 10-HCPT-OTMCS micelles were able to maintain the higher plasma concentration of the lactone form of 10-HCPT for up to 240 min. In contrast, previous study [30] showed that i.v. injection of 10-HCPT (10-HCPT solution, pH 9) at a dosage of 5 mg/kg following the same experimental condition, a much higher plasma concentration (C) of 28.2 μg/ml was observed in rabbits immediately. However, the concentration decreased rapidly with a much lower AUC_{0-240} value of 3.65 μg/ml h. Because the apparent distribution volume (V_0) of the rabbits in two experiments was very close to each other, and both the micelle-loaded system and the 10-HCPT injection could be described by a single compartment model, so the larger AUC_{0-240} of the micellar system may contribute to the long circulation characteristics of the nanoparticles and to the slower release of 10-HCPT, which was retarded by the stable polymeric micelle barriers. It was important that no significant lactone ring-opening phenomena were detected after i.v. administration of the micellar delivery system of 10-HCPT. These data suggested that the new micellar delivery system might increase the therapeutic efficiency and the decrease the side effects of camptothecins. Nevertheless, more detailed *in vivo* study on this novel 10-HCPT loaded micelle delivery system is currently under further investigation.

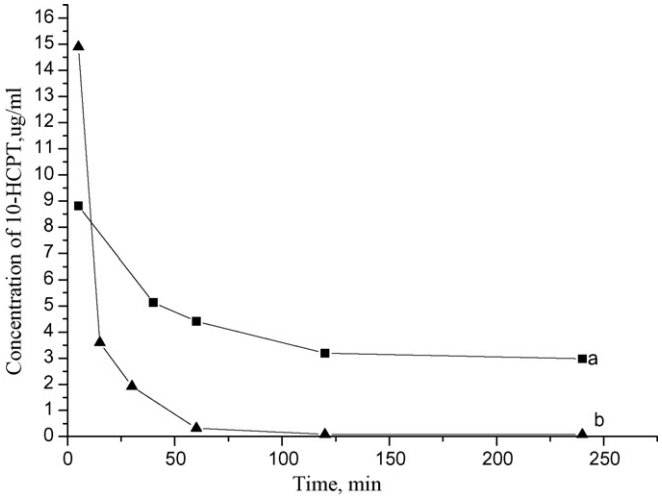

Fig. 6. Time courses of 10-HCPT levels in rabbit plasma after i.v. administration of (a) 1.7 mg/kg dose of 10-HCPT-OTMCS micelle (degree of octyl and trimethyl substitution is 8% and 54%, respectively, 32.5% (w/w) loading) (■) and (b) 5 mg/kg dose of 10-HCPT solution (▲) (adapted from Ref. [30]).Each point represents the mean ± S.D. of four rabbits per time point.

4. Conclusions

This research demonstrated the possibility to develop OTMCS micellar systems for delivery, solublization, stabilized lactone ring structure, and controlled release of 10-HCPT, a lipophilic camptothecin derivative. Such a micellar system with high encapsulation efficiency, satisfactory stability and drug

loading amount may be prepared by dialysis followed by filtering sterilization and lyophilization. The particle size ranging from 24 to 280 nm is suitable for intravenous use and can be well controlled by modulating the chemical structure of OTMCS and the micellar forming conditions. In addition, this study suggests the possible utilization of amphiphilic micellar chitosan derivatives as carriers for hydrophobic drugs for improving their delivery and releasing properties.

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