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Novel pH-sensitive chitosan-derived micelles loaded with paclitaxel

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ABSTRACT

A series of pH-sensitive graft copolymers, *N*-octyl-*N*-(2-carboxyl-cyclohexamethenyl) chitosan derivatives were synthesized and characterized by FTIR, ^{1}H NMR and elemental analysis, and their physical properties were measured with differential scanning calorimetry and X-ray diffraction spectrometry. The critical micelle concentrations (CMCs) of the modified chitosan determined by using pyrene as a hydrophobic probe in fluorescence spectroscopy were from 11 to 72 μg/ml. The graft polymers can form micelles solubilizing paclitaxel, with drug-loading rate ranging from 30.47% to 48.10% and entrapment efficiency from 42.22% to 59.24%. Cytotoxicities of carrier against tumor cells estimated that carriers were nearly non-cytotoxic. Additionally, the results of pH-sensitivity and drug release experiments showed that the micelles were highly sensitive to mild acidic conditions (pH 5.5) while reasonably stable at physiological conditions (pH 7.4). Therefore, chitosan-derived micelle may be a potential anti-tumor drug delivery system for chemotherapy of cancer.

1. Introduction

The selective control of drug concentration and distribution within the tumor microenvironment is one of the most important factors for achieving effective and safe cancer chemotherapy (Bae, Diezi, Zhao, & Kwon, 2007; Bae & Kataoka, 2009; Jain, 2001). Polymeric micelles from amphiphilic graft or block copolymers were recognized as one of the most promising anti-tumor drug carriers, characterized by excellent biocompatibility, high drug-loading content, and markedly improved bio-distribution (Nishiyama & Kataoka, 2006). They can increase the solubility of many poorly water-soluble anti-cancer drugs successfully (Kwon, 2003; Torchilin, 2004). In particular, the nano-scaled polymeric micelles exhibit tumor accumulation by enhanced permeability and retention (EPR) effect (Torchilin, 2001).

A number of natural or synthetic polymers have been used to form polymeric micelles. Among them, chitosan is the most attractive candidate due to its biochemical activity, biocompatibility, biodegradability and low toxicity, and it has been widely used in the pharmaceutical field (Kumar, Muzzarelli, Muzzarelli, Sashiwa, & Domb, 2004). Grafting the hydrophobic and hydrophilic segments to the chitosan backbone would give rise to amphiphilic graft copolymers, which can form self-assembled micelles in water. Chitosan-derived micelles were studied in our group (Chen, Ding, Qu, & Zhang, 2008; Qu, Yao, Zhang, Wu, & Ping, 2009; Qu, Zhu, Zhang, & Ping, 2009; Yao, Zhang, Ping, & Yu, 2007; Zhang et al., 2008). Especially, *N*-octyl-*O*-sulfate chitosan (NOSC) micelle was reported to be a safe and effective carrier for delivering paclitaxel (Zhang et al., 2008). However, the problem of polymeric micelle without adequate drug release upon micelle accumulation in the tumor tissues is usually revealed by clinical trials.

In order to improve the therapeutic efficiency and reduce side-effect of anti-cancer drugs, many scientists began to develop 'smart' drug delivery systems. Acidic pH is known to be a prominent microenvironment (Rapoport, 2007) in solid tumors, interstitial fluid in tumors has a lower pH than that in normal tissues (6.75 vs. 7.23) (Lee et al., 2008; Rapoport, 2007). This phenomenon has been employed in the design of numerous pH-sensitive polymeric micelles for the delivery of anti-cancer drugs to tumors (Kale & Torchilin, 2007; Lee et al., 2008; Lee, Na, & Bae, 2003). In addition, the micelles end up in the acidic compartment of endosomes and lysosomes (pH 5.0–5.5) after endocytosis, so pH-sensitive micelles may overcome the intracellular barriers created by endosomal or lysosomal membranes. Thus the arrival of the drug to its target is attributed to the degradation of pH-sensitive micelles releasing the encapsulated drug. Polymeric micelles formed by poly(ethylene glycol)–poly(aspartate hydrazide adriamycin) have been reported as an effective drug delivery nanocarrier (Bae et al., 2007). Its response to intracellular acidic compartments, such as endosomes and lysosomes, resulted in the selective release of anti-cancer drug at low pH (<6). Hruby, Konak, and Ulbrich (2005) presented a novel pH-sensitive micelle drug delivery system based on hydrazone-bound doxorubicin. But pH-sensitive micelle

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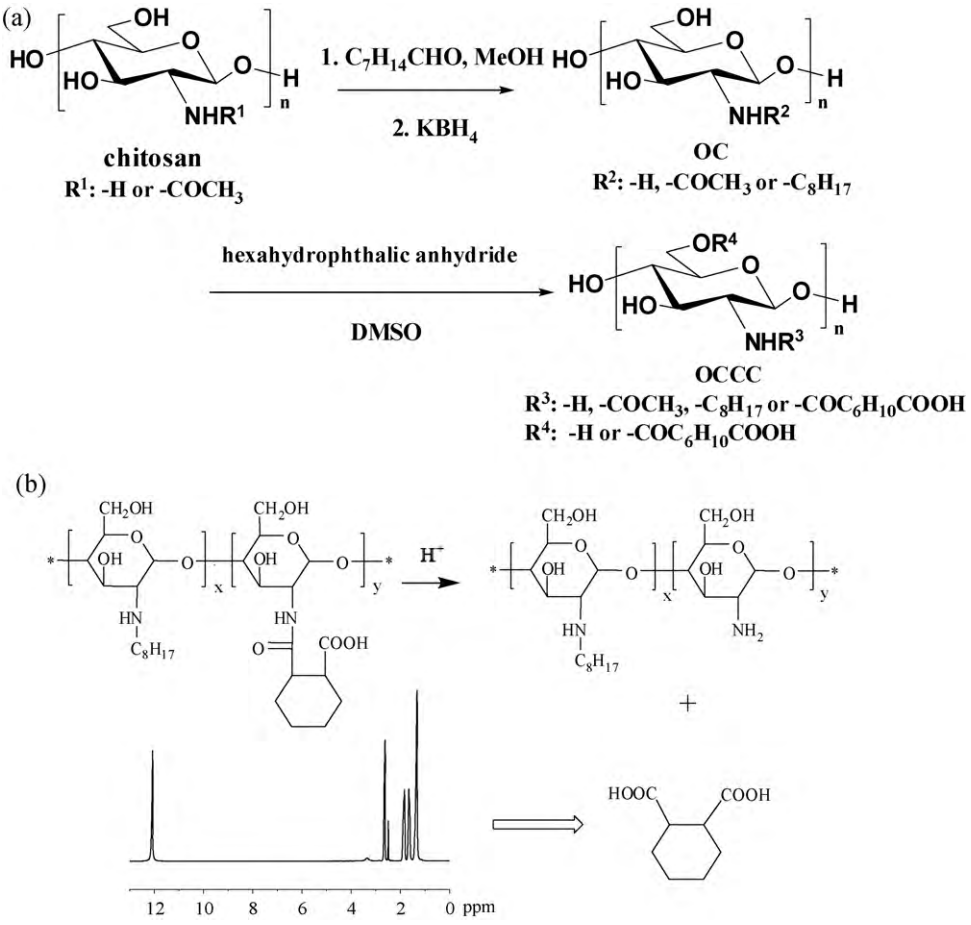

Scheme 1. (a) Synthesis route of OCCC and (b) hydrolyze route of OCCC-2.

delivery systems based on natural polymers have rarely been reported.

Amides with neighboring carboxylic acid groups were reported to exhibit pH-dependent hydrolysis. The amide of the secondary amine is almost instantly hydrolyzed at pH 5, slightly slower at pH 6, but only 50% even after 60 h at pH 7.4 (Xu et al., 2007). In our previous paper, *N*-octyl-*N*-(2-carboxylbenzoyl) chitosan (OCBC) as the material for drug carrier was synthesized (Li et al., 2009). It shows the pH sensitivity to mild acidic environment, but during the experiments there were many other things required to be improved, for example the water solubility of the polymers. Its relatively poor solubility possibly results from the hydrophobic benzyl group in the structure, so we come up with a way to make a modification of the polymer by replacing the benzyl group with other pH-sensitive groups, which are more hydrophilic. The hexahydrophthalic acid was hydrophilic due to the less hydrophobical cyclohexyl group compared with benzyl ring. In the current work, we describe a novel pH-sensitive drug delivery system using chitosan modified with amide linkage as a carrier for delivery of anti-cancer drug paclitaxel (PTX). Amphiphilic acylated chitosan was synthesized by the introduction of the octyl and carboxyl-cyclohexamethenyl moieties to part of amino groups of chitosan. The pH-sensitive micelle based on *N*-octyl-*N*-(2-carboxyl-cyclohexamethenyl) chitosan (OCCC) could remain stable at physiologic pH 7.4. In this research, the chemical structure of OCCC was confirmed by FTIR, ^{1}H NMR and elemental analysis. Furthermore, physical properties were measured with differential scanning calorimetry (DSC) and X-ray diffraction spectrometry (XRD) techniques. Transmission electron microscopy (TEM) techniques were exploited to evaluate the micelle-forming properties. The pH-sensitivity of the polymeric micelle was evaluated, and proved to be stable in neutral solution while highly sensitive to mild acidic environment. In addition, OCCC showed high capacity in solubilization of paclitaxel and pH-dependent drug release was investigated. Furthermore, the cellular cytotoxicity of OCCC and the paclitaxel-loaded micelle compared with Taxol®, the commercial paclitaxel for administration, were all evaluated in this paper. In conclusion, this study may lead to a novel pH-sensitive drug delivery system and improve the efficacy and safety for paclitaxel in chemotherapy.

2. Methods

2.1. Materials

Chitosan was provided by Nantong Shuanglin Biochemical Co. Ltd. (China), with a degree of deacetylation of 92% and viscosity average molecular weight of 70 kDa. Pyrene was purchased from Fluka Company (>99%). Paclitaxel was supplied by Taihua Natural Plant Pharmaceutical Co. Ltd. (China). HPLC/spectra-grade reagents were used as the mobile phase in HPLC analysis, and all commercially available solvents and reagents were used without further purification. Distilled and deionized water was used in all experiments.

2.2. Synthesis of amphiphilic chitosan derivative

N-Octyl-*N*-(2-carboxyl-cyclohexamethenyl) chitosan derivatives were prepared by introducing an octyl group to NH_2 on C_2 of the glucosamine unit in chitosan followed by a different degree of *N*-acylated as shown in Scheme 1a.

2.2.1. Preparation of N-octyl chitosan (OC) (Zhang, Ding, Yu, & Ping, 2007)

Chitosan (2.0 g, 12 mmol as monosaccharide residue containing 11 mmol amino groups, 70 kDa) was suspended in 60 ml of methanol solution with stirring at room temperature, and then octanal (5.3 ml and 33 mmol) was added to the reaction mixture. After 8 h, KBH_4 (2 g, 37 mmol) was slowly added to the solution in batch. After a further 24 h of continuous stirring, the reaction solution was neutralized with aqueous 1 M HCl solution, and the product was precipitated with methanol. The precipitate was filtered and repeatedly washed with methanol and water. The product was dried under vacuum at 60 °C overnight, and 3.80 g of *N*-octylchitosan (OC-2) yellow powder was obtained.

OC-1, OC-3, OC-4 and OC-5 were prepared following the same procedures using the chitosan (70 kDa) as starting material by reacting 5 h, 36 h,60 h and 72 h, respectively.

2.2.2. Synthesis of N-octyl-N-(2-carboxyl-cyclohexamethenyl) chitosan derivatives

OC-2 (0.5 g, 0.68 mmol) was dissolved in 25 ml of dimethyl sulfoxide (DMSO) solution with stirring at room temperature. Hexahydrophthalic acid (0.6 g, 2 mmol) was slowly added to the solution in batch, and this mixture was heated to 80 °C with stirring. After 24 h of reaction, the reaction solution was neutralized with aqueous 20% NaOH solution to pH 10 at 0 °C, and the filtrate solution was dialyzed against distilled water for 5 days using a membrane obtained from Sigma with a molecular weight cutoff range (MWCO) of 10,000. The solution was lyophilized, and 0.45 g of white powder, *N*-octyl-*N*-(2-carboxyl-cyclohexamethenyl) chitosan (OCCC-2) was obtained.

OCCC-1, OCCC-3, OCCC-4, and OCCC-5 were prepared following the same procedures using the OC-1, OC-3, OC-4 and OC-5 as starting materials, respectively.

2.3. Characterization of chitosan derivatives and measurement of physical properties

^{1}H NMR was performed on a Bruker (AVACE) AV-300 spectrometer. Chitosan was dissolved in the mixed solvent of D_2O and CF_3COOD, and chitosan derivatives were dissolved in D_2O.

FTIR spectra were recorded on Fourier-transform infrared spectrometer (Nicolet 2000) in KBr discs.

Elemental analysis was performed using an Element Vario EL III analyzer. Data from elemental analysis were used to calculate the degree of *N*-alkyl substitution.

XRD was obtained by using XD-3A powder diffraction meter with Cu Kα radiation in the range of 5–40 °C (2θ) at 40 kV and 30 mA.

DSC was obtained with NETZSCH DSC 204 equipment. The temperature range is 30–500 °C with a heating rate of 20 °C/min.

Turbidity measurements were conducted with 752N UV–vis spectrophotometer.

2.3.1. Measurement of critical micelle concentration (CMC)

The CMC of the OCCCs was determined by using pyrene (Fluka, >99%) as a hydrophobic probe in fluorescence spectroscopy (Shimadzu RF-5301 PC, Japan) (Chae, Son, Lee, Jang, & Nah, 2005; Wang, Liu, Weng, & Zhang, 2007). Briefly, a known amount of pyrene in acetone was added to each of the series of 10 ml vials and the acetone was evaporated, 10 ml of various concentrations of OCCCs (5×10^{-4} to 0.35 mg/ml) was added to the vials (the final concentration of pyrene was controlled to 1×10^{-4} mg/ml), then sonicated for 30 min at room temperature. The sample solutions were heated at 60 °C for 30 min to equilibrate pyrene and the micelle, and then left to cool overnight at room temperature. Fluorescence excitation spectra were measured at the excitation wavelength of 334 nm, and the emission wavelength was 350–450 nm for emission spectra. Both excitation and emission bandwidths were set at 5 nm. Based on the pyrene emission spectra and a decreasing I_{373}/I_{384} with increasing log concentrations of OCCCs. The CMC values were calculated by the crossover point at which I_{373}/I_{384} began to decrease rapidly.

2.3.2. In vitro pH-dependent hydrolysis of OCCCs

The pH-sensitivity of the chitosan derivative was evaluated by using the spectrophotometer. To assess the effect of the hydrolysis of the amide group in OCCCs, the changes of transmittance at λ 560 nm of aqueous polymer solutions within a wide pH range were monitored. The OCCC-2 (degree of substitution (DS) of *N*-octyl 36.4%) was dissolved in phosphate buffer solutions of different pH values (i.e. 5.0, 6.0, 6.5, 7.0 and 7.4). The solution pH was measured with a Delta 320 pH meter equipped with a Mettler-Toledo instrument (Shanghai).

2.4. Preparation and characterization of paclitaxel-loaded chitosan derivative micelles

Paclitaxel-loaded chitosan derivative micellar solutions were prepared by a dialysis method (Bae et al., 2007). Briefly, OCCC 7.5 mg was dissolved in 2.5 ml water, and 7.5 mg of PTX was dissolved in 0.25 ml ethanol (the feeding ratio of the drug to copolymer was 1:1). Then the paclitaxel solution was injected into OCCC solution with magnetic stirring at room temperature, the mixture was dialyzed against distilled water overnight at room temperature using dialysis membrane (MWCO 10 kDa). The micelle solution was filtered with a 0.22 μm pore-sized microfiltration membrane, then the PTX concentration was analyzed by high-performance liquid chromatography (HPLC) and the lyophilized micelle powder was obtained. The HPLC was equipped with a reverse-phase column (4.6 mm × 250 mm, Hanbon, Jiangsu, China) at 40 °C and with a UV spectrophotometer (Agilent Technologies, Palo Alto, CA, USA). The mobile phase was a mixture of methanol and water (75:25, v/v). The samples were delivered at a flow rate of 1.0 ml/min and detected at a wavelength of 227 nm. A certain volume of PTX-loaded micelle solution was taken and redissolved with 50-fold volume mobile phase, and 20 μl of dilution was injected into the HPLC system. The PTX-loading rate and entrapment efficient in micelle were calculated by the following equations:

$$\text{drug-loading rate } (\%) = \frac{C \times V}{W_{\text{freeze-dried micelle}}} \times 100; \quad (1)$$

$$\text{entrapment efficiency } (\%) = \frac{C \times V}{W_{\text{PTX}}} \times 100; \quad (2)$$

where C, V, $W_{\text{freeze-dried micelle}}$ and W_{PTX} represent the PTX concentration of micelle solution, the volume of micelle solution, the weight of freeze-dried micelle and the weight of PTX added, respectively.

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. TEM analysis was performed using the micelle solution with JEM-200CX (JEOL Ltd., Japan). The micelle solution was placed on a copper grid coated with framer film.

2.5. In vitro pH-dependent size change of blank polymeric micelle

The pH-sensitivity of blank polymeric micelle was evaluated by detecting the size change. The OCCC-2 (DS of *N*-octyl 36.4%) was dissolved in phosphate buffer solutions of different pH values (i.e. 5.0, 6.0, 6.5, 7.0 and 7.4), and the size of each blank polymeric micelle was detected by a Zetasizer 3000HS instrument (Malvern Instruments, Malvern, UK) with 633 nm He–Ne lasers at 25 °C after stirring.

2.6. In vitro pH-dependent release of PTX from polymeric micelle

In vitro release of PTX from micelles was conducted using 1% Tween-80 PBS solution as the dissolution medium with different pH values (7.4, 6.8, 6.5, 5.5, and 5.0) (Wei et al., 2009). The saturated solubility of PTX in the dissolution medium was measured to be 76 μg/ml. In vitro release behavior of PTX from drug-loaded OCCC-2 micelles was studied using the modified dialysis method, which was shown as follows: 1.0 ml of polymer micelles loaded with the same amount of PTX (1.4 mg) were placed into a dialysis membrane bag (MWCO 10 kDa). The whole bag was sunk in 150 ml of release medium as a sink condition (37 °C). The dialysis membrane bag was then placed in an incubator shaker, which was maintained at 37 °C and shaken horizontally at 60 rpm. At predetermined time intervals, 1.0 ml sample was withdrawn, and was filtered with a 0.45 μm pore-sized microfiltration membrane. Then PTX concentration was determined by HPLC. The mobile phase was a mixture of methanol and water (75:25, v/v). The samples were delivered at a flow rate of 1.0 ml/min and detected at a wavelength of 227 nm. All drug release tests were performed thrice.

2.7. Cytotoxicity

Cytotoxicities of OCCC-2 (DS of *N*-octyl 36.4%), PTX–OCCC-2 micelles and Taxol® against MCF-7 and KB cells were evaluated by MTT assay (Zhang, Hu et al., 2007). The cells were seeded at 5.0×10^4 ml^{-1} cells/well in a 96-well plate and grown for 24 h. After removing the growth medium, 100 μl DMEM containing different concentrations of OCCC-2, Taxol®, and PTX-micelle was added. The MCF-7 cells were further incubated for 72 h. Thereafter, the wells were washed three times with warm PBS. And the KB cells were further incubated for 48 h. As a control the cells were incubated with DMEM for 72 h or 48 h. The culture medium from each well was removed, and 100 μl DMEM and 10 μl MTT solution (5 mg/ml in PBS) were then added to each well. After 4 h further incubation, the culture medium was removed, and the formazan crystals in cells were solubilized with 150 μl DMSO. The UV absorbance at 570 nm was measured using a microplate reader (Bio-Rad, Model 680, USA).

3. Results and discussion

3.1. Synthesis and characterization of chitosan derivatives

The synthetic route of OCCC was briefly summarized in Scheme 1a.

The structure of these graft copolymers was characterized by ^{1}H NMR (Fig. 1a), and FTIR spectra (Fig. 1b).

Compared with chitosan, the ^{1}H NMR spectrum of OCCC (Fig. 1a) showed new-emerged peaks at δ (ppm) 0.8–1.85 which attributed to the $-CH_2$ and $-CH_3$ of the long-chain alkyl group. The results certified that the chitosan derivative carried *N*-octyl groups. The peaks at δ (ppm) 1.4–1.9 attributed to the aromatic carbon of the *N*-carboxylbenzoyl groups indicating the successful graft of these segments onto chitosan backbone.

FTIR analysis was performed to evaluate the *N*-alkyl substitution. Fig. 1b showed the FTIR spectra of OC and OCCC. Chitosan had the strongest absorbance at 1597 cm, which indicated the presence of the NH_2 group on C2 of the glucosamine unit. The intensity of this specific NH_2 signal was significantly decreased upon introducing the *N*-octyl group and was hardly observed for OCCC. In comparison with the FTIR spectra of chitosan and OC, introducing the *N*-alkyl into the molecule increased the intensity of peaks at 2860 and 2932 cm, suggesting the presence of *N*-alkyl substitution (Li, Liu, & Yao, 2002). Furthermore, the intensity of these peaks was greater in the FTIR spectra of OCCC-5 than that observed for OCCC-2 under the same experimental conditions, indicating the additional *N*-alkyl substitution. These data suggested the presence of methyl and long alkyl groups on nitrogen at the C2 position of the glucosamine unit in chitosan molecules. New peaks were observed at about 1450 cm in the FTIR spectra of OC and OCCC.

The DS of octyl group was calculated by comparing the C, N molar ratio of each derivative from elemental analysis (Miwa et al., 1998). Besides, the DS of octyl group was increased when the reaction time was prolonged. The DS of octyl group of OC-1, OC-2, OC-3, OC-4 and OC-5 was 28.8%, 36.4%, 43.0%, 52.9%, and 64.0%, respectively.

3.2. Physical properties of modified chitosan

XRD and DSC spectrometry were employed to study the physical properties of chitosan and its derivatives. XRD was conducted for OC-2 and OCCC-2 (DS of octyl group 36.4%) to further evaluate their crystallization behaviors (Fig. 1c). Two reflection falls at 2θ of 12° and 20° were observed in the X-ray diffraction spectrum of OC, whereas OCCC only had one broad peak at 2θ of about 22°. It is well accepted that the reflection fall at 2θ of 11° reflects the presence of crystal form I and the strongest reflection at 2θ of 22° corresponds to crystal form II. The data in Fig. 1c indicated that introduction of octyl and carboxylbenzoyl group to chitosan main chain decreased their ability of forming intermolecular hydrogen bonds and resulted in the part of OCCC becoming amorphous.

DSC thermograms of OC-2 and OCCC-2 are shown in Fig. 1d. The spectrum of OC-2 showed a broad endothermic peak around 85 °C and a sharp exothermic peak at 306 °C. The former endothermic peak may be caused by the water vapor that the OC-2 contained and some of the polymer had low molecular weight. The latter may be attributed to the decomposition of chitosan. The DSC result of OCCC-2 was similar to OC-2.

3.2.1. Measurement of critical micelle concentration (CMC)

At low concentration (C) of OCCCs (C < CMC), there were small or negligible changes in fluorescence intensity ratio of I_{373}/I_{384}. As the concentration increased, remarkable decrease of the intensity ratio (I_{373}/I_{384}) was observed (Fig. 2a). Fig. 2b shows the intensity ratio (I_{373}/I_{384}) of the pyrene excitation spectra versus the log concentrations of OCCC-2. Based on the intensity ratio data, the CMC value of OCCC-2 was calculated by the crossover point, all the CMC values of OCCCs with different DS of octyl group were listed in Table 1. The higher the DS of octyl group, the lower the CMC value. It has been reported that similar phenomenon was observed for micelles based on chitosan derivatives (Yao et al., 2007).

3.2.2. In vitro pH-dependent hydrolysis of OCCC-2

The pH-sensitive polymeric micelles based on OCCC-2 are expected to be stable for circulation in vivo, while rapidly releasing drug once entered into endosomes or lysosomes of tumor cell in response to acidic environment (pH 5.0). As it was known, the extracellular pH of tumor cell was slightly lower (pH 6.3–6.8) than the surrounding tissues and blood (pH 7.4) (Asokan & Cho, 2002; Stubbs, McSheehy, Griffiths, & Bashford, 2000). If micelle was sensitive to this pH 6.3–6.8, drug would be released prematurely outside tumor cell and undermine our expectation. So a wild range of pH (5.0, 6.0, 6.5, 7.0 and 7.4) was included in the experiment (Fig. 3a) to determine the pH-sensitivity of OCCC-2.

The pH-sensitivity of the chitosan graft polymer was evaluated by using the spectrophotometer. Because the hydrolysis of the amide group in OCCC (Scheme 1b) would lead to the formation of *N*-octyl chitosan (NOC) that is insoluble in acidic solution, the transmittances of OCCC-2 solution decreased with increasing degree of hydrolysis. OCCC-2 solution in pH 7.4 remained transparent to light

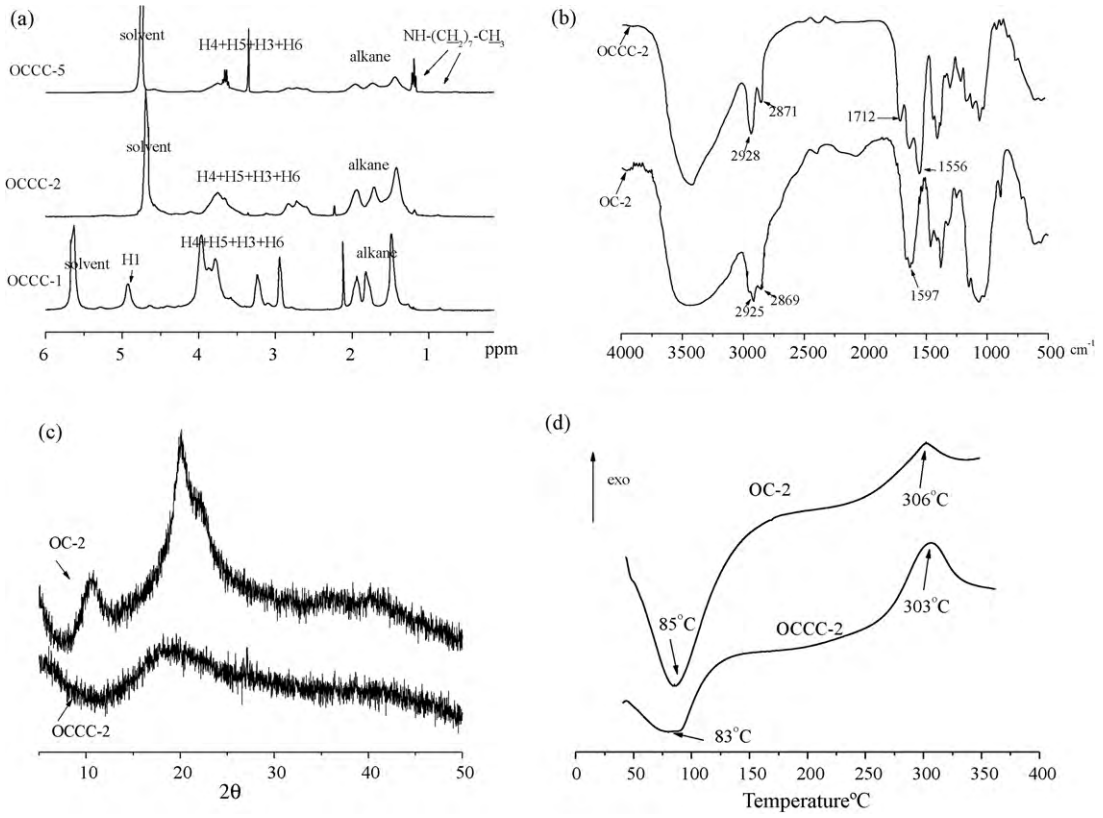

Fig. 1. ^{1}H NMR (D_2O) spectra (a), FTIR spectra (b), XRD patterns (c) and DSC thermograms (d).

(94.5% transmittance), while the transmittance of OCCC-2 solution in pH 5.0 decreased sharply (85.5% transmittance), suggesting that the hydrolyze rate of OCCC-2 was larger in phosphate buffer solution in pH 5.0 than in pH 7.4. From Fig. 3a the sensitivity of OCCC-2 was pH 5.5 that would not cause the premature release of PTX outside tumor cell.

The time-dependent hydrolysis process of OCCC-2 was studied in PBS at pH 5.5 (37.4 °C). Fig. 3b shows with the extension of the time, the transmittance drop, and slow downward trend after 60 min. The results indicated that the hydrolyze rates of OCCC in PBS were mainly dependent upon the time.

Decreasing the pH to 4.0 by CH_3COOH, then hydrolysis product was collected. ^{1}H NMR analysis demonstrated that molecule was cyclohexane-1,2-dicarboxylic acid (Scheme 1b).

3.3. Preparation and characterization of paclitaxel-loaded chitosan derivative micelles

Dialysis method was used to prepare drug-loaded micelle solutions based on chitosan derivates, and the drug-loaded micelle powder was obtained via lyophilization. Table 1 listed drug-loading rate, entrapment efficiency, particle size and zeta potential of PTX-loaded micelles. Drug-loading content and encapsulation efficiency are two critical characteristics for evaluating the capacity of a selected polymer to entrap and carry a selected drug. In this study, the encapsulation efficiency and drug-loading rate of micelles' preparation with an octyl substitution degree range of 28–64% range from 42.22% to 59.24% and from 30.47% to 48.10%, respectively (Table 1). The result demonstrated that drug-loading rate and encapsulation efficiency of these carriers were increased accompanied with the higher DS of octyl group. However, the graft polymers which had the higher DS of octyl group (43%, 53% and 64%) exhibited the lower solubility. The synthesized carriers aimed at encapsulating drug, the water-soluble graft polymer with the octyl DS of 36%, whose drug-loading rate and entrapment efficiency were the highest, was selected for further research.

The TEM micrograph of the PTX-loaded OCCC-2 micelle was presented in Fig. 4, showing that OCCC-2 was able to form homogeneous spherical micelles with the particle size around 150 nm, which was consistent with the measurement of Zetasizer 3000HS instrument.

3.4. In vitro pH-dependent size change of blank polymeric micelle

The phenomenon of size change accompanied with pH values was reported in some pH-sensitive nanocarriers (Boudier, Aubert-Pouessel, Gerardin, Devoisselle, & Begu, 2009; Sethuraman & Bae, 2007). In this paper, this phenomenon of pH-dependent size change

Table 1
The DS octyl group, CMC of OCCCs and characterization of PTX-loaded micelles ($n = 3$).

Sample	DS of octyl group	CMC (μg/ml)	Drug-loading rate (%, w/w)	Entrapment efficiency (%)	Particle size (nm)	Zeta potential (mV)
OCCC-1	28%	50	30.47 ± 3.15	42.22 ± 4.65	150.7 ± 8.7	−18.2 ± 2.3
OCCC-2	36%	42	31.47 ± 1.48	44.05 ± 7.11	145.9 ± 10.1	−14.8 ± 1.5
OCCC-3	43%	37	32.39 ± 5.48	50.11 ± 4.73	154.3 ± 1.7	−10.8 ± 2.6
OCCC-4	53%	11	33.38 ± 7.50	51.13 ± 11.7	153.5 ± 8.4	−19.3 ± 0.6
OCCC-5	63%	11	48.10 ± 7.50	59.24 ± 15.10	123.0 ± 12.8	−8.6 ± 2.0

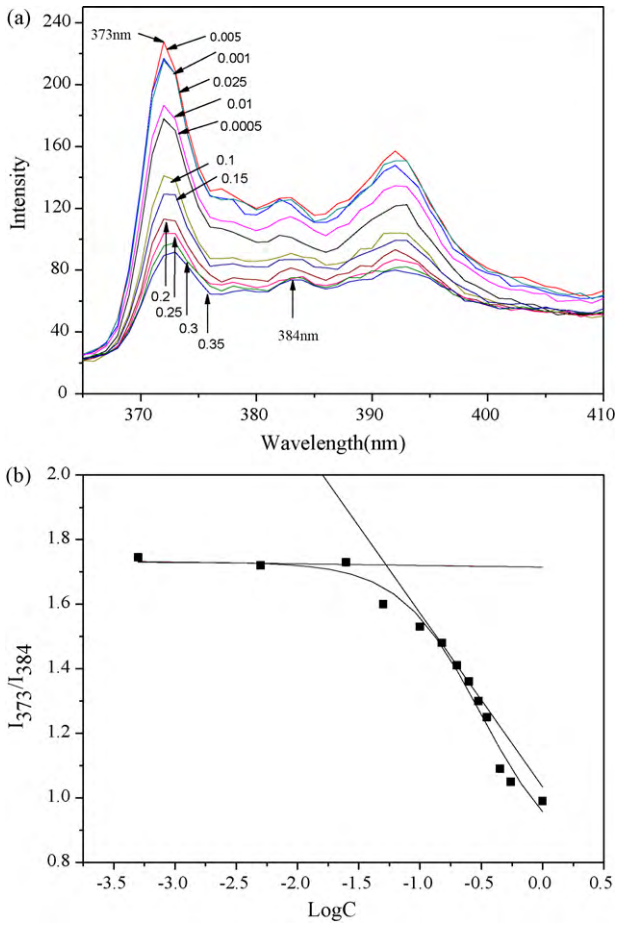

Fig. 2. (a) Pyrene emission spectra of OCCC-2 solutions (excitation wavelength of 334 nm). (b) The intensity ratio (I_{373}/I_{384}) of the pyrene emission spectra versus the log concentrations of OCCC-2.

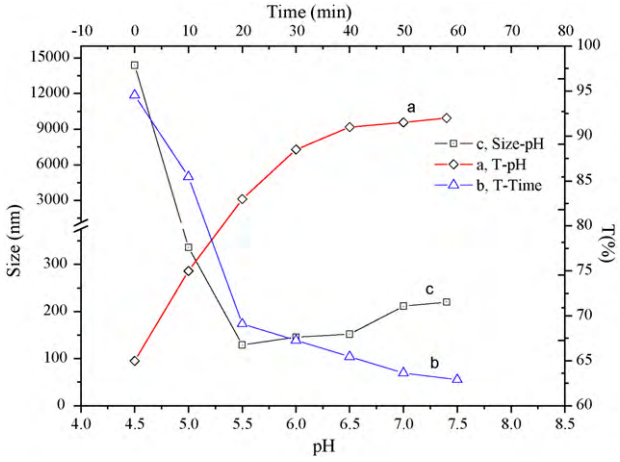

Fig. 3. (a) Changes of the transmittance (T) as a function of pH, (b) changes of the transmittance (T) as a function of the time of the OCCC-2 solution in pH 5.5 and (c) particle size of OCCC-2 blank micelle as a function of pH ($n = 3$).

was also observed. A wide range of pH values (4.5, 5.0, 5.5, 6.0, 6.5, 7.0 and 7.4) were included in the experiment (Fig. 3c). The size of blank micelle in pH 7.4 was 219 nm, while in pH 5.0 increased to 336 nm obviously, and the micelle became suspension completely at pH 4.5. The results indicated that the micelles formed by OCCC-2 were found to be highly sensitive to acidic pH and reasonably stable under physiologic pH.

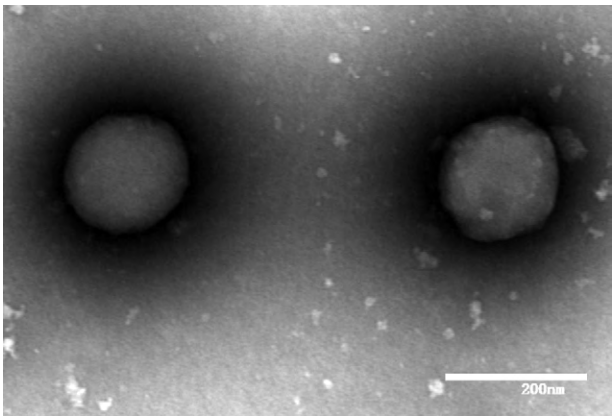

Fig. 4. TEM of PTX-loaded OCCC-2 micelle.

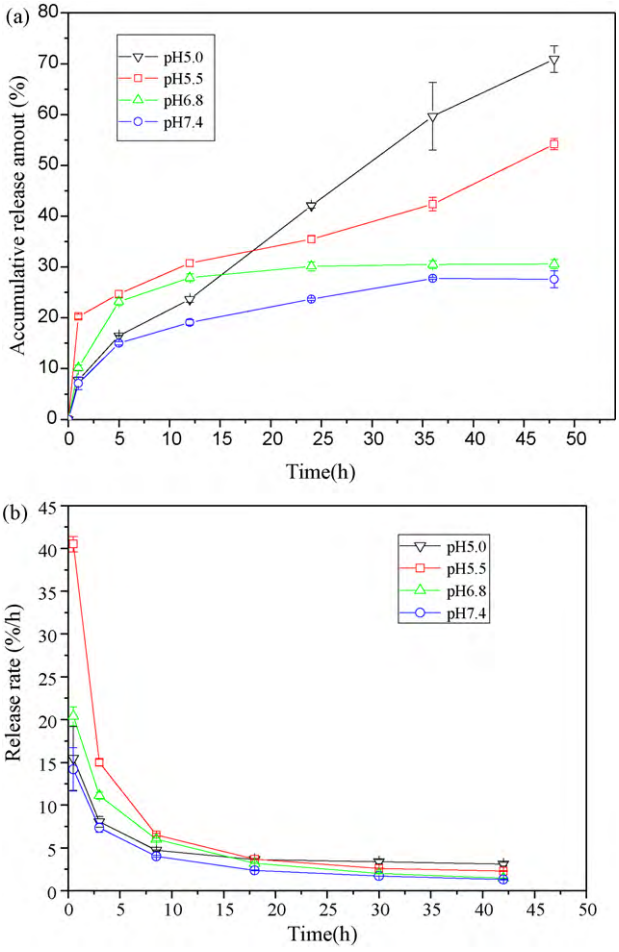

Fig. 5. (a) PTX accumulative release curve from OCCC-2 micelle for 48 h at different pH values, (b) PTX release rate curve at different pH values.

3.5. *In vitro pH-dependent release of PTX from polymeric micelle*

The micelle released PTX both time- and pH-dependently as the pH value increased from pH 7.4 to 5.0. Fig. 5a shows that the accumulative released amount of PTX at 48 h was markedly increased to 52% at pH 5.5 and 72% at pH 5.0, and there was an obvious significant difference between pH 7.4 and pH 5.5 ($p < 0.05$). So it was obvious that the acidic pH could increase the PTX released from micelle. In contrast, only 27% and 22% PTX released in pH 6.8 and 7.4 at 48 h ($p > 0.05$). In conclusion, the pH introduced OCCC-2 micelle releas-

Table 2
IC_{50} values of OCCC-2 (36.4%), Taxol® and PTX/OCCC-2 micelle against KB, MCF-7 cell lines ($n = 3$).

Sample	IC_{50} (μg/ml)	
	KB	MCF-7
OCCC-2	2630 ± 53	780 ± 30
Taxol®	0.13 ± 0.04	0.018 ± 0.005
PTX/OCCC-2 micelle	1.13 ± 0.15	0.137 ± 0.012

ing PTX was 5.5, which was the endosomal pH (Asokan & Cho, 2002), so it indicated that OCCC-2 micelle could release encapsulated drug in the endosomal pH.

Furthermore, the release rate curve (Fig. 5b) showed that the release rate at pH 5.5 increased markedly and there was a significant difference from the beginning ($p < 0.05$). This result indicated that OCCC-2 micelle at pH 5.5 resulted drug release difference at the beginning. However, the rapid hydrolysis at pH 5.0 made the hydrophilic layer broken and aggregation of hydrophobic precipitation in a short time, which resulted in the sustained release of inner hydrophobic drug, so it appeared at the lower release rate at pH 5.0 from the beginning. After many hours, the micelle completely destroyed contributing to the accelerated release rate at pH 5.0.

3.6. Cytotoxicities

The cytotoxicities of OCCC-2 (DS of octyl 36.4%), Taxol® and PTX-OCCC-2 micelle against KB, MCF-7 cell lines were evaluated using an MTT assay. Table 2 summarizes the 50% growth inhibition (IC_{50}) values of blank carrier and formulations. The IC_{50} values of KB and MCF-7 for OCCC-2 were 2.63 and 0.78 mg/ml, respectively. It suggested that OCCC-2 was nearly non-cytotoxic and persuasive for the potential application as a new drug carrier.

PTX, the first of a new class of microtubule stabilizing agents, is one of the most successful anti-cancer drugs and shows potency against a broad spectrum of cancers, especially carcinomas of the breast and ovary and lung (Panchagnula, 1998; Singla, Garg, & Aggarwal, 2002). Our results indicated that the IC_{50} values of both Taxol® and PTX-OCCC-2 micelle on MCF-7 were much lower than those on KB cells. Thus, it suggested that PTX is more sensitive against breast cancer than nasopharyngeal carcinoma cell, which was consistent with the clinical research.

Compared with Taxol®, the higher IC_{50} values of OCCC-2 micelle on the cells might be caused by the lower cellular uptake of micelle. This study suggested that both negative charge on the surface of OCCC-2 micelle and hydrophilic shell might result in the lower cellular uptake into cell. Furthermore, as it was shown in release experiment, only 22% of PTX was released at 48 h in pH 7.4. The sustained PTX release from micelle resulting in delayed anti-cancer effect on tumor cells might also contribute to the higher IC_{50} values.

4. Conclusion

In this study, a series of novel pH-sensitive graft copolymer, *N*-octyl-*N*-(2-carboxyl-cyclohexamethenyl) chitosan derivatives were synthesized. The chemical structures and some of physical properties were characterized by FTIR, ^{1}H NMR, elemental analysis, DSC, and XRD. The CMCs of the modified chitosan were found to be 11–72 μg/ml. The pH-sensitivity study showed that chitosan-derived amides micelle was stable in neutral solution pH 7.4 while highly sensitive to mild acidic environment pH 5.5. The graft chitosan could self-assemble to form micelles in the aqueous solution and had good solubilization ability of paclitaxel. The drug-loading rate ranged from 30.47% to 48.10% and entrapment efficiency ranged from 42.22% to 59.24%. Drug release study showed that the pH-sensitivity of drug releasing from the micelle was caused by pH 5.5. Cytotoxicities of OCCC-2 against tumor cells suggested that the graft polymer was nearly non-cytotoxic, and the higher IC_{50} of OCCC-2 micelle than Taxol® might be caused by the sustained release of PTX in micelle. Further evaluation of the micelle system in vivo is undertaken. In conclusion, the OCCC micelle was expected to be potential PTX delivery system for chemotherapy of cancer.

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