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Potential of amphiphilically modified low molecular weight chitosan as a novel carrier for hydrophobic anticancer drug: Synthesis, characterization, micellization and cytotoxicity evaluation

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ABSTRACT

Amphiphilically modified low molecular weight chitosan (LMWC) with long chain alkyl groups as hydrophobic moieties and carboxymethyl groups as hydrophilic moieties (*N*-octyl-*N*,*O*-carboxymethyl LMWC, OC-LMWC) was synthesized. Self-assembled polymeric micelles of OC-LMWC were prepared in aqueous environment. Critical micelle concentrations (CMC) of OC-LMWCs were varied from 8.7 to 27.7 mg/l. Paclitaxel (PTX) was successfully encapsulated into the hydrophobic cores of the nanoparticles. The drug loading content and entrapment efficiency were higher to 32.17% (w/w) and 80.61%, respectively. Differential scanning calorimetry (DSC), transmission electron microscope (TEM) observation and dynamic light scattering (DLS) measurements were carried out to determination the physicochemical properties of the micelles. MTT assay showed that the in vitro cytotoxic effect of the PTX-loaded micelles was comparable to that of the commercial formulation, but the blank micelles were far less than the Cremophor EL® vehicle. These results suggested that OC-LMWC micelles were promising carriers for hydrophobic anticancer agents.

1. Introduction

Over the past decades, polymeric micelles formed by self-assembly of amphiphilic block copolymers in aqueous solution have received widespread attention (Harish Prashanth & Tharanathan, 2007; Min et al., 2008; Rosler, Vandermeulen, & Klok, 2001). Polymeric micelles are composed of a hydrophobic core which serves as a reservoir for hydrophobic drugs and a hydrophilic shell which affects their pharmacokinetic behavior (Hyung Park et al., 2006). Hydrophobic drugs can be physically entrapped in the core of micelles and transported at concentrations that highly exceed their intrinsic water solubility. The hydrophilic blocks can form hydrogen bonds with the aqueous surroundings and form a tight shell around the micellar core. Most of polymeric micelles were prepared with synthetic materials. Recently, naturally derived biodegradable materials have attracted more attention for polymeric micelles due to their unique characteristics.

Chitin (poly *N*-acetyl-D-glucosamine) is the most abundant natural amino polysaccharide on Earth. Chitosan, the product of *N*-deacetylation of chitin, is attracting increasing attention in drug delivery systems due to its excellent biocompatibility, biodegradability, nontoxicity and low immunogenicity (Chen et al., 2006). However, the high molecular weight and thus high viscosity and low water solubility at neutral pH significantly limit its application (Harish Prashanth & Tharanathan, 2007; Kittur, Vishu Kumar, & Tharanathan, 2003). To date, the molecular weight of chitosan previously used for polymeric micelles drug delivery system was usually higher than 50 kDa, its diluted acid solutions were too viscous. This high viscosity may make it more difficult to prepare a good drug carrier for i.v. administration.

Fortunately, these drawbacks can be partially circumvented by using low molecular weight chitosan (LMWC) (Ercelen et al., 2006; Zhang et al., 2008b), which is water soluble in a wide pH range and thus be more easily to conduct a chemical modification. Moreover, LMWC showed superior biological activities compared to chitosan, including strong bactericidal activity, hypolipidemic and hypocholesterolemic effect (Tharanathan & Kittur, 2003; Vishu Kumar, Varadaraj, Lalitha, & Tharanathan, 2004), among these characteristics, the most attractive feature of LMWC is that they were shown to stimulate murine peritoneal macrophages and kill the tumor cells (Maeda & Kimura, 2004; Seo et al., 2000).

Paclitaxel (PTX) is one of the best anti-tumor drugs found in the past decades. It has been widely used in the treatment of various cancers. Because of it's extreme insolubility in water and most pharmaceutical solvents, Cremophor EL has to be used in the commercial Taxol® formulation (Cremophor EL:ethanol, 50:50). However, the use of Cremophor EL causes serious side effects such as nephrotoxicity, neurotoxicity and cardiotoxicity. These serious side

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effects have limited the clinical application of Taxol® and caused lots of inconvenience when it was clinical applied. Therefore, it's emergent to find a drug delivery system for paclitaxel without Cremophor EL. In recent years, several drug delivery systems, such as polymeric micelles (Wang, Li, Wang, Wu, & Fang, 2008; Xiangyang et al., 2007; Zhang, Ping, Zhang, & Shen, 2003; Zhang et al., 2008a), liposomes (Tong, Zhou, & Tan, 2006), parenteral emulsion (Sznitowska, Klunder, & Placzek, 2008) and amphiphilic nanoparticles (Bilensoy, Gurkaynak, Dogan, & Hincal, 2008), have been studied to increase solubility of paclitaxel and to minimize the side effects of the formulation, among these new delivery vehicles, polymeric micelles have attracted an increasing interest.

The purposes of present work were (1) to synthesize *N*-octyl-*N*,*O*-carboxymethyl LMWC (OC-LMWC) by two-step modification of LMWC with carboxymethyl and subsequent octyl group, (2) to prepare self-assembled polymeric micelles of OC-LMWC in aqueous medium and to characterize the physicochemical properties of the micelles, (3) to encapsulate the hydrophobic anticancer drug paclitaxel into the hydrophobic cores of the micelles, (4) to evaluate the cytotoxicity of both blank micelles and PTX-loaded micelles against HepG2 cells and to show the potential of OC-LMWC as a novel carrier for anticancer drug.

2. Materials and methods

2.1. Materials

Low molecular weight chitosan (Mw = 5 kDa, 10 kDa, 20 kDa) were purchased from Golden-Shell Biochemical Co., Ltd. Chloroacetic acid was obtained from Yancheng Kunye Chemical Co., Ltd. Octanal was purchase from Nanjing Skyrun Golden Harvest Perfume Manu Co., Ltd. Sodium borohydride was purchased from Sinopharm Chemical Reagent Co., Ltd. Pyrene was supplied by Fluka (Switzerland), Paclitaxel was from Chongqing Meilian Pharm Co., Ltd. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Sigma-Aldrich. Cremophor EL was a kind gift from BASF Corp (Ludwigshafen, Germany). All other chemicals and solvents were of analytical grade and were used without further purification.

2.2. Synthesis of OC-LMWC

Synthesis of OC-LMWC was shown in Fig. 1, low molecular weight *N*,*O*-carboxymethyl chitosan (LMW-CMC) was used as a starting precursor, which was prepared using a previously reported method with a little modification (Liu, Chen, Lin, & Liu, 2006). In brief, 2 g of chitosan was dissolved in 15 ml of 2% HAc solution and then 15 ml of isopropanol was added at room temperature while being stirred for 30 min. NaOH (8 g) was added and the mixture was stirred at 40 °C for 30 min with subsequently addition of 12 g chloroacetic acid dissolved in isopropanol. The supernate was discarded and 40 ml of distilled water was added after 2 h of reaction, the solution was adjusted to pH 7.0 with HAc and then dispersed in 500 ml of methanol followed by filtration, washing by methanol solution, and drying. LMW-CMC was obtained by dissolving the filter cake in 20 ml of distilled water and lyophilization. The obtained LMW-CMC derivated from different molecular weight chitosans were named as LMW-CMC-5K, LMW-CMC-10K, LMW-CMC-20K, respectively.

OC-LMWC was synthesized by conjugating of alkyl chains to LMW-CMC. Briefly, 1 g of LMW-CMC was dissolved in 10 ml of distilled water, 10 ml of methanol was added and stirred for 30 min followed by addition of 1.02 g octanal and 0.3 g of $NaBH_4$ dissolved in distilled water, the mixture was stirred at room temperature for 12 h and adjusted to pH 7.0 with 5 M HCl solution, then the mixture was dispersed in 500 ml of methanol followed by filtration, washing by methanol/water (80:20 and 90:10, v/v), methanol, hexane, acetone and drying in vacuum. The obtained products were named OC-LMWC-5K, OC-LMWC-10K, OC-LMWC-20K, respectively.

2.3. Characterization of OC-LMWC

FT-IR spectra were recorded with KBr pellets on a Nicolet Impact 410 spectrometer (Nicolet Analytical Instruments, Madison, WI). ^1H and ^{13}C NMR spectra were recorded with a Bruker Avance (AV-500, Biospin, Germany) spectrometer operating at 500 MHz. Chitosan was dissolved in the mixture of D_2O and DCl. OC-LMWC was dissolved in D_2O. Degree of substitution (DS), defined as the number of carboxymethyl groups or octyl groups per 100 glucosamine groups of chitosan, was determined by elemental analysis using a Vario EL III Element Analyzer.

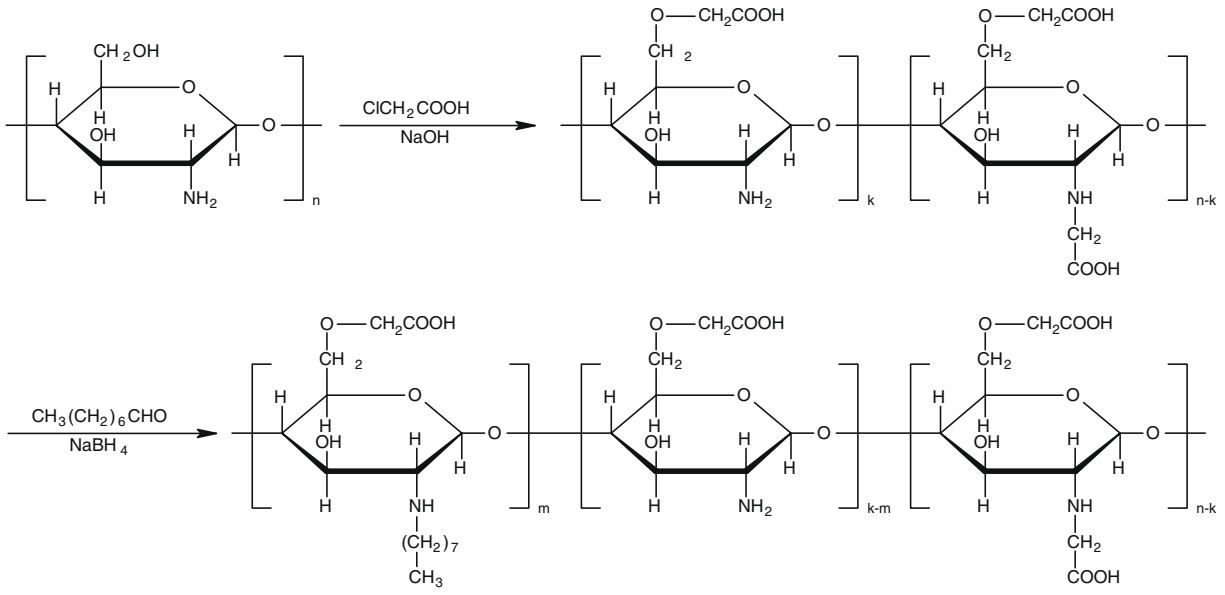

Fig. 1. Synthetic scheme of LMW-CMC and OC-LMWC.

2.4. Micellization of OC-LMWC and drug loading

2.4.1. Preparation of blank and PTX-loaded micelles

Both PTX-loaded and blank micelles were prepared by dialysis technique. Briefly, 17 mg of OC-LMWC was dissolved in distilled water at 50 °C and stirred for 30 min, the solution was added with or without 10 mg of PTX at the concentration of 30 mg/ml in alcohol followed by 15 min of stirring, the solution was ultrasonicated for 30 min (active every 1 s for a 3 s duration with the power of 200W) in ice-bath by a probe-type ultrasonicator (JY92-2D, Ningbo Scientz Biotechnology Co., Ltd., China). The resultant PTX-loaded micelles solution was dialysised (MWCO = 1 kDa) against 2000 ml of distilled water over night at room temperature and diluted to 10 ml with distilled water flowed by a centrifugation at 3000 rpm for 10 min, and then filtered by 0.8 μm microfiltration membrane, the blank micelles solution was directly filtered without further dialysis.

2.4.2. Determination of drug loading capability

Drug loading capability of the OC-LMWC micelles was determined using high-performance liquid chromatography (HPLC). PTX-loaded micelles solution (50 μl) was transferred into 10 ml volumetric flask and diluted with methanol to the mark. The solution was centrifuged at 10,000 rpm for 10 min, 20 μl of supernatant was injected into the chromatographic system.

The HPLC system (LC-2010C, Shimadzu, Japan) was equipped with a Lichrospher C18 column (4.6 × 250 mm, 5 μm) with a mobile phase of methanol and water (75:25), the flow rate and column temperature were set at 1 ml/min and 30 °C, respectively. The signals were recorded by UV detector at 227 nm. A calibration line was conducted to determination PTX concentration in the range of 0.5–25 mg/l, and the r^2-value of peak area against PTX concentration was at least 0.999. The encapsulation efficiency (EE, %) and the drug loading content (DL, wt.%) were calculated based on the following formulations:

$$\mathrm{EE}(\%) = \frac{\text{mass of PTX extracted from micelles}}{\text{mass of PTX added in}} \times 100$$

$$\mathrm{DL}(\mathrm{wt}\%) = \frac{\text{mass of PTX extracted from micelles}}{\text{mass of PTX extracted from micelles} + \text{mass of OC} - \text{LMWC added in}} \times 100$$

2.5. Determination of critical micelle concentration (CMC)

Fluorescence technique was employed to determinate the CMC of OC-LMWC micelles using pyrene as a hydrophobic fluorescence probe. Briefly, 1 ml of pyrene solution (6×10^{-6} M) in acetone were transferred into a 10 ml volumetric flask and then remove acetone from the solution by a flow of nitrogen gas. A series of concentration of OC-LMWC micelles solutions were added into the volumetric flasks containing acetone-free pyrene and diluted with distilled water to the mark to obtain the final pyrene and micelles concentrations of 6×10^{-7} M and $1 \times 10^{-4} \sim 2$ mg/ml, respectively. The solutions were sonicated for 30 min at 100W followed by incubation at 40 °C for 1 h and kept from light overnight at room temperature. Fluorescence spectra were recorded with a RF-5301 PC fluorescence spectrophotometer (Shimadzu, Japan) with the emission wavelength at 390 nm. The peak height intensity ratio (I_3/I_1) of third peak (I_3 at 338 nm) to the first peak (I_1 at 333 nm) against the logarithm of micelles concentration was plotted. CMC values of micelles were calculated at the intersection of two straight lines, one of which was fitted line of the part at low micelles concentrations and the other was the fitted line on the rapid rising part of the curve.

2.6. Dynamic light scattering and zeta potential measurement

The particle size and zeta potential were measured using a Malvern Zetasizer Nano-ZS90 (Malvern instruments, UK). All of the DLS measurements were performed at 25 °C and at a scattering angle 90°. The zeta potential values were calculated using the Smoluchowski equation which was automatic integrated with the instrument.

2.7. Morphology observation by TEM

The morphologies and size distributions were observed by TEM using a H-7000 (Hitachi, Japan) electron microscope operating at an accelerating voltage of 75 kV. Samples' negative staining was performed as follows: a drop of sample solution was placed onto a copper grid coated with carbon, the sample drop was taped with a filter paper to remove surface water and air-dried for 5 min followed by the application of 0.01% phosphotungstic acid to deposit the nanoparticles on the grid. The samples were air-dried before observation.

2.8. Differential scanning calorimetry (DSC) measurement

DSC experiments were performed using Netzsch 204 (Netzsch-Geraetebau GmbH, Germany) differential scanning calorimetry. Four samples including PTX, OC-LMWC-10K, the physical mixture of PTX and OC-LMWC-10K, PTX-loaded OC-LMWC-10K micelles were sealed on the nonhermetic aluminum pans and then scanned from 50 to 300 °C at a heating rate of 10 °C /min. The micelles solutions were lyophilized before the tests were carried out. Nitrogen was used as the purge gas at the flow rate of 20 ml/min.

2.9. Cell culture and cytotoxicity evaluation

2.9.1. Cell culture

HepG2 cells were obtained from American Type Culture Collection (ATCC) and were maintained in RPMI-1640 medium at 37 °C in a 5% CO_2 humidified environment. For the MTT assay, cells were plated on 96-well plates to confluence and were allowed to adhere overnight.

2.9.2. Cytotoxicity evaluation

In vitro cytotoxicity of PTX-loaded and PTX-free OC-LMWC micelles was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay with human hepatoma

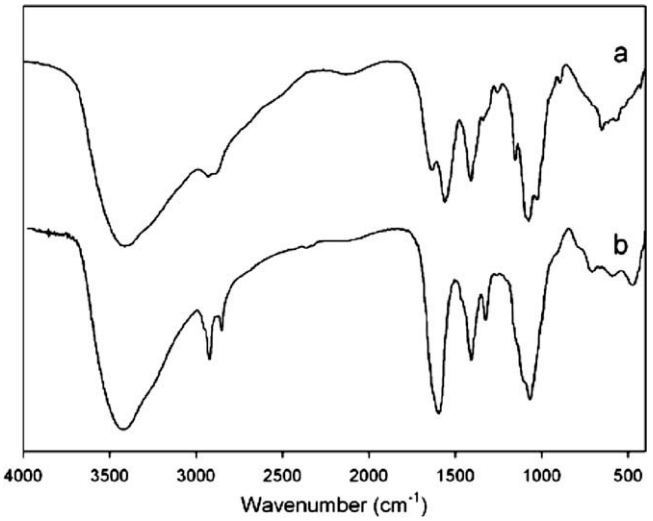

Fig. 2. FT-IR spectra of (a) LMWC and (b) OC-LMWC.

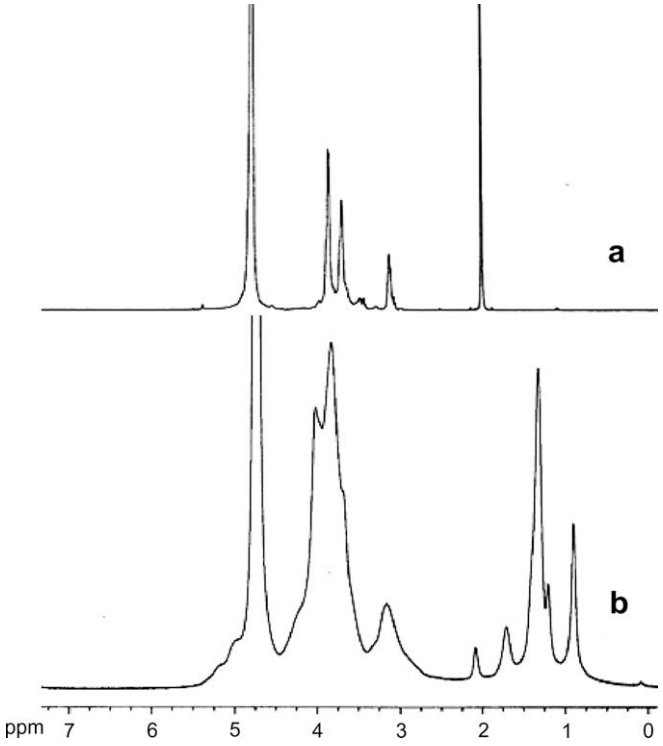

Fig. 3. ^{1}H NMR spectra of (a) LMWC in D_2O/DCl and (b) OC-LMWC in D_2O.

HepG2 cell lines (Diaz, Vargas, & Gatjens-Boniche, 2006). For comparison, commercial formulations (Taxol) with or without PTX were prepared in our laboratory according to previous report (Soga et al., 2005). Briefly, 12 mg of PTX was dissolved in 1.0 ml ethanol and 1.0 ml of Cremophor EL was added followed by a sonication for 30 min, formulation without PTX was prepared as the same procedure but without the addition of PTX. The solutions were further diluted with culture medium to obtain corresponding PTX concentration ranging from 0.0001 to 100 μg/ml. As control sample, a certain amount of PTX was dissolved with methanol and diluted with culture medium to give PTX concentration of 0.0001 to 1 μg/ml.

The cultured cells were incubated for 72 h in the presence of various concentrations of the above samples. Then 20 μl of MTT solution (5 mg/ml) was added followed by an incubation for 4 h and shaken at room temperature. MTT was solubilized with dimethylsulfoxide (DMSO), the optical density (OD) was measured at a test wavelength of 570 nm and a reference wavelength of 630 nm with a microplate reader (Multiskan MK3, Thermo Labsystems, USA), each sample and concentration was tested in triplicate, and six wells with only culture medium served as blank, the relative cell viability (%) was calculated as (OD of treated cells/OD of nontreated cells) × 100.

3. Results and discussion

3.1. Structural characterization of OC-LMWC

The structure of synthesized polymer was determined by FT-IR, ^{1}H and ^{13}C NMR. The degree of substitution (DS) of carboxymethyl groups and octyl groups was estimated by elemental analysis. FT-IR spectra of LMWC and OC-LMWC were shown in Fig. 2a and b, compared to FT-IR spectrum of LMWC, the stronger absorptions at 1601 and 1409 cm^{-1} in Fig. 2b could be respectively assigned to the asymmetry and symmetry stretch vibration of COO−. The very strong absorption at 1074 cm^{-1} shown in Fig. 2b was attributed to the ether bond in OC-LMWC which indicated that the hydrogen of 6-OH was substituted by carboxymethyl group. These results were consistent with previous report (Prabaharan, Reis, & Mano, 2007). Moreover, Fig. 2b showed new absorption bands at 2921 and 2851 cm^{-1} which can be assigned to the octyl group of OC-LMWC. All these differences indicated that both carboxymethyl groups and octyl groups were successfully conjugated to LMWC.

As shown in Fig. 3a, the peaks in the ^{1}H NMR spectrum of the D-glucosamine unit of LMWC were nearly identical to those in previous reports (Chen et al., 2004; Liu, Hsieh, Fan, Yang, & Chung, 2007; Pang, Chen, Park, Cha, & Kennedy, 2007), the peaks could be assigned as follows: 2.0 (–CH_3, acetyl group), 3.1 (2-H), 3.5–3.9 (3-H–6-H). Compared to Fig. 3a, Fig. 3b showed a serial

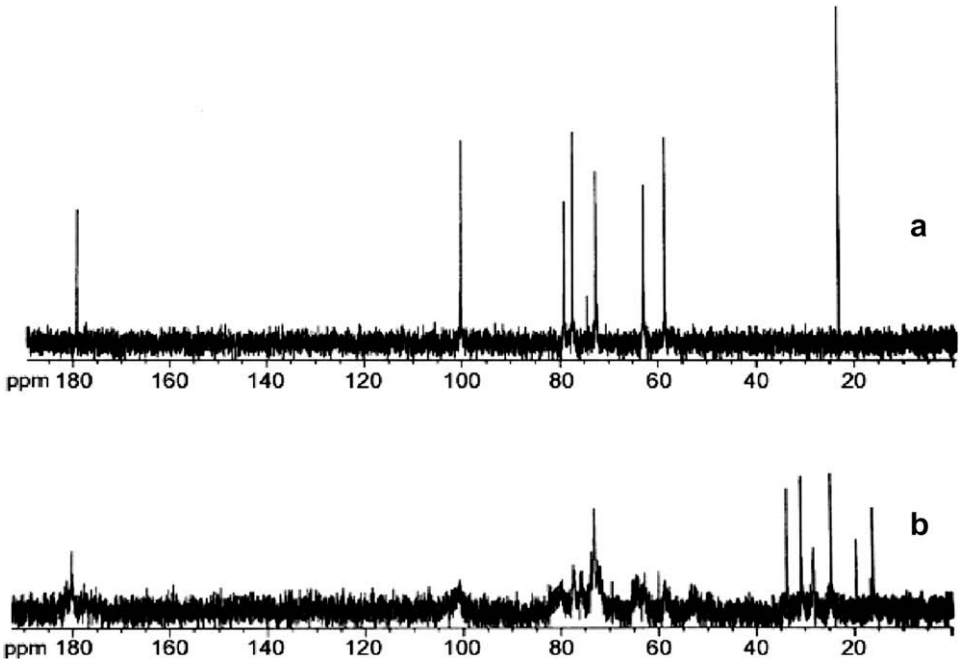

Fig. 4. ^{13}C NMR spectra of (a) LMWC in D_2O/DCl and (b) OC-LMWC in D_2O.

Table 1
Physicochemical properties of PTX-free and PTX-loaded nanoparticles.

Sample[a]	DS (%)[b]	DS (%)[c]	CMC (mg/l)	Size (nm)	PI	Zeta (mV)
OC-LMWC-5K	138.4	57.3	8.7	233	0.092	−45.7
OC-LMWC-10K	147.7	55.4	27.7	198	0.267	−50.5
OC-LMWC-20K	128.2	58.5	25.1	202	0.22	−44.1
	EE (%)	DL (wt.%)				
PTX-OC-LMWC-5K	80.61	32.17		158	0.151	−35.2
PTX-OC-LMWC-10K	99.18	36.85		134	0.14	−43.5
PTX-OC-LMWC-20K	97.77	36.50		147	0.176	−24.2

[a] OC-LMWC-xK indicates the molecular weight of raw material of chitosan is $x * 1000$.
[b] Degree of substitution of carboxymethyl groups.
[c] Degree of substitution of octyl groups.

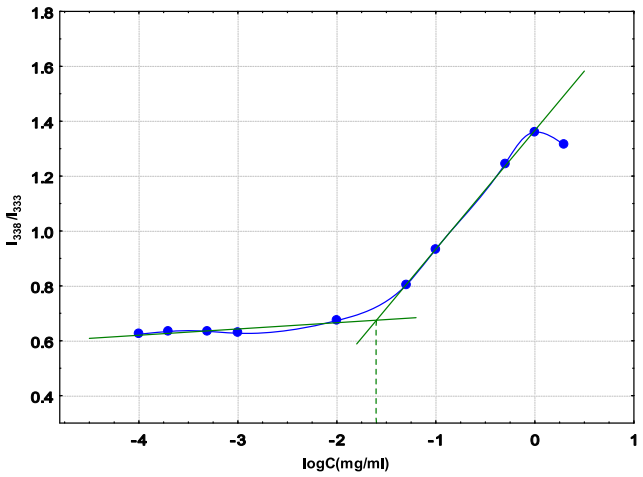

Fig. 5. Plot of the quotient of vibrational band intensities (I_{338}/I_{333}) from excitation of pyrene vs. log(C) of OC-LMWC-20K in distilled water.

of characteristic peaks of octyl group between 0.9 and 1.8 ppm of which peaks at 0.9 ppm and 1.2–1.6 ppm could be assigned to $-CH_3$ and $-CH_2-$ of octyl group, respectively. The very strong peak at 4.2 ppm was attributed to the carboxymethyl hydrogen protons ($-CH_2-COOD$). Fig. 4 showed the ^{13}C NMR spectra of LMWC and OC-LMWC. As reported in a previous work (Taboada, Cabrera, & Cardenas, 2004), the assignments and chemical shifts of chitosan were as follows: 100.1 (C1), 58.5 (C2), 72.6 (C3), 79.1 (C4), 77.4 (C5) and 62.8 (C6), peaks at 179.2 and 23.1 ppm were contributed to the carbonyl and methyl group of acetyl groups. Fig. 4b showed a serial of peaks at 16–35 ppm which could be confirm the attachment of octyl groups onto backbone of LMWC. In conclusion, all the data of FT-IR, ^1H NMR and ^{13}C NMR demonstrated the successful synthesis of OC-LMWC.

Degrees of substitution of carboxymethyl groups and octyl groups were calculated from the C/N ratios, which were obtained from the elemental analysis. All results were shown in Table 1, DS of carboxymethyl groups were higher than 100%, which indicated that carboxymethyl groups were conjugated at not only 2-NH_2, but 6-OH as well. DS of octyl groups were varied from 55.4% to

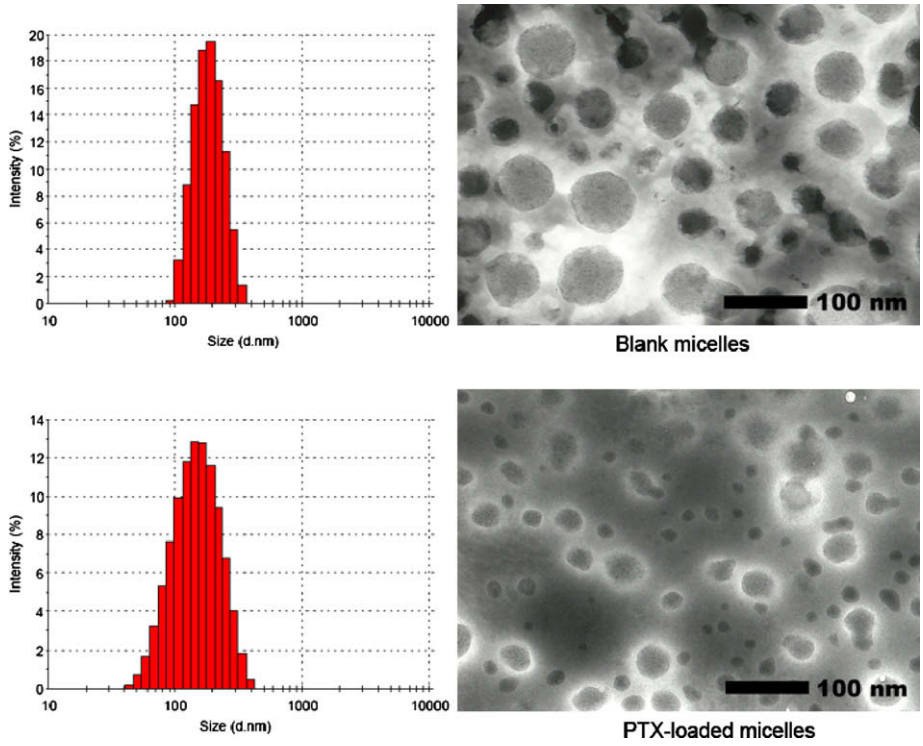

Fig. 6. Particle size distributions and transmission electron microscope (TEM) images of blank and PTX-loaded micelles of OC-LMWC-10K.

58.5% which were slightly influenced by the molecular weight of chitosan. As summarized in Table 1, DS of carboxymethyl groups increased with slight decreasing DS of octyl groups, this might be due to the simultaneously conjugation of carboxymethyl groups and octyl groups at 2-NH_2. In addition, DS of carboxymethyl groups was greatly higher than that of octyl groups, this is because carboxymethyl groups were conjugated at both 2-NH_2 and 6-OH while octyl groups were conjugated only at 2-NH_2 as shown in Fig. 1.

3.2. PTX loading into OC-LMWC micelles

PTX was incorporated into OC-LMWC micelles using dialysis method, the encapsulation efficiency (EE) and drug loading (DL) amount were measured by HPLC. The loading capabilities of OC-LMWC with different molecular weights were evaluated. As shown in Table 1, EE and DL of OC-LMWC-10K and OC-LMWC-20K were obviously higher than that of OC-LMWC-5K. Among these chitosan derivatives, OC-LMWC-10K showed the highest PTX loading ability, the DL was 36.85% and the EE was quite high at 99.18%. The results indicated a stable incorporation of PTX into the hydrophobic core of OC-LMWC micelles. Moreover, nearly 10 mg of PTX encapsulated in the lyophilized micelles was easily reconstituted in 5 ml of water providing a solubility of approximately 2 mg/ml of PTX in aqueous solution which is about 2000 times higher than its intrinsic water solubility (<1 μg/ml) (Carstens et al., 2008). The remarkably increasing of solubility indicates that OC-LMWC could be a promising carrier for hydrophobic drugs.

3.3. Critical micelle concentration determination

Polymeric micelles can be formed only when the concentration of polymer is higher than the CMC which plays an important role in the stability of the micelles system. Polymeric micelles are generally more stable than low molar mass surfactant micelles due to their remarkable lower CMC. In order to determinate the CMC of OC-LMWC, fluorescence measurements were carried out using pyrene as a probe. Fig. 5 shows a typical plot of I_{338}/I_{333} vs. log(C) of OC-LMWC, a substantial increase of the intensity ratio is observed when the concentration is higher than a certain value (CMC) which indicating the formation of the micelles. Therefore, the interception of two straight lines is estimated as CMC. As shown in Table 1, the CMC values of OC-LMWC with different molecular weights were ranging from 8.7 to 27.7 mg/l, the result indicated that the molecular weight of OC-LMWC was not significantly affect the CMC value. Moreover, the CMC value were significantly lower than some reported high molecular chitosan derivatives, such as glycol chitosan bearing 5β-cholanic acid with CMC of 47–219 mg/l (Kwon, Park, Chung, Kwon, & Jeong, 2003) and glycol chitosan bearing deoxycholic acid with CMC of 38–260 mg/l (Lee, Jo, Kwon, Kim, & Jeong, 1998). The results suggested that the micelles form from OC-LMWC can remain stable in solution even after extreme dilution and preserve stability without dissociation after intravenous injected into the much larger volume of blood for systemic circulation.

3.4. Characterization of OC-LMWC micelles

Particle size and zeta potential of PTX-free and PTX-loaded micelles were measured by DLS method. As shown in Table 1, all the blank micelles showed similar average diameters and zeta potentials, ranging from 198 to 233 nm and −44.1 to −50.5 mV, respectively. Fig. 6 showed the size distribution profile of OC-LMWC-10K micelles before and after loading of PTX. After the PTX loading in micelles, their sizes were slightly decreased, meanwhile, the size distribution was a little wider, and the zeta potentials were decreased from −44.1 to −50.5 mV to −24.2 to −43.5 mV. Zeta potential or particle surface charge is an important measure of the stability of micelles system, relatively high surface charge could provide a repelling force between the particles, thus increase the stability of the solution. As shown in Table 1, all the micelles presented relative high negative zeta potentials, it's safe to conclude that the charged particles could repel each other and prevent aggregation or precipitation and show a good stability. In addition, no significant influence of molecular weight of chitosan on the particle size and zeta potential was observed. TEM observations were presented in Fig. 6, both blank and PTX-loaded micelles exhibited regular spherical shape, but the size estimated from the TEM was smaller than that from DLS due to the dehydration and shrinkage during drying process (Yang, Zhao, Wei, El Ghzaoui, & Li, 2007). It's interesting to observe a decrease of particle size after PTX was loaded, which could be attributed to the formation of more tight hydrophobic cores after the encapsulation of hydrophobic PTX into the micelles.

3.5. Differential scanning calorimetry analysis

To confirm the existence form of PTX in the polymeric micelles, DSC analysis was carried out for PTX, blank micelles, PTX-loaded

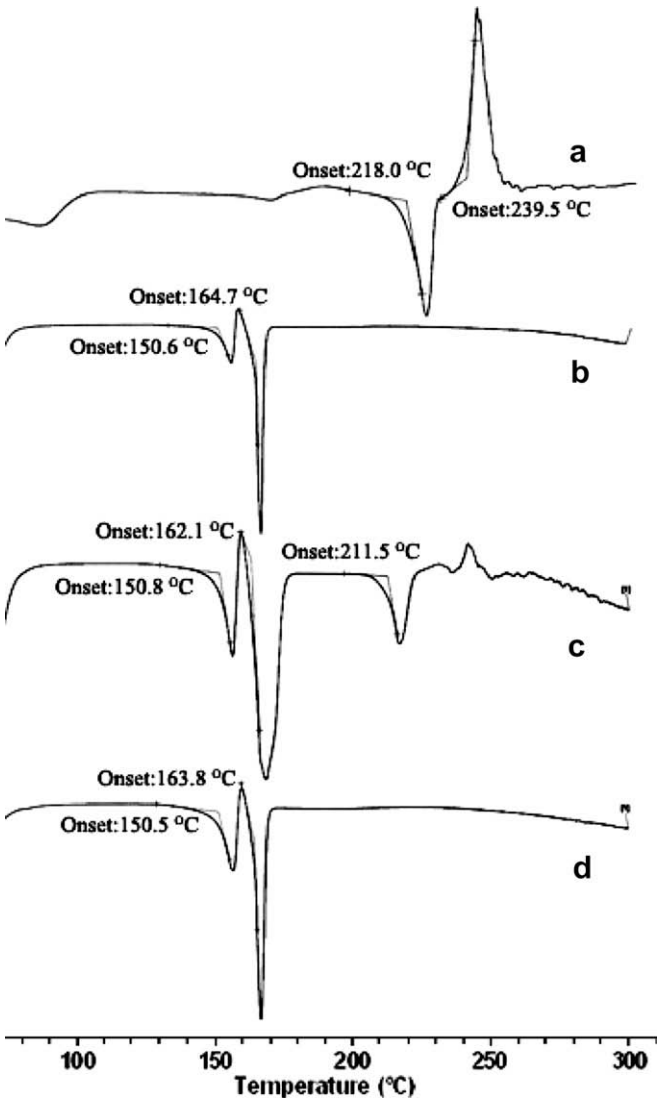

Fig. 7. DSC thermograms of: (a) PTX; (b) OC-LMWC-10K; (c) physical mixture of OC-LMWC-10K and PTX (63:37 w/w); (d) PTX-OC-LMWC-10K (36.85 wt.% drug loading).

micelles and the physical mixture of blank micelles and PTX. As shown in Fig. 7, calorimetric curves of PTX exhibited a melting peak at 218.0 °C and a decomposition peak at 239.5 °C, blank micelles presented a sharp endothermal peak at 164.7 °C. The physical mixture of them showed all characteristic peaks of each component only with slightly shift. The PTX-loaded micelles exhibited a similar calorimetric curve to the blank micelles, and no obvious melting peak of PTX appeared in the curve of PTX-loaded micelles which suggested that PTX presented a solid dispersion state in the lyophilized OC-LMWC micelles system.

3.6. In vitro cytotoxicity

Cytotoxicity of PTX-loaded micelles against the human hepatoma HepG2 cells was evaluated using MTT method. For comparison, the cytotoxicity of blank micelles, Taxol® and its vehicle (Cremophor EL:ethanol, 50:50), further abbreviated as Taxol vehicle) and free PTX were evaluated. As shown in Fig. 8a, blank micelles did not show any toxicity even at the highest test concentration, while a strong cytotoxicity was observed when the cells were incubated in the presence of a high concentration of Taxol vehicle. Therefore, it can be expected that OC-LMWC micelles are far less toxic than Cremophor EL vehicle.

As shown in Fig. 8b, PTX-loaded micelles showed comparable cytotoxic activity as Taxol in the presence of various concentrations of PTX, indicating that PTX remains its biologically activity after incorporation into OC-LMWC micelles. Taxol showed a strong cytotoxicity at 100 and 10 μg/ml, which could be partially attributed to the using of Cremophor EL vehicle. In contrast, the cytotoxicity of PTX-loaded micelles was more likely induced by PTX itself. Cytotoxicity of free PTX at the concentration of 10 and 100 μg/ml was not tested because of low solubility of PTX in cell culture medium. It should be mentioned that PTX-loaded micelles exhibited a higher cytotoxic activity than free PTX at the concentrations of 1 and 0.1 μg/ml, this might be attributed to the enhancement of fluidity of the cell membrane induced by OC-LMWC polymer, as a re-

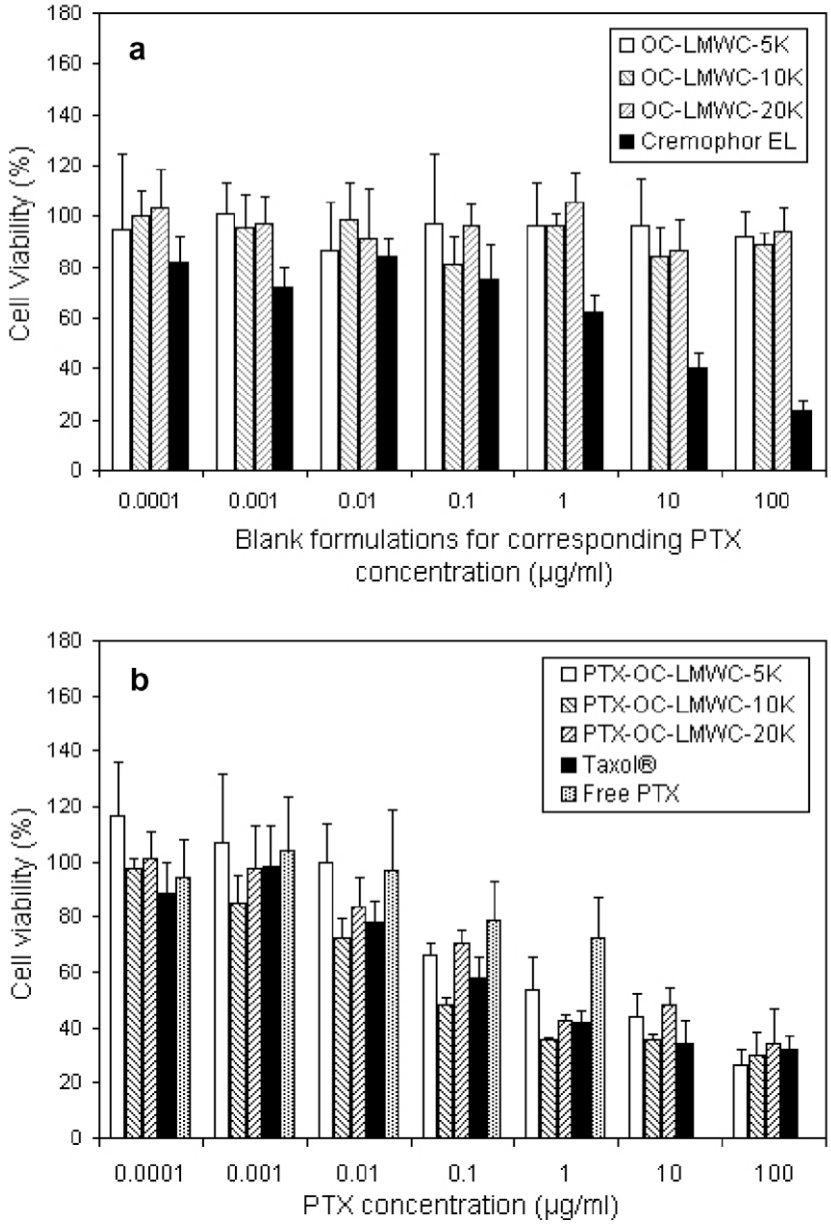

Fig. 8. (a) Cell viability in the presence of blank formulations for corresponding PTX concentration varies form 0.0001 to 100 μg/ml; (b) Cell viability in the presence of PTX-loaded OC-LMWC formulations, Taxol and free PTX.

sult, more drug could be easily taken up by the cells. From these results of cytotoxicity test, we can conclude that PTX-loaded polymeric micelles formulation was equipotent to the commercial PTX formulation, meanwhile, OC-LMWC was less cytotoxic than Taxol vehicle in the absence of drug.

4. Conclusions

In this study, a novel amphiphilic derivative of low molecular weight chitosan was successfully synthesized. The amphiphilic derivative of LMWC, named OC-LMWC with a very low level of CMC at about 25.1 mg/l, could self-assemble in aqueous environment to form nano-vehicles for hydrophobic drugs. Paclitaxel was stably encapsulated into the core of OC-LMWC micelles using member dialysis method. The drug loading content and entrapment efficiency were 32.17% (w/w) and 80.61% respectively. TEM and DLS studies showed that both blank and PTX-loaded micelles were of spherical shape with high negative zeta potentials of about −44.1 and −24.2 mV, respectively. The particle sizes of blank micelles were about 202 nm and their size distribution showed a narrow pattern. After encapsulation with PTX, the particle sizes were slightly decreased. The in vitro cytotoxic effect of the PTX-loaded micelles was comparable to that of the commercial formulation Taxol®, but the blank micelles were far less than the Cremophor EL vehicle. No significant differences were observed between the OC-LMWCs based on LMWCs with different molecule weights. The result of this research showed the potential of OC-LMWC as an alternative and promising carrier for PTX and other similar hydrophobic anticancer chemicals.

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