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2008 Nanotechnology 19 145101

(http://iopscience.iop.org/0957-4484/19/14/145101)

Nanotechnology **19** (2008) 145101 (8pp) doi:10.1088/0957-4484/19/14/145101

Self-assembled nanoparticles of cholesterol-modified *O*-carboxymethyl chitosan as a novel carrier for paclitaxel

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Received 29 November 2007, in final form 15 January 2008
Published 4 March 2008
Online at stacks.iop.org/Nano/19/145101

Abstract

Self-assembled nanoparticles of cholesterol-modified *O*-carboxymethyl chitosan (CCMC) were prepared to be used as a novel carrier for paclitaxel (PTX) in this study. CCMC-6.9 was synthesized by the covalent conjugation of cholesterol to *O*-carboxymethyl chitosan with the succinyl linkage and the degree of substitution (DS) of the cholesterol moiety was 6.9%. CCMC-6.9 formed self-assembled nanoparticles with a size of 209.5 nm in aqueous media. Paclitaxel-loaded CCMC-6.9 self-assembled nanoparticles were prepared using a dialysis method and their characteristics were analyzed by dynamic laser light scattering (LLS), transmission electron microscopy (TEM) and ultraviolet spectroscopy (UV). PTX-loaded CCMC-6.9 self-assembled nanoparticles were almost spherical in shape and their size increased from 245.6 to 355.3 nm with PTX-loading content increasing from 18.7% to 34.9%. *In vitro* release of PTX from CCMC-6.9 self-assembled nanoparticles was carried out by the dynamic dialysis method. PTX continuously released in phosphate buffered saline (PBS) solutions for 84 h at 37 °C and its release was sensitive to the pH of the release media. The biodistribution of PTX-loaded CCMC-6.9 self-assembled nanoparticles was studied in female Balb/c mice. Compared with PTX in the solution of Cremophor EL (polyethoxylated castor oil)/ethanol (PTX-Cre), CCMC-6.9 self-assembled nanoparticles significantly increased the uptake of PTX in plasma, liver and spleen, but decreased the uptake in heart and kidney. These results suggest that CCMC-6.9 self-assembled nanoparticles can effectively solubilize PTX and modify its tissue biodistribution, which may be advantageous in enhancing the therapeutic index and reducing the toxicity of PTX.

1. Introduction

Polymeric amphiphiles consisting of hydrophilic and hydrophobic segments can form nanosized self-assemblies with a hydrophobic core and a hydrophilic shell due to the intra- and/or intermolecular interactions of hydrophobic segments in the aqueous media [1, 2]. Many investigations [3–7] have reported that these self-assemblies, used as the drug delivery system, can reduce the unwanted toxic side effects, prolong the circulation time, reduce the uptake by the reticuloendothelial system (RES) and enhance the therapeutic index of drugs. Hydrophobically modified polysaccharides, as a novel kind of polymeric amphiphiles, have attracted an increasing

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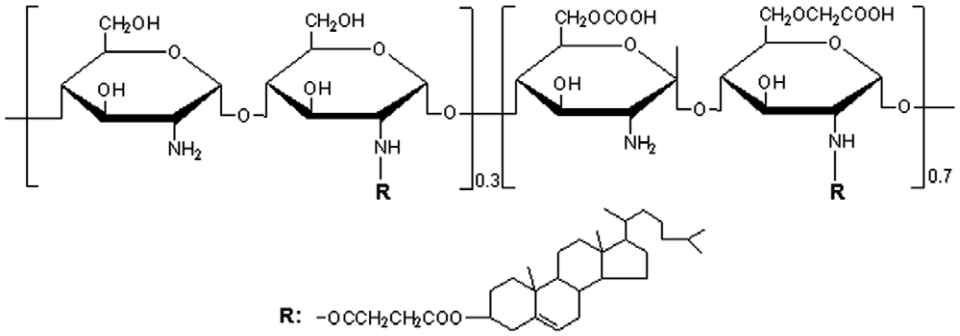

Figure 1. Chemical structure of cholesterol-modified *O*-carboxylmethyl chitosan (CCMC).

interest recently [8, 9] since their self-assembled nanoparticles in the aqueous media are suitable for trapping hydrophobic substances [10] and some bioactive macromolecules [11] in biotechnology and medicine.

Chitosan, a homopolymer of (1, 4)-linked 2-amino-2-deoxy-β-glucan, is produced by deacetylation of chitin, which is the second most abundant and renewable natural polysaccharide after cellulose. Since chitosan shows many good properties such as biocompatibility, biodegradability, positively charged, nontoxicity and bioadhesivity, it can be used as an ideal biomedical material [12, 13]. Hydrophobically modified chitosan derivatives such as alkylated chitosan [14, 15] and deoxycholic acid-modified chitosan [16, 17] have been focusing on recently due to their strong amphiphilic property and their formation of self-assembled nanoparticles in the aqueous media, which is very useful for biotechnological and pharmaceutical applications. We have previously reported the synthesis and characteristics of self-assembled nanoparticles of cholesterol-modified *O*-carboxymethyl chitosan (CCMC, figure 1) [18] and their interaction with bovine serum albumin (BSA) [19]. In addition, the hydrophobic core of these self-assembled nanoparticles formed by the hydrophobic interactions among the cholesterol moieties was also considered to act as a reservoir of hydrophobic substances. In this study, paclitaxel (PTX) was chosen as a model drug to assess the potential of CCMC self-assembled nanoparticles as a carrier for hydrophobic anticancer drugs by the *in vitro* and *in vivo* investigations.

PTX is known to have highly effective activity against various tumors and has been used clinically in the treatment of metastatic breast cancer, ovarian cancer and several other malignancies [20, 21]. Because the water solubility of PTX is very low, the commercial preparation of PTX is formulated in a solution composed of a 50:50 (v/v) mixture of Cremophor EL (polyethoxylated castor oil) and dehydrated alcohol, which is diluted 5–20 fold in normal saline or dextrose solution (5%) before administration. However, serious side effects, such as hypersensitivity reactions, nephrotoxicity and neurotoxicity, attributable to Cremophor EL have been reported [22, 23]. Therefore, CCMC-6.9 self-assembled nanoparticles, being used as a carrier, was hoped to increase the aqueous solubility, enhance the therapeutic index and decrease the toxicity of PTX.

2. Experimental details

2.1. Materials

Cholesterol-modified *O*-carboxymethyl chitosan (CCMC-6.9) was synthesized and analyzed according to the method previously reported [18]. Briefly, the cholesterol moiety was conjugated to *O*-carboxymethyl chitosan (CMC, molecular weight was 6.78×10^4 Daltons and DS of carboxymethyl groups was 70%) by the succinyl linkage. The degree of substitution (DS) of cholesterol moiety, defined as the number of cholesterol moieties per 100 glucosamine units of chitosan, was 6.9%. Paclitaxel (PTX) was obtained from Xian Tiancheng Drugs & Bioengineering Co., Ltd (China). Cremophor EL was obtained from BASF Corp (Parsippany, NJ). Pyrene was obtained from Aldrich and purified by recrystallization from absolute ethanol. All reagents for high performance liquid chromatography (HPLC) analysis, including acetonitrile and methanol, were HPLC grade. Other chemical reagents in the study were analytical grade.

Six to eight-week-old female Balb/c mice were supplied by the Laboratory Animal Center of Tianjin Medical University, (Tianjin, China). The animals were used following the guidelines of the Ethical Committee for Animal Experiments of Tianjin Medical University. The animals were acclimatized at a temperature of $25 \pm 2\,^{\circ}C$ and a relative humidity of $70\% \pm 5\%$ under natural light/dark conditions for at least 24 h before dosing.

2.2. Preparation of CCMC-6.9 self-assembled nanoparticles and drug loading

CCMC-6.9 self-assembled nanoparticles were prepared by the method previously reported [18, 19]. Briefly, CCMC-6.9 was dispersed in distilled water under gentle shaking at $37\,^{\circ}C$ for 48 h, followed by sonication using a probe type sonifier (Automatic Ultrasonic Processor UH-500A, China) at 100 W for 2 min. The sonication step was repeated three times until the desired size value had been reached. To prevent the sample solution from heat built-up during the sonication, the pulse function was used (pulse on 2.0 s, pulse off 2.0 s).

PTX-loaded CCMC-6.9 self-assembled nanoparticles were prepared by a dialysis method [24]. Briefly, the different amounts of PTX dissolved in methanol were slowly added to CCMC-6.9 self-aggregated nanoparticle solutions

under stirring, and then the methanol solvent was removed by dialysis (Millipore dialysis tube, molecular weight cutoff 12–14 kDa, USA) to obtain PTX-loaded CCMC-6.9 self-assembled nanoparticles. The dust and impurity in the sample solution were removed by passing through a filter (0.45 μm, Millipore).

2.3. Characterization of PTX-loaded CCMC-6.9 self-assembled nanoparticles

The mean diameter and particle size distribution of CCMC-6.9 self-assembled nanoparticles with/without PTX loading were determined by laser light scattering (LLS) with a Brookhaven BI-200SM/BI-9000AT goniometer (USA) at a scattering angle of 90°, a wavelength of 532 nm and a temperature of 25 $\pm$ 0.1 °C.

The morphology of CCMC-6.9 self-assembled nanoparticles with/without PTX loading was observed by transmission electron microscopy (TEM). In practice, the sample solution was dropped onto the carbon-coated 200 mesh copper grid. Then, the grid was air-dried and imaged using a Tecnai G^2 20 S-Twin TEM (FEI Company, USA) at 80 kV.

2.4. Determining the loading capacity of PTX

The loading capacity of PTX was determined according to the method proposed by Huo MR [25]. To calculate the drug loading content (drug/carrier) and the drug loading efficiency (loaded drug/initially added drug), PTX-loaded CCMC-6.9 self-assembled nanoparticles were dispersed in methanol, and then the UV absorption of PTX at the wavelength (λ) of 227 nm was measured with a Beckman DUR 640 UV spectrophotometer (USA).

2.5. PTX releases from CCMC-6.9 self-assembled nanoparticles in vitro

PTX release behavior was studied *in vitro* by the dynamic dialysis method reported by Han [26] in phosphate buffered saline (PBS) solution with pH values of 4.0, 7.2 and 9.0, respectively. Briefly, the solutions of PTX-loaded CCMC-6.9 self-assembled nanoparticles were respectively placed into Visking dialysis tubing (molecular weight cutoff 12–14 kDa, Millipore, USA) and dialyzed against 50 ml of the above PBS solutions at 37 $\pm$ 0.2 °C in an air-bath shaker at 50 rpm. Then, 0.5 ml of the release media was collected and replaced with an equal volume of the fresh release media at predefined time intervals. The release amounts of PTX were determined by the high performance liquid chromatography (HPLC) method described below. PTX release from the solution of Cremophor EL/dehydrated alcohol (50/50 v/v) was also conducted under the same conditions as the control.

2.6. Tissue biodistribution study

Female Balb/c mice were administered either PTX-loaded CCMC-6.9 self-assembled nanoparticles or PTX in the Cremophor EL/ethanol solution (PTX-Cre) at a single dose of 5 mg PTX/kg via the tail vein. At 0.083, 0.25, 0.5, 1, 2, 4, 8 and 12 h after drug injection, each animal ($n = 6$ for each time point) was killed and heart, liver, spleen, lung and kidney as well as blood samples were collected. Tissue samples were washed in ice-cold saline, blotted with paper towels to remove excess fluid, weighed and stored at -50 °C until assessed for drug concentration by HPLC.

2.7. HPLC analysis

The analyses of PTX levels *in vitro* and *in vivo* were carried out using a HPLC system consisted of a LC-10AT_{VP} pump, a manual injector with a 20 μl fixed loop and a SPD-10A_{VP} UV–vis detector (Shimadzu, Kyoto, Japan) at 227 nm. A reversed-phase column (Lichrospher C_{18}, 4.6 mm $\times$ 150 mm, 5 μm, Merck, Darmstadt, Germany) was used at room temperature and 20 μl samples were injected into this column for all the analyses. The mobile phase consisted of methanol, acetonitrile and water (25:35:40, v/v/v) and its flow rate was 1.0 ml min^{-1}. The retention time of PTX was approximately 13.2 min.

Tissue samples were homogenized in the mixed solution of acetonitrile and water (50:50 v/v). 50 μl diazepam (1 μg ml^{-1}) as an internal standard was added into 200 μl of plasma or tissue samples, and vortexed for 1 min. The drug and the internal standard were then extracted into 4 ml of tert-butyl methyl ether by vortex mixing for 5 min. After centrifugation at 6000*g* for 10 min, 3 ml of the clear supernatant was removed and evaporated under a gentle stream of nitrogen. The residue was then dissolved in 200 μl mobile phase and centrifuged at 10 000*g* for 5 min before HPLC analysis.

2.8. Statistical evaluation

The area under the curve (AUC) based on statistical moment theory was calculated by the trapezoid method. The results were presented as means with standard deviations (SD) and were analyzed for statistical significance by Student's *t*-test. The level of significance was defined at $p < 0.05$.

3. Results and discussion

3.1. Preparation of CCMC-6.9 self-assembled nanoparticles and drug loading

CCMC-6.9 formed monodisperse self-assembled nanoparticles with a relatively narrow size distribution by probe sonication in distilled water. The mean diameter of these nanoparticles determined by the dynamic laser light scattering (LLS) was 209.5$\pm$0.6 nm, and the polydispersity index was 0.165$\pm$0.031 (figure 2(b)). The TEM image (figure 2(a)) shows that CCMC-6.9 self-assembled nanoparticles were regular spherical in shape and the size was slightly smaller than the size determined by LLS. This is mainly due to the process involved in the preparation of the sample. TEM depicted the size in the dried state of the sample, whereas LLS measured the size in the hydrated state of the sample, so that the size measured by LLS was a hydrodynamic diameter and had a larger value because of the solvent effect [18, 27].

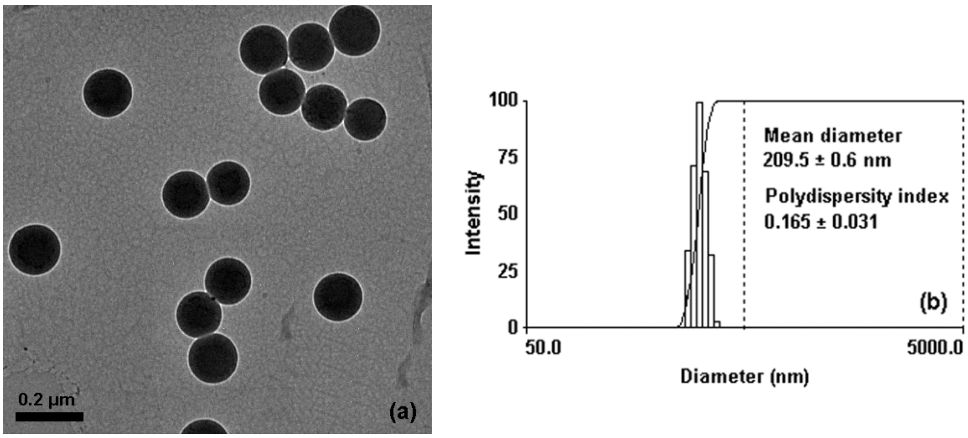

Figure 2. TEM image (a) and the size distribution (b) of CCMC-6.9 self-assembled nanoparticles in distilled water. The concentration of CCMC-6.9 was 0.5 mg ml^{-1}.

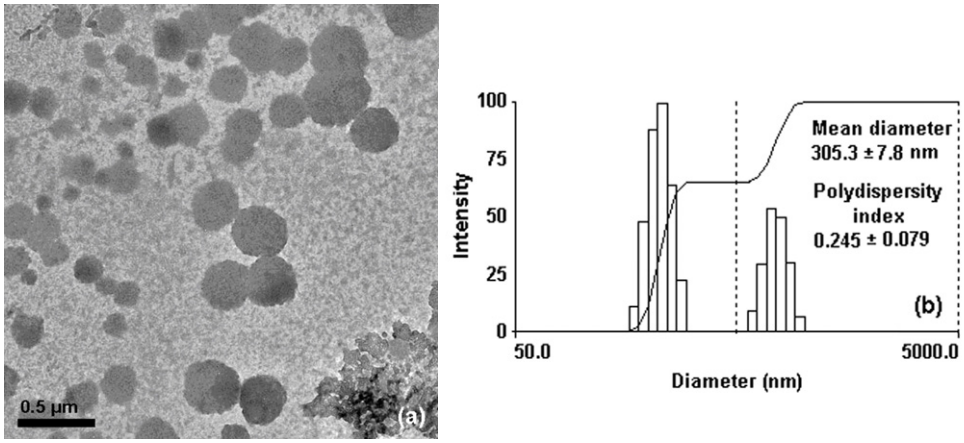

Figure 3. TEM image (a) and the size distribution (b) of PTX-loaded CCMC-6.9 self-assembled nanoparticles with a PTX-loading content of 30.4% in distilled water. The concentration of CCMC-6.9 was 0.5 mg ml^{-1}.

The anticancer drug, paclitaxel (PTX), is known to have the highly hydrophobic property [22], so that we hoped to use CCMC-6.9 self-assembled nanoparticles as a carrier for PTX to increase its aqueous solubility. The dialysis method was used to prepare PTX-loaded CCMC-6.9 self-assembled nanoparticles in this study. PTX dissolved in methanol was first added to the nanoparticle solution, and then was dialyzed against aqueous medium. In the dialysis process, methanol was gradually removed, which resulted that PTX spontaneously transferred from the aqueous medium into the hydrophobic cores of CCMC-6.9 self-assembled nanoparticles due to the driving force of hydrophobic interaction. TEM image (figure 3(a)) shows that PTX-loaded CCMC-6.9 self-assembled nanoparticles were almost spherical in shape. However, compared with blank nanoparticles (figure 2), PTX-loaded CCMC-6.9 self-assembled nanoparticles had a bumpier surface, a larger size and a broader size distribution which showed 'two' main peaks (figure 3(b)). We believed these differences were due to the physical entrapment of PTX and the agglomeration of nanoparticles during the drug loading process.

The characteristics of PTX-loaded CCMC-6.9 self-assembled nanoparticles are listed in table 1. The loading content increased from 18.7% to 34.9% with the weight ratio of drug to carrier increasing from 1/5 to 1/2 as expected. However, the loading efficiency (92.8%–90.2%) scarcely changed with the weight ratio ranging from 1/5 to 1/3, and then significantly decreased to 67.8% with the weight ratio increasing to 1/2, which indicated that the ability of CCMC-6.9 self-assembled nanoparticles to carry PTX reached saturation. When PTX was entrapped in CCMC-6.9 self-assembled nanoparticles, its maximum solubility in water was about 0.7 mg ml^{-1}, 100 times more than PTX water solubility, which indicated that CCMC-6.9 self-assembled nanoparticles could effectively solubilize PTX. Furthermore, it is also shown from table 1 that the size of PTX-loaded CCMC-6.9 self-assembled nanoparticles increased from 245.6 to 355.3 nm with the PTX-loading content increasing from 18.7% to 34.9%. Therefore, considering the size and the drug loading property, we believed that 1/3 (drug/carrier) was the optimal weight ratio to prepare PTX-loaded CCMC-6.9 self-assembled nanoparticles.

Table 1. Characteristics of PTX-loaded CCMC-6.9 self-assembled nanoparticles.

Drug/carrier[a] (w/w)	Diameter[b] (nm)	Polydispersity index[b]	Loading content[c] (%, w/w)	Loading[d] efficiency (%, w/w)
1/5	245.6 ± 5.8	0.105 ± 0.053	18.7 ± 0.57	92.8 ± 1.60
1/4	279.9 ± 6.5	0.138 ± 0.017	23.6 ± 1.05	92.4 ± 2.57
1/3	305.3 ± 7.8	0.254 ± 0.079	30.4 ± 0.50	90.2 ± 1.45
1/2	355.3 ± 15.6	0.305 ± 0.059	34.9 ± 1.65	67.8 ± 4.55

[a] The weight ratio of PTX to CCMC-6.9 self-assembled nanoparticles.
[b] The size and size distribution (mean value ± standard deviation) of PTX-loaded CCMC-6.9 self-assembled nanoparticles determined by LLS three times.
[c] (Loading PTX/CCMC-6.9 self-assembled nanoparticles) ×100% (mean value ± standard deviation) determined by UV method three times.
[d] (Loading PTX/total PTX) ×100% (mean value ± standard deviation) determined by ultraviolet (UV) method three times.

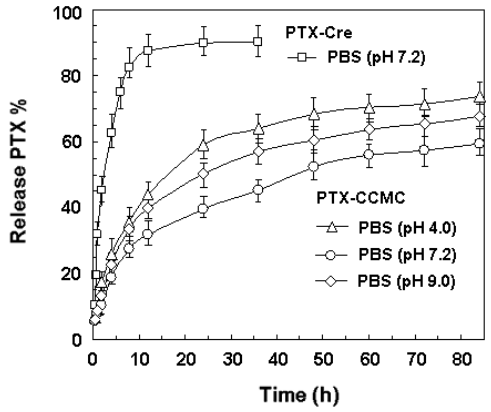

Figure 4. PTX release profiles from PTX preparations at 37 ± 0.2 °C in PBS solutions. PTX-Cre: PTX in the solution of Cremophor EL/dehydrated alcohol; PTX-CCMC: PTX-loaded CCMC-6.9 self-assembled nanoparticles.

3.2. In vitro drug release study

PTX release behavior from CCMC-6.9 self-assembled nanoparticles was studied *in vitro* by the dynamic dialysis method proposed by Han [26] in PBS solution with the pH values of 4.0, 7.2 and 9.0, respectively. PTX release from the solution of Cremophor EL/dehydrated alcohol (50/50 v/v) was also investigated as the control. The maximum concentrations ($\leqslant$4.0 μg ml^{-1}) of PTX in PBS solutions in the release experiments were less than the solubility (6–10 μg ml^{-1}) of PTX in these release media, so that the sink condition for PTX could be assured. The cumulative PTX release profiles are shown in figure 4. PTX rapidly released from the solution of Cremophor EL/dehydrated alcohol in PBS solution (pH = 7.2), and about 87.5% PTX was released in 12 h. However, during the same time period, only 32.0% PTX released from CCMC-6.9 self-assembled nanoparticles in PBS solution (pH = 7.2), and approximately 59.3% PTX was released during 84 h, which suggested that CCMC-6.9 self-assembled nanoparticles had the potential as a sustained-release carrier for PTX.

Furthermore, it is also shown from figure 4 that PTX released from CCMC-6.9 self-assembled nanoparticles related to the pH value of the release media. The PTX release rate from CCMC-6.9 self-assembled nanoparticles in PBS solution (pH = 7.2) was slightly slower than that in PBS solution (pH = 4.0 or 9.0). We believed this was because CCMC-6.9 was a novel ampholytic polyelectrolyte due to the presence of carboxyl and amino groups in its molecule, and its isoelectric point (pI) determined by the turbidimetry method [28] was about 7.0. Therefore, when the pH value of the PBS solution was 4.0 or 9.0, CCMC-6.9 self-assembled nanoparticles absorbed water to swell due to the ionization of amino groups or carboxyl groups, which resulting in the increase of permeability of their non-covalently cross-linked hydrogel structures, so that the PTX release rate increased accordingly. The similar drug release mechanism from other pH-sensitive hydrogel systems has already been reported [11, 29, 30].

3.3. Tissue biodistribution study

Tissue biodistribution of PTX after intravenous administration of CCMC-6.9 self-assembled nanoparticles to mice was investigated with PTX-Cre as control. Mice showed no obvious toxic reaction to administration of any formulation. PTX concentration–time profiles in different tissues, including plasma, heart, liver, spleen, lung and kidney, are shown in figure 5. It was observed from the plasma PTX concentration–time profile (figure 5(a)) that the time of distribution phase was short and the concentration decreased quickly in this phase, such as a 95% decrease of PTX concentration during 2 h. Compared with PTX-Cre, a 97% decrease of PTX concentration in mice plasma during 2 h, there was a slightly decreased clearance of PTX, but CCMC-6.9 self-assembled nanoparticles did not exhibit obvious long-circulating properties *in vivo*, which was different from other polymeric micelle formulations previously reported [3, 4, 31–33]. This may be due to the structural difference between CCMC-6.9 self-assembled nanoparticles and other polymeric micelles. As shown in figure 6(a), the amphiphilic block copolymer can form micelles with a core–shell structure by self-assembly in aqueous media. The hydrophilic shell of the micelle consists of flexible and long-chain-length hydrophilic blocks, so that there is a big space hindrance for the opsonin in the blood to adhere onto the micelle surface, and thereby decrease the recognition

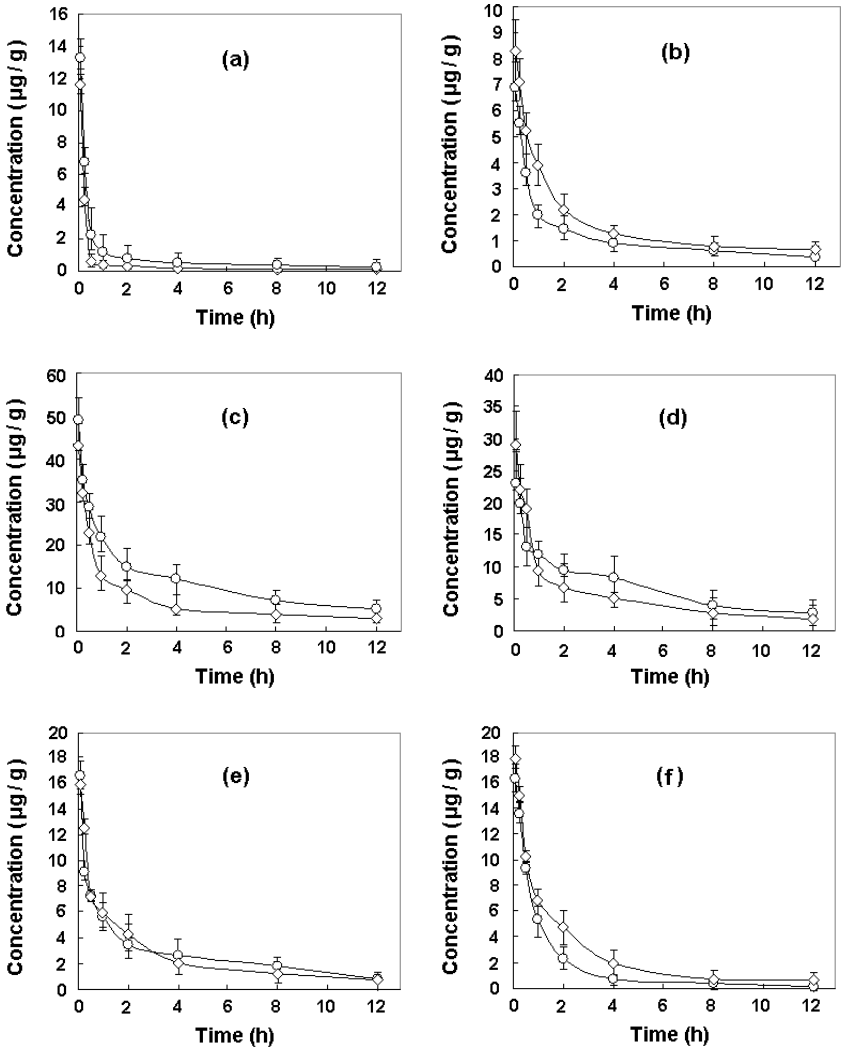

Figure 5. PTX concentration–time profiles of PTX-loaded CCMC-6.9 self-assembled nanoparticles (–○–) and PTX in a solution of Cremophor EL/dehydrated alcohol (–◇–) in mice plasma (a) and tissues of the heart (b), liver (c), spleen (d), lung (e) and kidney (f) (each point represents the mean ± SD of 6 mice).

and subsequent phagocytosis by the mononuclear phagocytes, which resulted in a long circulation time of micelles in blood. However, a CCMC self-aggregated nanoparticle has a poly-core model structure (figure 6(b)) which has been reported before [11, 18]. The hydrophobic micro-domains are formed by the association of cholesterol moieties and the backbones of the *O*-carboxymethyl chitosan coil to form the hydrophilic shells outside these hydrophobic micro-domains; thus a minimal energy state is attained in aqueous media. Furthermore, inter- and/or intramolecular hydrogen bonds between polar groups of sugar monomers will stabilize the poly-core structure of CCMC self-aggregated nanoparticles. Thus, the hydrophilic backbones of *O*-carboxymethyl chitosan tightly packed on the nanoparticle surface, so that there is little space hindrance for the adherence of opsonin, and for the recognition and phagocytosis by the mononuclear phagocytes, which resulted in the rapid decrease of PTX concentration in the plasma.

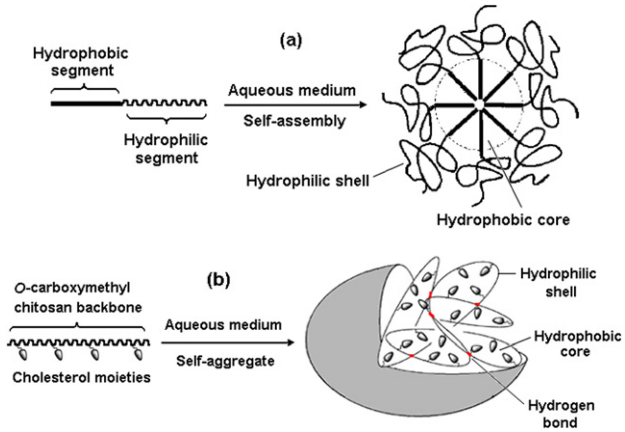

Figure 6. Schematic representations of self-assembly micelle of amphiphilic block copolymer (a) and CCMC self-aggregated nanoparticle (b) [18].

The AUC of two PTX preparations in different tissues were calculated by the trapezoid method. As shown in figure 7, the order of AUC from highest to lowest for PTX-loaded CCMC-6.9 self-assembled nanoparticles was

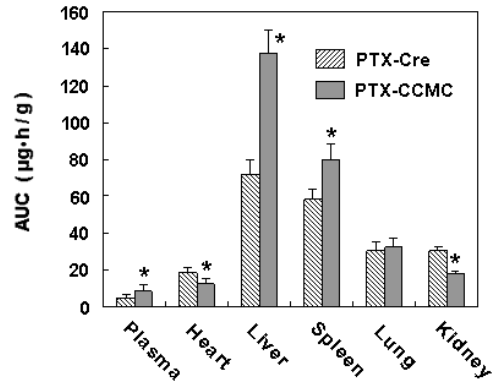

Figure 7. Comparative tissue distribution (AUC, 0–12 h) of PTX formulations. PTX-Cre: PTX in a solution of Cremophor EL/dehydrated alcohol; PTX-CCMC: PTX-loaded CCMC-6.9 self-assembled nanoparticles. Data represent the mean ± SD; paired *t*-test, * $P < 0.05$.

liver > spleen > lung > kidney > heart > plasma, similar to the corresponding order for the control. However, the PTX AUC of CCMC-6.9 nanoparticles was significantly higher in plasma, liver and spleen, but lower in heart and kidney, compared to the control. We believed that the higher PTX AUC in plasma than the control in tissue biodistribution was due to the solubilizing effect of CCMC-6.9 self-assembled nanoparticles on PTX. The PTX AUC of CCMC-6.9 self-assembled nanoparticles in liver was 137.9 μg h g^{-1}, a 91% increase compared to the PTX AUC of control (71.9 μg h g^{-1}), and more than 10% PTX remained in the liver after 12 h, which indicated that CCMC-6.9 self-assembled nanoparticles showed a high targeting efficiency and a long retention time in liver. This result was consistent with the study reported by Huo [25]. Moreover, it is well known that the adverse side effects of PTX in clinical practice mainly include myelosuppression, cardiotoxicity, renal toxicity and some allergic reactions, and therefore the lower PTX AUC in heart and kidney than the control in tissue biodistribution will be advantageous to reduce the toxicity of PTX.

4. Conclusions

In this study, CCMC-6.9 self-assembled nanoparticles were prepared and their potential for being used as the carrier for PTX was systemically investigated *in vitro* and *in vivo*. CCMC-6.9 self-assembled nanoparticles efficiently loaded PTX and significantly enhanced PTX solubility in water. The release *in vitro* of PTX from CCMC-6.9 self-assembled nanoparticles was sensitive to the pH of the release media, and the release rate was slower than that of PTX in the solution of Cremophor EL/dehydrated alcohol (PTX-Cre). The tissue distribution of PTX entrapped in CCMC-6.9 self-aggregated nanoparticles after intravenous injection into mice changed compared to PTX-Cre. The uptake of PTX significantly increased in plasma and liver, but decreased in heart and kidney. It is concluded that CCMC-6.9 self-assembled nanoparticles can effectively solubilize PTX and modify the biodistribution of PTX, which is advantageous to enhance the therapeutic index and reduce the toxicity of PTX. A further study on the anti-tumor effects of PTX-loaded CCMC-6.9 self-assembled nanoparticles is in progress now.

Acknowledgments

This work was supported by the Major State Basic Research Program of China (no. 2006 CB 933300), the Doctoral Fund of Ministry of Education of China (no. 96002323) and the Natural Science Foundation of Tianjin Medical University (no. 2007 ky 08).

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