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Formation, microstructure, biodistribution and absence of toxicity of polymeric micelles formed by N-octyl-N,O-carboxymethyl chitosan

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ABSTRACT

In this study, N-octyl-N,O-carboxymethyl chitosans (octyl-CM-Chitosan) with different molecular weights and degrees of alkylation were synthesized. The critical micelle concentration of octyl-CM-Chitosan was in the range of 7.5–30.4 mg/L, depending on their chemical compositions. Micelles formed by octyl-CM-Chitosan were spherical, with a particle size of <200 nm and high negative zeta potentials of ~30 mV. Additionally, multi-core microstructure of octyl-CM-Chitosan micelle was confirmed by using fluorescence quenching technique. With the decrease of molecular weight and increase of alkylation, both of the hydrophobicity of inner-core and aggregation number of alkyl chains per microdomain increased. Fluorescein-labeled octyl-CM-Chitosan micelles could easily avoid the mononuclear phagocyte system (MPS) clearance and had the potential for passive targeting to non-MPS tissues. The absence of toxicity of octyl-CM-Chitosan micelles as intravenous materials was confirmed by hemolysis, cell viability assays and histopathological evaluations. These results suggest that octyl-CM-Chitosans are promising materials for intravenous administration.

1. Introduction

Polymeric micelles have attracted increasing attention in recent years as a promising vehicle for poorly soluble drugs due to their nano-size, thermodynamic stability and core–shell structure (Matsumura & Kataoka, 2009; Nishiyama & Kataoka, 2006). However, only a limited number of amphiphilic polymers are suitable as drug delivery vehicles, because of the requirement for biocompatibility and biodegradability. In recent years, self-assemblies based on naturally occurring polymers have been of particular interest (Miyazaki & Tabata, 2009). Chitosan, the second most plentiful biomass prepared from N-deacetylation of chitin, has attracted significant interest in drug or gene delivery systems because of its well-known non-toxicity, biocompatibility and biodegradability (Chen et al., 2009; Liu et al., 2007). Several hydrophobically modified chitosan such as stearic acid-modified chitosan with 4.96% degree of N-substitution (Hu, Zhao et al., 2006), deoxycholic acid-modified chitosan with 5.4–11.1% degree of N-substitution (Lee, Kwon, Jo, & Jeong, 2005), linolenic acid-modified chitosan with 1.8% degree of N-substitution (Liu, Desai, Chen, & Park, 2005), and linoleic acid-modified chitosan with 3.3–6.1% degree of N-substitution (Cheong et al., 2009) have been synthesized and demonstrated to form micelle-like structures by self-aggregation in aqueous environment. These micelles showed good loading capacities for anionic macromolecules, e.g., gene (Cheong et al., 2009; Hu, Zhao et al., 2006) or protein (Hu, Li, Yuan, & Zeng, 2006) However, only a small part of the hydrophobic segment can be conjugated to chitosan backbone because of the extremely low solubility of chitosan in neutral media, which leads to low loading capacities of the micelles for nonionic hydrophobic drugs (Du, Wang, Yuan, Wei, & Hu, 2009; Hu, Ren, Yuan, Du, & Zeng, 2006). Moreover, the outer shell of hydrophobically modified chitosan micelles is weakly hydrophilic, therefore, they are rapidly phagocytosed by the mononuclear phagocyte system (MPS). This does not allow sufficient time in the circulation for efficient extravasation of the micelles to target non-MPS cells (e.g. tumor cells).

To overcome the above disadvantages, chitosan can be amphiphilically modified by attaching both hydrophobic and hydrophilic segments to the backbone. The introduction of hydrophilic groups significantly increased the hydrophilicity of chitosan, thus, a greater number of hydrophobic groups were successively conjugated to the chitosan backbone as hydrophobic moieties. In our previous studies, we found that alkyl chain-modified succinyl chitosan (Xu et al., 2007), carboxymethyl chitosan (Huo et al., 2007; Zhang, Huo, Zhou, Yu, & Wu, 2009) and glycol chitosan (Huo et al., 2010) showed excellent loading capacities for paclitaxel and doxorubicin. Kwon's group synthesized cholanic acid (Nam et al., 2009) and deoxycholic acid-modified glycol chitosan (Kim et al., 2005), Hu's group designed stearic acid-modified chitosan-g-polyethylene glycol (PEG) (Hu, Meng et al.,

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2008), Ngawhirunpat designed cholic acid-modified chitosan-g-mPEG (Ngawhirunpat et al., 2009), Zhang's group designed alkyl chain-modified sulfate chitosan (Zhang et al., 2008), PEG conjugated N-octyl-O-sulfate chitosan (Qu, Yao, Zhang, Wu, & Ping, 2009) and pH-sensitive amphiphilic chitosan derivatives such as N-octyl-N-(2-carboxylbenzoyl) chitosan (Li, Liu et al., 2009), N-octyl-N-(2-carboxyl-cyclohexamethenyl) chitosan (Liu, Li, Jiang, Zhang, & Ping, 2010) and N-octyl-N′-imino-O-carboxymethyl chitosan (Li, Zhang, & You, 2009) which significantly improved the cytoplasmic delivery of drugs. All these studies suggested that chitosan amphiphilic derivatives exhibited higher promising as efficient carriers for hydrophobic drugs, as compared to the hydrophobically modified chitosan.

The microstructure of nanocarriers can significantly influence physicochemical and biological properties such as particle size, morphology, and even *in vivo* behavior. Hu's group found that micelles formed from hydrophobically modified chitosan possess special spatial structure with multi-hydrophobic cores (Hu, Ren et al., 2006; Hu, Wu, Du, You, & Yuan, 2008), which was significant different from the simple core–shell structure of micelles formed from classic diblock copolymers. However, little research has been focused on the microstructure of amphiphilically modified chitosan, much less the influence of chemical compositions of chitosan amphiphilic derivatives on their microstructure. Moreover, when the drug is loaded into the hydrophobic inner-core of the micelles, its *in vivo* behavior depends heavily on the carriers' *in vivo* fate. Thus, it is important to study the *in vivo* fate of micelles itself for their efficient delivery of encapsulated drug. However, to the best of our knowledge, there were no reports available in the literature with regard to the *in vivo* behavior of carrier itself. Hence, the present study is focused on the formation, microstructure and biodistribution of micelles formed from the amphiphilic chitosan derivatives, N-octyl-N,O-carboxymethyl chitosan (octyl-CM-Chitosan) and explore the influence of molecular weight of chitosan backbone and degree of alkylation on them. Moreover, hemolysis, cytotoxicity and histopathological effect on the various organs were also evaluated to investigate the feasibility of octyl-CM-Chitosan as an intravenous (*i.v.*) material.

2. Materials and methods

2.1. Materials

Chitosan was purchased from the Zhejiang Yuhuan Biochemical Co. Ltd. (Zhejiang, China) with deacetylation degree of 90–95% and weight 87, 190 and 480 kDa, respectively. Chloroacetic acid was obtained from Yancheng Kunye Chemical Co. Ltd. (Jiangsu, China). Octaldehyde was purchased from Nanjing Skyrun Golden Harvest Perfume Manu Co. Ltd. (Jiangsu, China). Sodium borohydride was purchased from Sinopharm Chemical Reagent Co., Ltd. Pyrene and cetylpyridinium chloride were purchased from Fluka Company (>99%). 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). Tween 80 was obtained from Jiangsu Chenpai Pharmaceutical Co. Ltd. (Jiangsu, China). FITC was purchased from Sigma Chemical Company (St. Louis, USA). All other reagents were analytical grade and used without further purification. Distilled and deionized water was used in all experiments.

2.2. Animals

Kunming mice were obtained from New Drug Screening Center of China Pharmaceutical University. The animals were pathogen free and allowed to access food and water freely. The experiments were carried out in accordance with the Chinese council on animal care guidelines.

2.3. Synthesis of chitosan amphiphiles

In this study, octyl-CM-Chitosans with different molecular weights and degrees of alkylation were synthesized as we previous reported (Zhang et al., 2009) (Scheme 1). Chitosan weight 87, 190 and 480 kDa was, respectively, carboxymethylated by reacting with chloroacetic acid in the mixture of isopropanol and NaOH solution. Octyl-CM-Chitosan were synthesized via Schiff bases formed by the reaction between the primary amino groups of carboxymethyl chitosan (CM-Chitosan) and octaldehyde followed by reduction of the Schiff base intermediates with sodium cyanoborohydride (Muzzarelli, Tanfani, Emanuelli, & Mariotti, 1983). The degree of alkylation was controlled by the feed molar ratio of CM-Chitosan and octaldehyde. Degree of carboxymethylation or alkylation, defined as the molar ratio of carboxymethylated or alkylated repeating units to all repeating units of chitosan, was determined by elemental analysis by using a Vario EL III analyzer (Elementar, Hanau, Germany).

2.4. Preparation of micelles

Fifty milligrams of octyl-CM-Chitosan was dissolved in 9 mL distilled water with stirring for 1.5 h at 50 °C and sonicated in an ice-bath by a probe-type ultrasonicator (JY92-2D; Ningbo Scientz Biotechnology Co., Ltd., China) at 100 W for 10 min. The solution was centrifuged at 3000 rpm for 10 min and filtered with a 0.45-μm-pore filtration membrane and stored in a refrigerator at 4 °C until use.

2.5. Fluorescence measurement

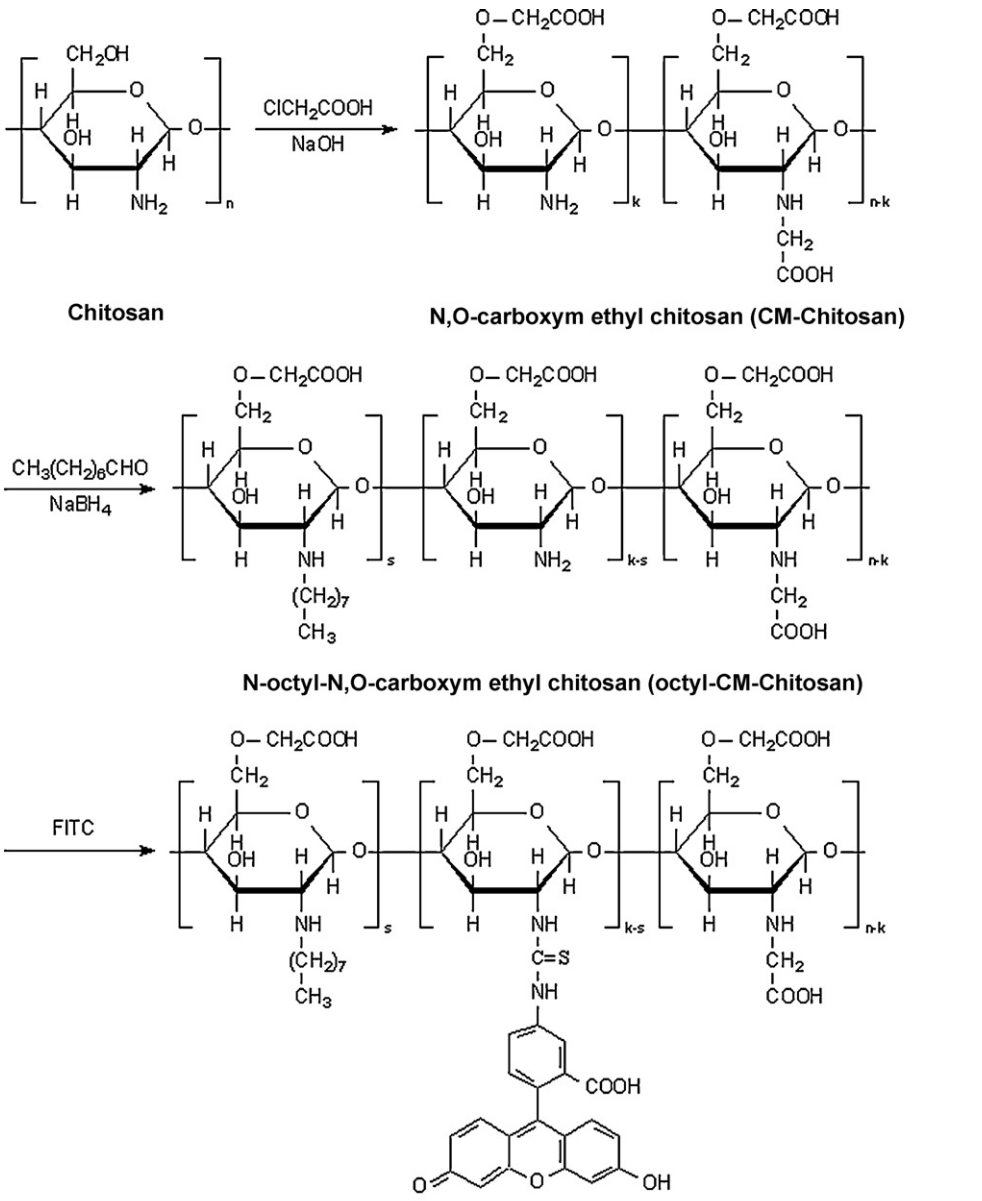
The critical micelle concentration of octyl-CM-Chitosan in distilled water was estimated by fluorescence spectroscopy using pyrene as a hydrophobic fluorescence probe (Wilhelm et al., 1991). Fluorescence spectra of pyrene were recorded with an RF-5301 PC fluorescence spectrophotometer (Shimadzu, Japan) with the emission wavelength at 390 nm. The slit widths for emission and excitation were set at 3.0 and 1.5 mm, respectively. The aggregation number of alkyl chains per hydrophobic microdomain (N_{alkyl}) was estimated by the steady-state fluorescence quenching method, where cetylpyridinium chloride was used as a fluorescence quencher for quenching of pyrene fluorescence (Akiyoshi, Deguchi, Tajima, Nishikawa, & Sunamoto, 1997). The concentration of octyl-CM-Chitosan was fixed to 0.046 mg/mL (PBS, pH 7.4).

2.6. Particle size and zeta potential

The lyophilized powders were reconstituted with 5% dextrose injection solution. The particle size and zeta potential were measured using a Malvern Zetasizer 3000 system (Malvern Instruments Ltd., Malvern, UK). All of the dynamic light scattering (DLS) measurements were performed at 25 °C and at a scattering angle of 90°. The zeta potential values were calculated using the Smoluchowski equation.

2.7. Morphology observation

Transmission electron microscopy (TEM) and atomic force microscopy (AFM) were employed to observe the micelle mor-



Scheme 1. Synthetic scheme of octyl-CM-Chitosan and FTC-octyl-CM-Chitosan.

phology and particle size. TEM of octyl-CM-Chitosan micelles was obtained using an H-7000 (Hitachi, Tokyo, Japan) electron microscope operating at an accelerating voltage of 75 kV. Negative staining of samples 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 an application of 0.01% phosphotungstic acid to get nanoparticles deposited on the grid. The samples were air-dried before observation.

The morphology of micelles was also investigated by AFM (SPI 3800/SPA 300HV, Seiko Instruments Inc., Japan) in tapping mode. A drop of properly diluted micelles was placed on the surface of a clean silicon wafer and dried under nitrogen flow at room temperature. To minimize any possible contamination of the surface by ambient air, each sample was freshly prepared just before the AFM experiments.

2.8. In vivo studies

2.8.1. Conjugation and self association of N-fluorescein thiocarbamyl-N-octyl-N,O-carboxymethyl chitosans

N-fluorescein thiocarbamyl N-octyl-N,O-carboxymethyl chitosans (FTC-octyl-CM-Chitosan) was synthesized as follows (Scheme 1). Briefly, octyl-CM-Chitosan (200 mg) was dissolved in 12 mL of 0.5 M sodium carbonate buffer. FITC (10 mg) dissolved in 8 mL of 0.5 M sodium carbonate buffer was added dropwise to the above OGC solution. After a stirring of 24 h at room temperature, the reaction mixture was divided into 7 mL fractions. Each fraction was purified by gel-filtration with a Sephadex® G-50 column (3.5 cm × 30 cm) using 0.5 M carbonate buffer as elution solvent. The purified FITC labeled octyl-CM-Chitosan collections were dialyzed against distilled water and then lyophilized. The FTC content was determined from the fluorescence intensity at 520 nm with excitation at 495 nm using an RF-5301 PC fluorescence

spectrophotometer (Shimadzu, Japan) in PBS at pH 7.4. The FTC-octyl-CM-Chitosan micelles were prepared as described in Section 2.4 and stored in the dark. The particle sizes of FTC-octyl-CM-Chitosan micelles were evaluated by DLS.

2.8.2. Biodistribution of micelles formed from chitosan amphiphiles

The biodistribution of FTC-octyl-CM-Chitosan in normal mice was examined after *i.v.* administration. The recovery ratios of FTC-octyl-CM-Chitosan from each tissue homogenate and plasma were determined as described previously (Kato, Onishi, & Machida, 2000). FTC-octyl-CM-Chitosan was administered to normal mice at a dose of 50 mg/kg by injection into the tail vein after fasting for 12 h. The mice were sacrificed at predetermined time points after administration, blood samples were withdrawn and several tissues (heart, liver, spleen, lung and kidney) were excised, weighed, and then washed and homogenized in PBS. After centrifugation of the homogenate or blood, the supernatant was diluted with PBS, and then measured fluorometrically at 520 nm, with excitation at 495 nm. The concentration of FTC-octyl-CM-Chitosan in the sample was corrected by the recovery ratios. The amount of FTC-octyl-CM-Chitosan in plasma was estimated using the reported volume of mouse plasma, 48.8 mL/kg (Tajima, 1989). The tissue distribution was calculated from the concentration and tissue weight. The pharmacokinetic parameters were calculated using non-compartmental analysis with WinNonlin software (Professional Edition, version 5.2; Pharsight, Mountain View, CA). The area under the concentration–time curve (AUC) was calculated using the linear trapezoidal rule.

2.9. Polymer toxicity characterization

2.9.1. Hemolysis test

The hemolysis test was performed as described previously (Le Garrec et al., 2004). The concentration of human red blood cells (RBCs) was fixed to 1% (v/v) and the final concentrations of octyl-CM-Chitosan, Tween 80 and Cremophore EL ranged from 0.2 to 4 mg/mL. The quantity of hemoglobin released was determined using a UV-2450 spectrophotometer (Shimadzu, Japan) at 416 nm. To obtain 0% and 100% hemolysis, saline and distilled water was added to the RBC suspension. The degree of hemolysis was determined by the method of Cheon Lee, Kim, Chan Kwon, Chung, and Young Jeong (2003).

2.9.2. In vitro cytotoxicity

In vitro cytotoxicity of octyl-CM-Chitosan micelles was evaluated by MTT assay with mouse primary hepatocytes. The isolation of mouse primary hepatocytes was performed as described previously (Shinkai, Sumi, Fukami, Ishii, & Kumagai, 2006). Primary mouse hepatocytes were suspended in William's medium E containing 10% fetal bovine serum, penicillin (100 U/mL), streptomycin (100 μg/mL), insulin (1 μg/mL), and dexamethasone (0.1 μM) at 37 °C in a 5% CO_2 humidified atmosphere. For the MTT assay, cells were plated on 96-well plates and washed three times with PBS, followed by addition of fresh serum-free medium. The cells with various concentrations of octyl-CM-Chitosan micelles were incubated for 24 h, and the MTT assay was performed and the percentage cell viability was determined.

2.9.3. Histopathological evaluation

The histopathological effects of blank octyl-CM-Chitosan micelles on various organs such as heart, liver, spleen, lung, and kidney were investigated after intravenous administration of octyl-CM-Chitosan solution at a high dose level of 280 mg/kg. The histopathological changes in each organ were observed with an Olympus BX-40 light microscope (Tokyo, Japan) at day 8 after treatment.

2.10. Statistical analysis

Statistical analysis was performed by Student's *t* test for two groups, and one-way ANOVA for multiple groups. All results were expressed as mean ± SD unless otherwise noted. $p < 0.05$ was considered statistically significant.

3. Results and discussion

3.1. Synthesis of chitosan amphiphilic derivatives

Octyl-CM-Chitosans with different molecular weights and degree of alkylation were synthesized in this study. The degrees of carboxymethylation and alkylation were calculated from the elemental analysis data by comparing the C and N molar ratio obtained from elemental analysis using the following equations (Senso, Franco, Oliveros, & Minguillon, 2000):

$$\text{Degree of carboxymethylation (\%)} = \frac{\text{C/N(mol)}_{\text{CM-Chitosan}} - \text{C/N(mol)}_{\text{chitosan}}}{2} \times 100 \quad (1)$$

$$\text{Degree of alkylation (\%)} = \frac{\text{C/N(mol)}_{\text{octyl-CM-Chitosan}} - \text{C/N(mol)}_{\text{CM-Chitosan}}}{8} \times 100 \quad (2)$$

All results are shown in Table 1. *X* in octyl-CM-Chitosan-*X*-*Y* indicates the molecular weight of parent chitosan, and *Y* is the degree of alkylation. The degree of carboxymethylation was >100%, which indicated that they were conjugated at not only 6-OH, but also 2-NH_2. The degree of alkylation varied from 37.9% to 61.6%, which was controlled by the feed ratio of octaldehyde to CM-Chitosan.

Synthesis of CM-Chitosan was carried out under strong alkaline conditions and the process is usually accompanied by the hydrolysis of the chitosan chain. Molecular weight of CM-Chitosan was determined by the Roberts and Domszy equation from viscosity measurement (Nishimura, Nishi, Tokura, Nishimura, & Azuma, 1986). The number of glucose units of chitosan and CM-Chitosan was calculated based on the molecular weight and degree of carboxymethylation, respectively. As shown in Table 1, it was found that the extent of scission depended significantly on the molecular weight of parent chitosan during carboxymethylation. The number of glucose units of CM-Chitosan was approximately 35% of that of parent chitosan with the longest backbone.

3.2. Self-assembly of chitosan amphiphilic derivatives

3.2.1. Formation of micelles

Micelles can be formed only when the concentration of polymer is higher than the critical micelle concentration, which play an important role in the stability of the micellar system. Polymeric micelles are generally more stable than low-molecular-weight surfactant micelles because of their markedly lower critical micelle concentration. The critical micelle concentrations of octyl-CM-Chitosans were determined using fluorescence probe technique (Wilhelm et al., 1991). As shown in Table 1, the critical micelle concentration of octyl-CM-Chitosan was in the range of 7.5–30.4 mg/L, depending on the composition. As the degree of alkylation increased, the critical micelle concentration of octyl-CM-Chitosan significantly decreased. A greater degree of hydrophobic substitutions in the macromolecules of octyl-CM-Chitosan might facilitate self-aggregation, which favors hydrophobic interactions and thus, the formation of dense polymer micelles. Molecular weight also had

Table 1
Chemical compositions and microscopic parameters of octyl-CM-Chitosan.

Sample[a]	MW of chitosan (Da)	MW of CM-Chitosan (Da)	Unit number of chitosan	Unit number of CM-Chitosan	Degree of carboxymethylation (%)	Degree of alkylation (%)	Critical micelle concentration (mg/L)	K_v	N_{alkyl}	Size (nm)	Polydispersity index	Zeta potential (mV)
Octyl-CM-Chitosan-87-40	8.7×10^4	6.4×10^4	593	264	118.4	37.9	30.4	1.1×10^5	1.9	189.3	0.131	−29.5
Octyl-CM-Chitosan-87-50	8.7×10^4	6.4×10^4	593	264	118.4	48.9	15.8	1.3×10^5	3.6	172.7	0.109	−29.7
Octyl-CM-Chitosan-87-60	8.7×10^4	6.4×10^4	593	264	118.4	61.6	7.5	1.6×10^5	5.0	165.6	0.129	−30.2
Octyl-CM-Chitosan-190-60	1.9×10^5	1.0×10^5	1293	427	119.2	60.5	11.2	4.3×10^4	3.5	188.2	0.148	−28.3
Octyl-CM-Chitosan-480-60	4.8×10^5	2.8×10^5	3265	1159	115.8	56.0	16.5	3.5×10^4	2.3	198.5	0.114	−26.9

[a] Octyl-CM-Chitosan-*X*–*Y* where *X* indicates the molecular weight of parent chitosan in kDa, and *Y* indicates the degree of alkylation.

a significant effect on octyl-CM-Chitosan self-aggregation. Low-molecular-weight octyl-CM-Chitosan may have a superior ability to form micelles, which might be attributed to the lower degree of chain stiffness of the backbones.

It was also found that the critical micelle concentration of octyl-CM-Chitosan was significantly lower than that of low-molecular-weight surfactants (e.g., 1.0×10^3 mg/L for deoxycholic acid and 2.3×10^3 mg/L for sodium dodecyl sulfate in water (Lee, Jo, Kwon, Kim, & Jeong, 1998), and comparable or even lower than that of chitosan amphiphiles, such as deoxycholic-acid-modified glycol chitosan with a critical micelle concentration of 47–219 mg/L (Kim et al., 2005) and deoxycholic-acid-modified chitosan with a critical micelle concentration of 13.2–44.7 mg/L (Lee et al., 1998). The low critical micelle concentration of octyl-CM-Chitosan was attributed to the higher degree of alkylation with stronger hydrophobicity. The results suggest that the octyl-CM-Chitosan micelles may remain stable in solution even after extreme dilution, and preserve their stability without dissociation after *i.v.* injection into the much larger volume of blood in the systemic circulation.

3.2.2. Dynamic light scattering and morphological observation

The particle size and polydispersity index of the micelles were estimated by DLS. The amphiphilic octyl-CM-Chitosan micelles, with different molecular weights and number of hydrophobic segments, were all <200 nm (Table 1). The results indicated that the particle size decreased as the degree of alkylation increased, which indicated the formation of more complete hydrophobic cores as a result of the enhanced interaction between hydrophobic alkyl chains. The increase in the molecular weight of the chitosan backbone was demonstrated to increase the size of the micelles because of the greater rigidity of the chitosan chain. It is also worth noting that the size of the micelles was not significantly affected by changes in the polymer concentration in the range of 0.2–2 mg/mL. This implied that the interparticle interaction between micelles was almost negligible. The polydispersity index of octyl-CM-Chitosan micelles was low (<0.15), which suggested a narrow size distribution.

Zeta potential or particle surface charge is an important measure of the stability of micelles. A relatively high surface charge may provide a repelling force between the particles, thus increasing the stability of the solution (Kwon et al., 2003). As shown in Table 1, all the micelles had relatively high negative zeta potentials of around −30 mV. It was obvious that the large negative zeta potential of octyl-CM-Chitosan micelles was attributed to the presence of ionized carboxyl groups on the surface, which indicated that negatively charged carboxymethyl chitosan covered the micelles. It is reasonable to conclude that the charged particles may repel each other and prevent aggregation or precipitation and show good stability.

TEM and AFM were used to visualize directly the size and the morphology of the micelles. The spherical morphologies of micelles were confirmed in Fig. 1A and B. The observed micelles size was approximately 100–150 nm, which was smaller than the hydrodynamic diameter obtained from the DLS experiment. This observation was due to the collapse of micelles during drying processes of TEM and AFM samples.

3.2.3. The hydrophobicity of the inner core of micelles

Since water-insoluble drugs are physically incorporated and stabilized in the hydrophobic inner core through hydrophobic interaction, the hydrophobicity of the inner core of self-organized structures is thought to be an important factor in their use as drug delivery vehicles. The hydrophobicity of octyl-CM-Chitosan micelles can be expressed with an equilibrium constant (K_v) of pyrene. In principle, a higher K_v value implies a greater hydrophobicity. The value of K_v was calculated by the method of Lee et al.

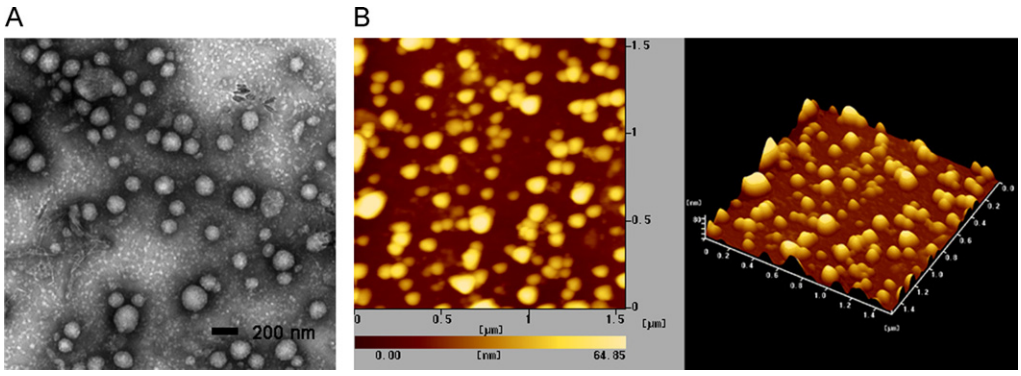


Fig. 1. (A) Transmission electron microscopy and (B) atomic force microscopy images of octyl-CM-Chitosan-87-60 micelles, where 87 is the molecular weight of parent chitosan in kDa, and 60 is the degree of alkylation.

(1998). The K_v values, as summarized in Table 1, were in the range from 3.5×10^4 to 1.6×10^5. The results indicated that the hydrophobicity of the inner core of micelles increased as the degree of alkylation increased. While, the K_v value decreased with the molecular weight of octyl-CM-Chitosan, which indicated the presence of weaker hydrophobic microdomains inside the micelles. We concluded from this evidence that the longer polymer chains showed stiffer properties, and therefore, a complete hydrophobic core was not readily formed.

3.2.4. The aggregation number of alkyl chains

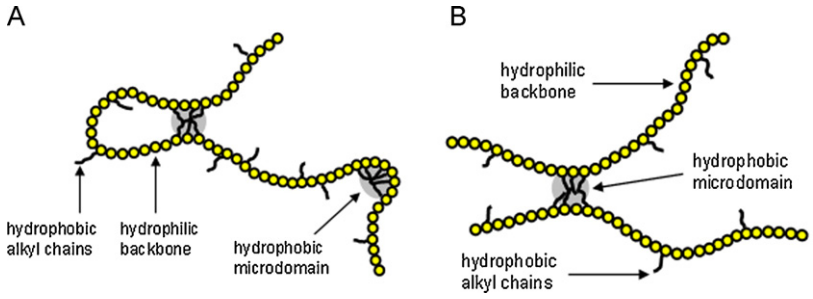
To identify the microscopic structure of micelles, the aggregation number per hydrophobic microdomain (N_{alkyl}) was determined using a fluorescence quenching method which has been used successfully to determine the aggregation number of surfactant micelles or polymeric assembles (Lee & Jo, 1998). The N_{alkyl} values of octyl-CM-Chitosans are listed in Table 1. It was found that 1.9–5.0 alkyl chains formed one hydrophobic domain, which was dependent on the degree of alkylation and molecular weight of octyl-CM-Chitosan. The limited motility of alkyl chains and stiffness of the CM-Chitosan backbone might lead to a decrease in N_{alkyl}. Considering the number of alkyl chains in one polymer chain, it may be concluded that there exist several hydrophobic microdomains in a micelle. For example, since octyl-CM-Chitosan-87-60 carries 163 alkyl chains per polymer chain, one polymer chain of octyl-CM-Chitosan-87-60 may form at least 32 independent hydrophobic microdomains in the interior of a micelle. The hydrophobic microdomains were formed by inter/intramolecular association of alkyl chains (Scheme 2). Each octyl-CM-Chitosan micelle contained multiple hydrophobic microdomains surrounded by a highly hydrated polysaccharide phase. The large number of microdomains with high hydrophobicity in the octyl-CM-Chitosan micelles may provide the necessary environment and space to incorporate poorly water-soluble drugs.

3.3. Pharmacokinetic and tissue distribution

Octyl-CM-Chitosan-87-40, octyl-CM-Chitosan-87-60 and octyl-CM-Chitosan-480-60 were labeled with FITC to indicate the *in vivo* behavior of octyl-CM-Chitosan in mice. The quenching of FTC-octyl-CM-Chitosan *in vivo* and the liberation of free fluorescein from FTC-octyl-CM-Chitosan were considered to be negligible referencing to previous results (Gerlowski & Jain, 1986). The FITC content of FTC-octyl-CM-Chitosan-87-40, FTC-octyl-CM-Chitosan-87-60 and FTC-octyl-CM-Chitosan-480-60 was calculated to be 0.34, 0.24 and 0.41 wt%, respectively. The particle size of FTC-octyl-CM-Chitosan-87-40, FTC-octyl-CM-Chitosan-87-60 and FTC-octyl-CM-Chitosan-480-60 micelles was 194.1, 171.2 and 197.4 nm in PBS, respectively, which was similar to that of octyl-CM-Chitosan micelles without FITC labeling.

The amounts of FTC-octyl-CM-Chitosan distributed in plasma and tissues of normal mice after a 50 mg/kg *i.v.* injection are shown in Fig. 2. The FTC-octyl-CM-Chitosan micelles were maintained at a high level in plasma, while there was little distribution in the liver, and scare distribution in other tissues such as heart, spleen, lung and kidney. The maximum distributed amount of FTC-octyl-CM-Chitosan-87-40 micelles in liver, spleen, heart, lung and kidney was 9.7%, 0.8%, 0.1%, 1.2% and 1.0% of dose, respectively. The maximum distributed amount of FTC-octyl-CM-Chitosan-87-60 micelles in liver, spleen, heart, lung and kidney was 11.4%, 0.8%, 0.1%, 1.3% and 0.8% of dose, respectively. The maximum distributed amount of FTC-octyl-CM-Chitosan-480-60 micelles in liver, spleen, heart, lung and kidney was 14.4%, 1.4%, 0.1%, 0.4% and 1.0% of dose, respectively. The low distribution of octyl-CM-Chitosan in tissues might be attributed to the lower interaction with tissues caused by the high negative charge on the octyl-CM-Chitosan surface.

The pharmacokinetic parameters for plasma and various tissues are shown in Table 2. Octyl-CM-Chitosan with different degree of alkylation showed similar body distributions and there were no significant difference between the pharmacokinetic parame-



Scheme 2. The (A) inter- or (B) intra-molecular association of hydrophobic alkyl chains.

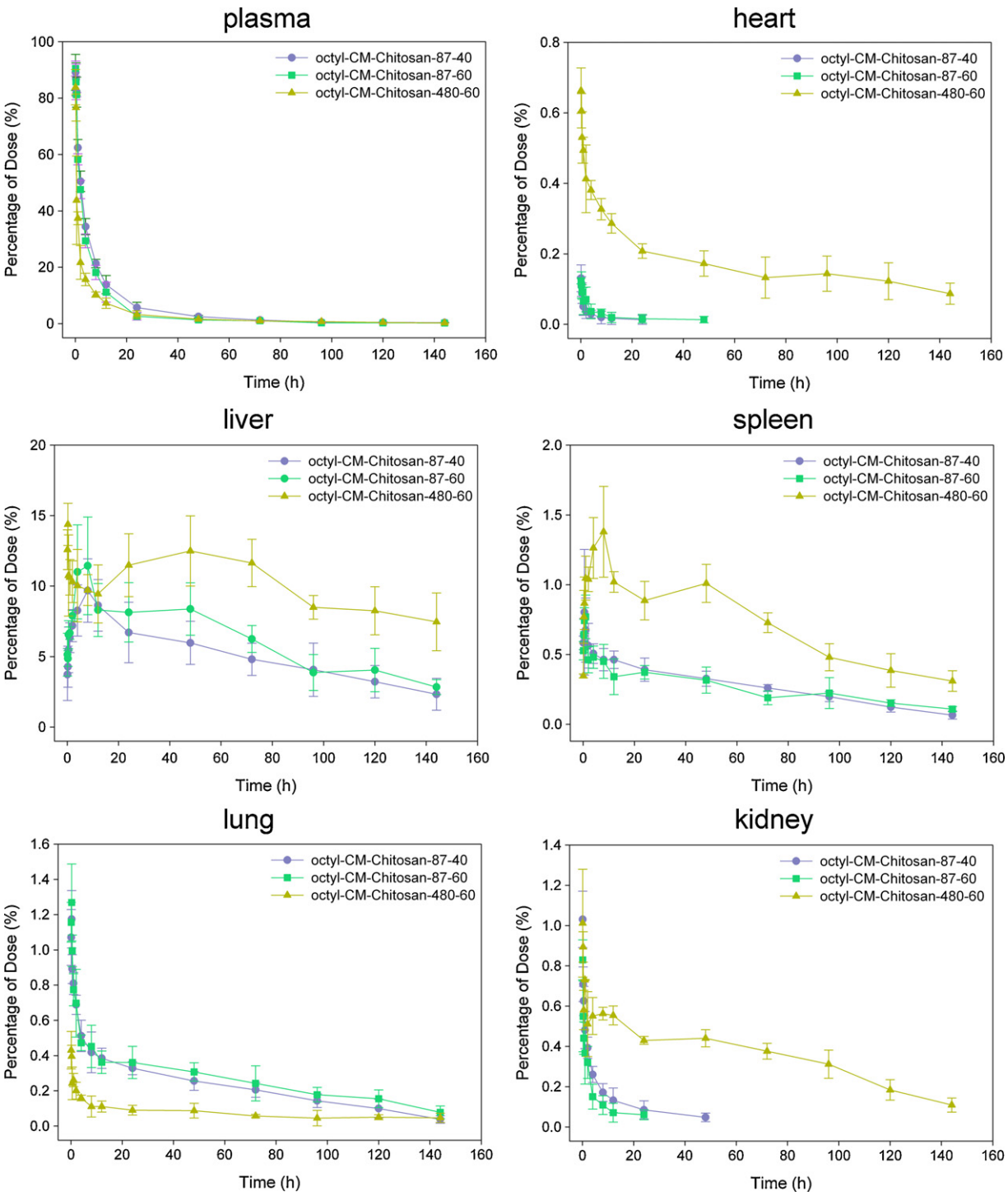


Fig. 2. Mean FTC-octyl-CM-Chitosan concentration–time profiles in plasma and organs after intravenous administration of FTC-octyl-CM-Chitosan to mice with dose 50 mg/kg (data were given as mean ± SD, $n = 6$).

ters of octyl-CM-Chitosan-87-40 and -60 groups. While, molecular weight had a significant effect on the *in vivo* behavior of octyl-CM-Chitosan. Octyl-CM-Chitosan with a higher molecular weight had a stronger interaction with blood components, leading to a decrease of plasma $AUC_{0\to\infty}$ and an increase of CL and V_d. Although there were no significant differences between the maximum distribution of octyl-CM-Chitosan in various tissues, octyl-CM-Chitosan with higher molecular weight exhibited a significantly longer retention time in tissues than octyl-CM-Chitosan with a lower molecular weight. The mean retention time (MRT) in heart, liver, spleen, lung and kidney was 2.9-, 1.6-, 2.4-, 1.9- and 3.2-fold longer for high-molecular-weight than low-molecular-weight octyl-CM-Chitosan, respectively. This might be attributed to a stronger interaction with tissue cells and a slower elimination rate. The results implied that octyl-CM-Chitosan micelles with low molecular weight could easily avoid mononuclear phagocyte system (MPS) trapping and had a long circulation time, which afforded passive targeting potential to the non-MPS disease regions, such as tumor tissues.

Table 2
Pharmacokinetic parameters for (A) plasma and (B) tissues after *i.v.* administration of FTC-octyl-CM-Chitosan to mice with dose 50 mg/kg.

Parameter	Octyl-CM-Chitosan-87-40[g]	Octyl-CM-Chitosan-87-60	Octyl-CM-Chitosan-480-60
A			
$t_{1/2}$ (h)[a]	29.1	31.9	29.6
C_0 (μg/mL)[b]	925.7	952.5	859.5
$AUC_{0\to t}$ (μg/g h)[c]	7222.5	5601.0	4124.9
CL (mL/h/kg)[d]	6.78	8.8	11.8
V_d (mL/kg)[e]	150.0	162.6	325.4
MRT (h)[f]	22.1	18.6	27.5

Tissues	Octyl-CM-Chitosan-87-40		Octyl-CM-Chitosan-87-60		Octyl-CM-Chitosan-480-60	
	$AUC_{0\to t}$ (μg/g h)	MRT (h)	$AUC_{0\to t}$ (μg/g h)	MRT (h)	$AUC_{0\to t}$ (μg/g h)	MRT (h)
B						
Heart	62.7	35.5	119.1	43.7	2386.4	126.9
Liver	7669.6	94.3	7968.6	100.7	11446.1	161.0
Spleen	3790.1	61.2	3645.9	77.7	10933.8	182.6
Lung	2451.8	79.7	2427.8	63.4	691.0	120.3
Kidney	527.6	29.6	264.8	20.2	3824.6	65.1

[a] $t_{1/2}$, elimination half-life.
[b] C_0, extrapolated peak plasma concentration.
[c] AUC, area under the plasma concentration–time curve.
[d] CL, total body clearance.
[e] V_d, volume of distribution.
[f] MRT, the mean retention time.
[g] Octyl-CM-Chitosan-*X*–*Y* where *X* indicates the molecular weight of parent chitosan in kDa, and *Y* indicates the degree of alkylation.

3.4. Polymer toxicity characterization

Octyl-CM-Chitosan micelles were aimed to be used as *i.v.* injectable nanocarriers for hydrophobic drugs. *In vivo* study showed that octyl-CM-Chitosan was mainly distributed in blood and liver in mice after *i.v.* injection. Thus, the toxicity of octyl-CM-Chitosan micelles was evaluated by hemolysis test with human red blood cells, MTT assay with mouse primary hepatocytes and histopathological effects on various organs.

The level of hemolysis of octyl-CM-Chitosan was compared with that of Tween 80 and Cremophore EL, which are typical surfactants used for increasing the solubility of hydrophobic drugs and *i.v.* administration. Fig. 3A shows hemolysis at various concentrations of octyl-CM-Chitosan, Tween 80 and Cremophore EL. As the concentration increased, hemolysis induced by Tween 80 increased dramatically. At a concentration of 4 mg/mL, hemolysis by Tween 80 reached 68.5%. Cremophore EL was non-hemolytic *in vitro* towards human RBCs (1.3% hemolysis at 4 mg/mL). Since the maximum tolerated dose of Cremophore EL for *i.v.* administration is 75 mg/day, compared with 500 mg/day for Tween 80 (Miwa et al., 1998), Cremophore EL is considered more dangerous than Tween 80, which might account for the serious side effects of Cremophore EL, such as hypersensitivity and neurotoxicity. The hemolysis of octyl-CM-Chitosan was slightly higher than that of Cremophore EL, but there were no significant difference ($p > 0.05$), which suggested that octyl-CM-Chitosan micelles were not toxic towards erythrocytes after *i.v.* injection. The chemical compositions of octyl-CM-Chitosan showed no significant effects on hemolysis.

Cell viability assay was performed to evaluate *in vitro* cytotoxicity of octyl-CM-Chitosan micelles towards mouse primary hepatocytes. As shown in Fig. 3B, octyl-CM-Chitosan micelles did not show any toxicity at a concentration of 10–200 μg/mL. The cell viability of primary hepatocytes was up to 85%, even at the highest concentration tested (300 μg/mL) which clearly indicated that octyl-CM-Chitosan had very low toxicity in mouse primary hepatocytes. No significant differences were observed among the octyl-CM-Chitosan micelles with different molecule weights and degrees of alkylation.

To investigate the histopathological effect of octyl-CM-Chitosan-87-60 on the various organs, including heart, liver, spleen, lung, and kidney, mice were *i.v.* administered with octyl-CM-Chitosan micelles at a high dose of 280 mg/kg. The histopathological

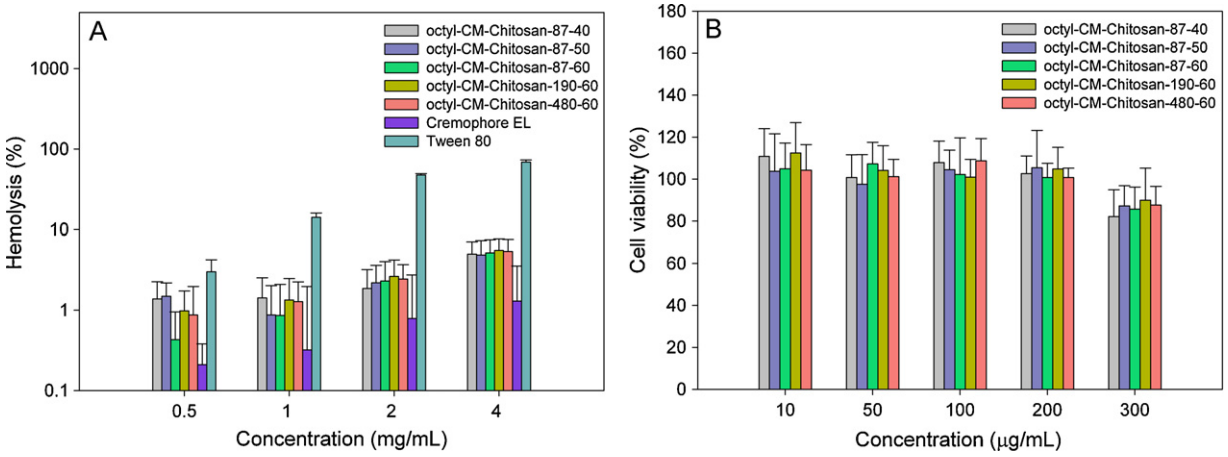


Fig. 3. *In vitro* cytotoxicity of octyl-CM-Chitosan micelles towards (A) red blood cells and (B) mouse primary hepatocytes ($n = 6$).

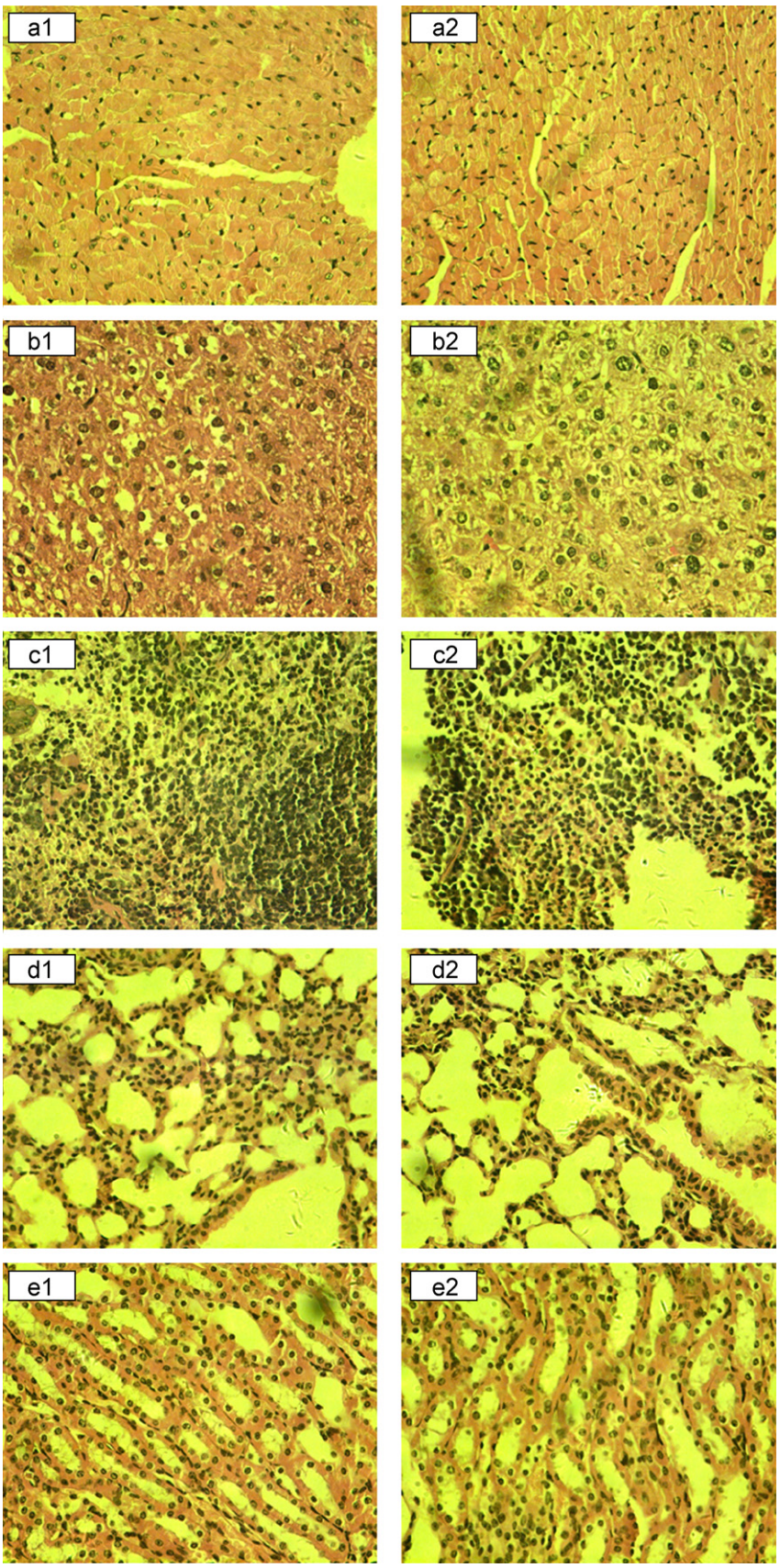


Fig. 4. Light micrographs of each organ in mouse treated with or without the octyl-CM-Chitosan micelles (100×): (A1) normal heart muscle, (A2) micelles-treated heart muscle, (B1) normal liver and (B2) micelles-treated liver, (C1) normal spleen, (C2) micelles-treated spleen, (D1) normal lung, (D2) micelles-treated lung, (E1) normal kidney, (E2) micelles-treated kidney.

changes in each organ were observed under light microscopy on day 8 after treatment with octyl-CM-Chitosan-87-60 (Fig. 4). No histopathological changes were observed in the octyl-CM-Chitosan-treated groups compared to the control group, which indicated that octyl-CM-Chitosan micelles had no significant toxicity for the main organs.

4. Conclusions

In conclusion, octyl-CM-Chitosans with lower molecular weight and higher degree of alkylation had a superior ability to form micelles. Octyl-CM-Chitosan micelles were spherical, with a particle size of <200 nm and high negative zeta potentials of ~30 mV. Additionally, multi-core microstructure of octyl-CM-Chitosan micelle was confirmed and both of molecular weight of chitosan backbone and degree of alkylation showed significant effect on the microstructure. In addition, octyl-CM-Chitosan micelles with low molecular weight could easily avoid the mononuclear phagocyte system (MPS) clearance and had the potential for passive targeting to non-MPS tissues. The absence of toxicity of octyl-CM-Chitosan added evidence to use the chitosan amphiphile as an *i.v.* material. These results demonstrate that octyl-CM-Chitosan micelles are a promising *i.v.* injectable nanocarrier for hydrophobic drugs.

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