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Preparation, characterization, and tissue distribution in mice of lactosaminated carboxymethyl chitosan nanoparticles

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ABSTRACT

Galactose group was coupled with carboxymethyl chitosan for liver specificity. The chemical structure of lactosaminated carboxymethyl chitosan (LAC-CMC) was characterized by FT-IR and ^{1}H NMR techniques. Glycyrrhizic acid was chosen as model drug and encapsulated within LAC-CMC nanoparticles through ionic gelification. Transmission electron microscope (TEM) and dynamic light scattering (DLS) were employed to characterize the nanoparticles for morphology and size. The effects, including LAC-CMC molecular weight (MW), glycyrrhizic acid concentration and LAC-CMC concentration on the physicochemical properties of the nanoparticles were studied. Glycyrrhizic acid release from the nanoparticles exhibited a biphasic pattern, initial burst release and consequently sustained release. Glycyrrhizic acid-loaded nanoparticles modify the tissue distribution profile of the glycyrrhizic acid solution, the kidney excretion rate is reduced and drug accumulation in the liver is increased. The experimental results show that the novel lactosaminated carboxymethyl chitosan nanoparticles may be used as a potential drug delivery system with hepatic targeting properties.

1. Introduction

In recent years, significant effort has been devoted to nanoparticle drug delivery systems, including nanospheres, nanocapsules, nanoliposomes, and so forth (Jung et al., 2000). Nanoparticle drug delivery systems can pass through the smallest capillary vessels because of their ultra-tiny volume and avoid rapid clearance by phagocytes so that their duration in blood stream is greatly prolonged. In addition, they can be used to provide targeted delivery of drugs, to improve oral bioavailability, to sustain drug in target tissue, and to improve the stability of therapeutic agents against enzymatic degradation (Lisa, 1995; Soppimath, Aminabhavi, Kulkarni, & Rudzinski, 2001). Polymeric nanoparticles play an important role on controlling drug release and delivering drug to the desirable action site passively.

Among polymeric nanoparticles, chitosan-based nanoparticles as drug delivery systems have received much attention (Chen et al., 2008; Elzatahry & Mohy, 2008; Shi, Du, Yang, Zhang, & Sun, 2006; Wang et al., 2009). Chitosan has been reported as a nontoxic, biocompatible and biodegradable polysaccharide, effective endocytotic uptake and low cytotoxicity were shown for chitosan nanoparticles using different cell culture models (Behren, Vila-Pena, Alonso, & Kissel, 2002; Huang, Khor, & Lim, 2004). However, chitosan is soluble only under acidic conditions, which limits some of its applications. The limited solubility of chitosan in water can be overcome by chemical modification (Jayakumar, Prabaharan, Reis, & Mano, 2005; Jayakumar, New, Tokara, & Tamura, 2007). An important chemical modification method is carboxymethylation. Carboxymethyl chitosan, a water-soluble chitosan derivative, has already been used extensively in a wide range of biomedical applications due to its unique chemical, physical, biological properties and its excellent biocompatibility. It was reported that carboxymethyl chitosan is nontoxic in vitro and in vivo (Kennedy, Constain, McAlister, & Lee, 1996; Lv et al., 2007).

Chronic hepatic disease such as hepatitis, hepatocirrhosis and hepatoma was a major class of serious disease that threating the health of people. At present, some drugs can reach the anticipative curative effect, but drugs were difficult to be transferred to the target site specifically and accurately by the drug delivery system, which caused toxicity, side-effect, a low bioavailability and limited clinic application. An effective approach to overcome this critical issue is the development of targeted drug delivery systems that release the drugs at the desired site of action. This could increase patient compliance and therapeutic efficacy of pharmaceutical agents through improved pharmacoki-

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netics and distribution (Gregoriadis, 1977; Langer, 1998; Poste & Kirsh, 1983). There are a lot of advantages in targeting delivery of drugs, which include the accumulation of drug in the action site, increase in therapeutic efficacy, reduction of therapeutic dose and toxicity, etc. (Lin, Chen, & Liu, 2009). It is well known that hepatocytes can recognize the asialoglycoprotein receptor (ASGP-R) among the liver-associated cell surface receptors and that ASGP-R is present in several human hepatoma cell lines (Ashwell & Harford, 1982; Fall & Schwartz, 1988). The receptor shows a high binding capacity and efficient cellular uptake of galactosylated ligands. Several studies indicated that the delivery system of galactose receptor-mediated endocytosis would be useful for drug targeting to hepatocyte and hepatoma cells (Mitsuaki et al., 1994; Nishikawa et al., 1993). Kato, Onishi, and Machida (2001) synthesized lactosaminated N-succinyl-chitosan by reductive amination between N-succinyl-chitosan and lactose in the presence of sodium cyanoborohydride, as a liver-specific drug carrier. Galactosylated chitosan-graft-poly (ethylene glycol) (Park et al., 2001), galactosylated chitosan-graft-dextran (Park et al., 2000) and galactosylated chitosan-graft-poly(vinyl pyrrolidone) (Park et al., 2003) as a hepatocyte-targeting DNA carrier were proved to have excellent specificity to liver cells. Recently, Yang et al. (2009) prepared lactose-conjugated PEG-graft-chitosan for liver-targeted delivery of diammonium glycyrrhizinate. Due to good water solubility, safe toxicity and excellent biocompatibility of CMC and liver specificity of galactose group, LAC-CMC may lead to a carrier that possesses the effects of inclusion and hepatic targeting properties. Therefore, the aim of this study is to synthesize and characterize novel LAC-CMC as hepatic targeting drug carriers. LAC-CMC was synthesized by reductive amination reaction. The galactose groups were easily introduced into the amino groups of carboxymethyl chitosan. LAC-CMC nanoparticles were prepared by gelification with calcium ions. The factors affecting nanoparticles formation were discussed and physicochemical properties have been characterized. The release of the nanoparticles in vitro and the tissue distribution in mice were studied to assess the merits of LAC-CMC nanoparticles as drug carriers for glycyrrhizic acid delivery.

2. Experimental

2.1. Materials

The carboxymethyl chitosan samples with different MWs and constant substitution degree (85%) were purchased from Qingdao Xunbo Biotechnology Co. Ltd (China). Glycyrrhizic acid was purchased from Nanjing Zelang Medicine Co. Ltd. (China). The mice, weighing 18–22 g, were obtained from Hubei Experimental Animal Center (Wuhan, China). All other reagents were of analytical grade.

2.2. Synthesis and purification of LAC-CMC

The LAC-CMC was synthesized by a reductive amination reaction (Zhang, Ping, & Ding, 2005). Briefly, CMC (0.5 g) was dissolved in 15 ml distilled water and 45 ml methanol was added, then α-lactose (4 g) was added. After stirring for 4 h, potassium borohydride (1.5 g), dissolved in 15 ml distilled water, was mixed with the solution, which was stirred at room temperature for 72 h. At the end of reaction, a part of the methanol was removed on a rotary evaporator under vacuum. The residual solution was dialyzed (molecular weight cutoff 12,000) against distilled water for 72 h and then lyophilized. Molecular weights of the prepared LAC-CMC were measured by gel-permeation chromatography (GPC), as 1.6, 4.7, 8.6, 17.5 and 34.6×10^4, respectively.

2.3. Measurement

The ^{1}H NMR spectra were recorded in D_2O on a Bruker (AVACE) AV-500 spectrometer. FT-IR spectra were performed on a Fourier-transform infrared spectrometer (Thermo Nicolet) in KBR disc.

2.4. Preparation of drug-free and drug-loaded LAC-CMC nanoparticles

LAC-CMC nanoparticles were prepared through ionic gelification with calcium ions. LAC-CMC was dissolved in double distilled water at different concentration. Then $CaCl_2$ solution was added to LAC-CMC solution dropwise under mild magnetic stirring, and the system changed spontaneously from a clear solution to an opalescent emulsion (Tyndall effect), which was further measured by TEM as nanoparticles. The glycyrrhizic acid loading nanoparticles were formed in the same way by adding $CaCl_2$ solution to LAC-CMC solution containing glycyrrhizic acid with various concentrations.

CMC nanoparticles were prepared under similar reaction conditions.

2.5. Physicochemical characterization

TEM (JEM-100CX11, JEOL) was used to observe the morphology of the LAC-CMC nanoparticles. Samples were placed onto copper grill and dried at room temperature, then examined without being stained.

The particle size of nanoparticles measured by Nano-ZS ZEN3600 (MALVERN Instrument) gives information about the average particle size. All measurements were performed at room temperature.

To determinate the encapsulation efficiency (EE), the glycyrrhizic acid loading nanoparticles were separated from the aqueous suspension medium by ultra centrifugation with $20{,}000 \times g$ (KDC-160HR) at 4 °C for 30 min. The amount of free glycyrrhizic acid in the clear supernatant was determined using Shimadzu HPLC system (Japan) equipped with a reverse-phase column C18 (250 mm × 4.6 mm). The mobile phase was a mixture of acetonitrile and 25 mM sodium acetate (1:3, v/v). The flow rate was maintained at 1 ml/min and the eluent was monitored with UV detector at 254 nm. All measurements were performed in triplicate.

The glycyrrhizic acid encapsulation efficiency of the nanoparticles was calculated with the following equation:

$$\mathrm{EE}\ (\%) = (\text{the weight of drug added into the system} - \text{the weight of free drug in the supernatant after centrifugation}) \times \frac{100}{\text{the weight of drug added into the system}}$$

2.6. Release experiment in vitro

3.0 ml nanoparticles suspension was enclosed in dialysis bags (cellulose membrane, molecular weight cutoff 12,000), and incubated in 30 ml phosphate buffered saline (PBS, pH 7.4) at 37 °C under mild agitation. At predetermination time intervals, 1.0 ml sample was withdrawn from the dialysis medium and analyzed for glycyrrhizic acid with HPLC as described above. After sampling, the dialysis medium was replaced by fresh PBS. Each experiment was done in triplicate.

2.7. Tissue distribution in mice

One hundred and sixty-five mice were divided into three groups randomly and fasted for 12 h with free access to water.

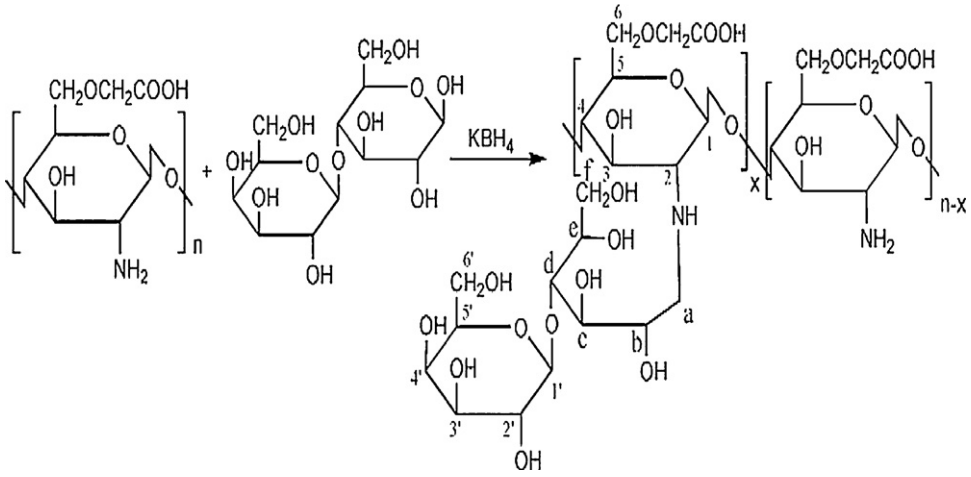

Fig. 1. Chemical structure of LAC-CMC.

The glycyrrhizic acid solution and CMC nanoparticle suspension (the added initial glycyrrhizic acid concentration was 1.0 mg/ml, the glycyrrhizic acid concentration in nanoparticle suspension was 0.58 mg/ml) equivalent to 50 mg/kg of glycyrrhizic acid were i.v. injected into the tail vein of mice, respectively (the control group). The LAC-CMC nanoparticle suspension (the added initial glycyrrhizic acid concentration was 1.0 mg/ml, the glycyrrhizic acid concentration in nanoparticle suspension was 0.67 mg/ml) equivalent to 50 mg/kg of glycyrrhizic acid was i.v. injected into the tail vein of another group of mice (the test group). The heart, liver, spleen, lungs, kidneys of each mouse were rapidly excised following sacrifice at the time intervals of 15, 30, 60 min and 2, 4, 6, 8, 12, 24, 48, and 96 h, respectively, immediately washed twice with normal saline (0.9%NaCl), wiped with filter paper, weighed and homogenized with 1.0 ml of normal saline (0.9% NaCl). The methanol (1 ml) was added to the homogenate (0.5 ml), after centrifuging for 15 min

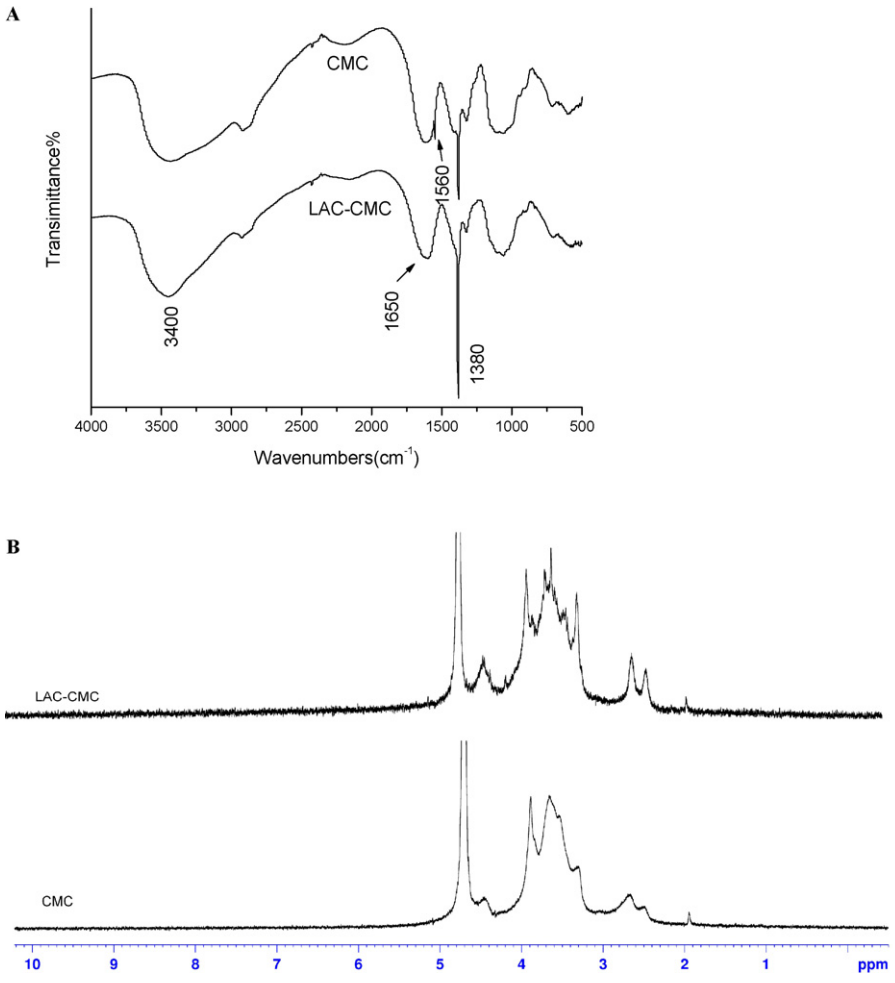

Fig. 2. FT-IR spectrum (A) and ^{1}H NMR spectrum (B) of LAC-CMC and CMC.

(12000 rpm), 20 μL of supernatant was injected into the HPLC system for analysis.

3. Results and discussion

3.1. Preparation and characterization of LAC-CMC

Carboxymethyl chitosan was coupled with lactose bearing galactose group for liver specificity. Fig. 1 shows the chemical structure of LAC-CMC. The basic chemical structure of LAC-CMC was confirmed with ^{1}H NMR and FT-IR spectrum. As shown in Fig. 2A, the absorption band at 3400 cm^{-1} is assigned to stretching vibrations of the amino and hydroxyl groups. Compared with the spectra of CMC, the peak intensity at 3400 cm^{-1} in the spectrum of LAC-CMC was increased due to the increase of hydroxyl groups. The band at 1560 cm^{-1} is attributed to amino groups. In contrast, the absorption peak for the N–H band was greatly weakened. This confirmed that lactosaminated groups were introduced into the amino groups of CMC.

The ^{1}H NMR spectra of CMC and LAC-CMC are given in Fig. 2B. The ^{1}H NMR (D_2O) spectra of LAC-CMC showed signals at $\delta = 2.61$ and 2.44, which were attributed to H_a and H_2, respectively. The peaks at $\delta = 3.27$–4.13 correspond to H_3, H_4, H_5, H_6, H_2, H_3, H_4, H_5, H_6, H_b, H_c, H_e, H_f. The other protons of LAC-CMC were assigned to $\delta = 4.32$ (H_1'), $\delta = 4.41$ (H_1). This suggested that the lactosaminated reaction had occurred and the substitution degree was determined as 0.20.

3.2. Physicochemical characterization of the nanoparticles

The morphology of LAC-CMC nanoparticles was examined by TEM in Fig. 3. LAC-CMC nanoparticles (Fig. 3A) and glycyrrhizic acid-loaded LAC-CMC nanoparticles (Fig. 3B) also take near spherical shape.

3.3. Factors influencing the preparation of nanoparticles and encapsulation of glycyrrhizic acid

3.3.1. Effect of LAC-CMC molecular weight

Fig. 4A shows the influence of LAC-CMC molecular weight on the size. A gradual increase in the particle size with the increase in molecular weight was noted. As shown in Fig. 4B, the EE of glycyrrhizic acid increased from 40% to 70% with increased MW of LAC-CMC. Compared with short molecular chains of low molecular weight LAC-CMC, the relatively longer chain makes the encapsulation of glycyrrhizic acid much easier due to some physical interaction such as adsorption and entrapment.

3.3.2. Effect of glycyrrhizic acid concentration

Fig. 5A demonstrated that the particle size of glycyrrhizic acid-loaded nanoparticles increased as the concentration of glycyrrhizic acid increased from 0.2 to 1 mg/ml. As shown in Fig. 5B, encapsulation efficiency of the nanoparticles were effected by the initial glycyrrhizic acid concentration in the LAC-CMC solution. The increase of glycyrrhizic acid concentration led to an increase of encapsulation efficiency.

3.3.3. Effect of LAC-CMC concentration on encapsulation efficiency

In terms of the effect of LAC-CMC concentration on entrapment, it seems that higher LAC-CMC concentration might contribute to higher entrapment ability. However, it was reported that too high CMC concentration made encapsulation extremely difficult (Vandenberg, Drolet, Scott, & Noüe, 2001). Fig. 6 exhibits the effect of LAC-CMC concentration on glycyrrhizic acid encapsulation efficiency. Glycyrrhizic acid encapsulation efficiency increased within the LAC-CMC concentration range of 0.5–2 mg/ml and decreased after that point.

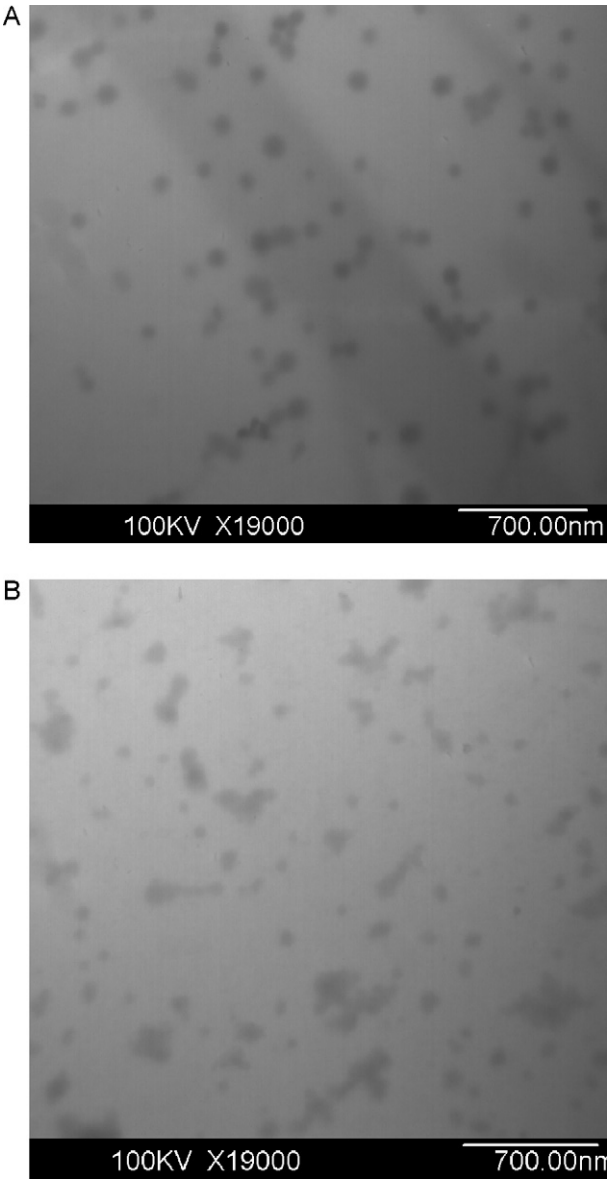

Fig. 3. TEM of LAC-CMC nanoparticles (A) and glycyrrhizic acid-loaded LAC-CMC nanoparticles (B).

To explain the above phenomena, the formation mechanism of LAC-CMC nanoparticles should be mentioned. The formation depends on the electrostatic polyelectrolyte interaction between negative LAC-CMC and positive calcium ions. In this process, $CaCl_2$ acts as an ionic crosslinking reagent. In the crosslinking, there is an optimal ratio between LAC-CMC and $CaCl_2$ to form the nanoparticles. In Fig. 7, 2 mg/ml LAC-CMC versus 1 mg/ml $CaCl_2$ should be the optimal ratio. Beyond the optimal ratio, with a fixed $CaCl_2$ concentration, the ratio of $CaCl_2$ to LAC-CMC lessened along with raising LAC-CMC concentration and hence less and less nanoparticles formed. For this reason, moderate concentrations and ratios of $CaCl_2$ and LAC-CMC are key to obtain a large number of nanoparticles.

The LAC-CMC nanoparticles used for experiment of release behavior and tissue distribution in mice were prepared as: LAC-CMC was dissolved in double distilled water with a concentration of 2.0 mg/ml, then glycyrrhizic acid was added to the solution at 1.0 mg/ml. $CaCl_2$ solution with concentration of 1.0 mg/ml was

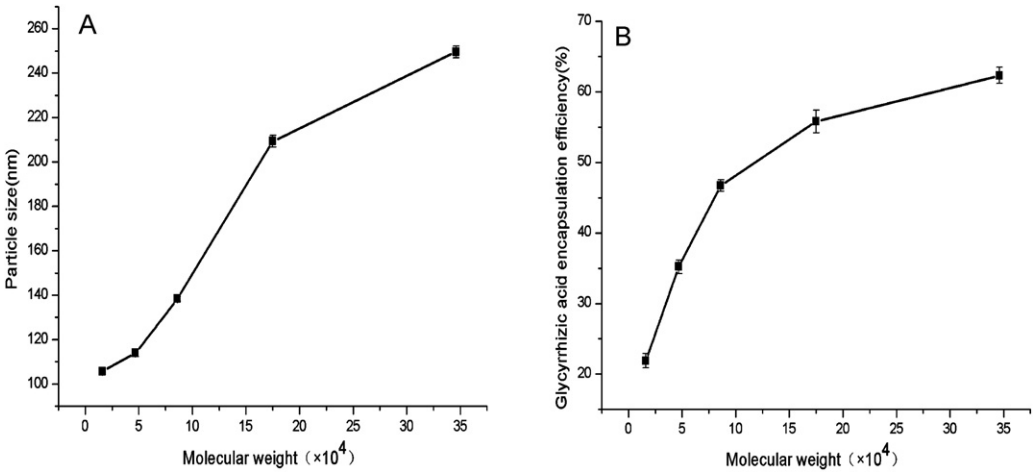

Fig. 4. The particle size (A) and glycyrrhizic acid encapsulation efficiency (B) of LAC-CMC nanoparticles with various MW.

added to LAC-CMC solution containing glycyrrhizic acid dropwise under mild magnetic stirring, and the system changed spontaneously from a clear solution to an opalescent emulsion (Tyndall effect), the EE of glycyrrhizic acid was 67.2%, the loading capacity was 17.5%.

3.4. The release behavior in vitro of the nanoparticles

The in vitro release profile of glycyrrhizic acid loaded LAC-CMC with various MWs nanoparticles in 0.01 M PBS (pH 7.4) is shown in Fig. 7. The particles with MW 4.7×10^4 showed an initial burst of 55% was observed within the first 2 h of incubation. However, after the initial burst release, drug release from the particle became rather low and glycyrrhizic acid release ranged from 55% to 69% in 3 days. For nanoparticles prepared from higher MW, reduced initial burst release was observed. It is obvious that the release of lower MW is much faster, and this was as expected because nanoparticles with lower MW has smaller size and larger volume-to surface area, which speeded glycyrrhizic acid initial release from surface (Shi et al., 2006). The release involves two different mechanisms of drug diffusion and polymer matrix degradation (Zhou, Deng, & Li, 2001). The burst release of drug was probably due to a part of the drug adsorbed or close to the surface of the nanoparticles that would be immediately released during the initial stage (Magenheim, Levy, &

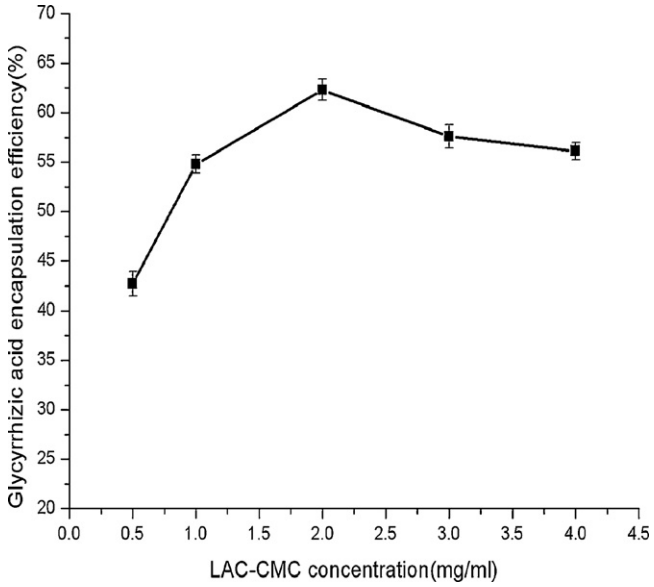

Fig. 6. Effects of LAC-CMC concentration on glycyrrhizic acid encapsulation efficiency.

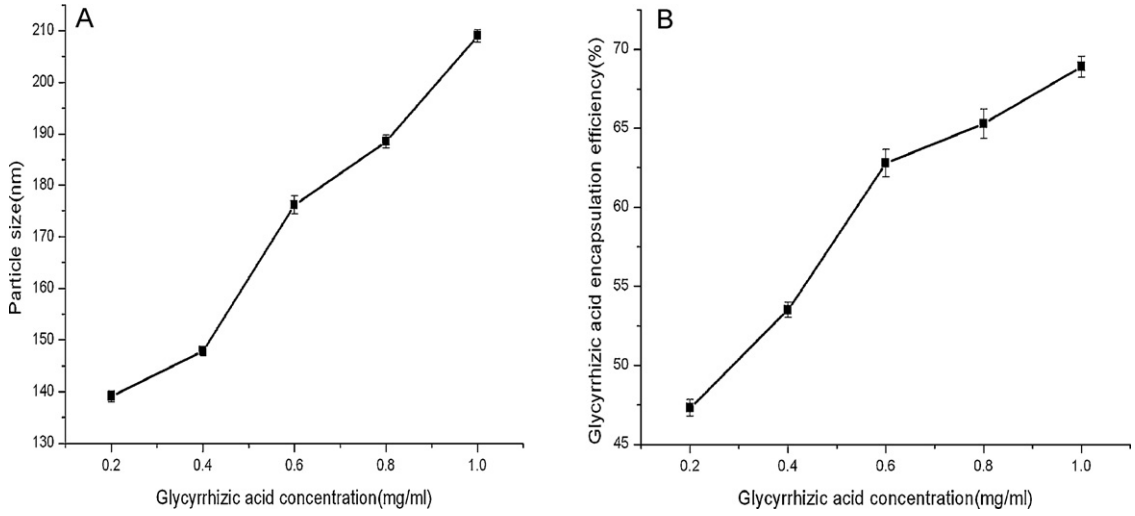

Fig. 5. The particle size (A) and glycyrrhizic acid encapsulation efficiency (B) of glycyrrhizic acid-loaded nanoparticles as a function of the final glycyrrhizic acid concentration added to LAC-CMC nanoparticles.

Benita, 1993). The initial phase of release is attributed to the glycyrrhizic acid located at the surface of the particles. After the initial burst release, the drug release profile displayed a typical sustained fashion. The sustained release would mainly depend on the drug diffusion and the copolymer matrix degradation that was a slower process. It has been shown high MW polymer have slow degradation rate, preventing the drug from diffusing from the copolymer matrix into the aqueous solution due to increased chain packing and rigidity (Zhang & Neau, 2002).

3.5. The tissue distribution in mice

LAC-CMC nanoparticles, CMC nanoparticles and glycyrrhizic acid solutions were taken up by the heart, liver, spleen, lungs, and kidneys of mice. Fig. 8 shows the glycyrrhizic acid levels in the heart, liver, spleen, lungs and kidneys at different time points after i.v. administration of LAC-CMC nanoparticles, CMC nanoparticles and glycyrrhizic acid solutions. The distribution of glycyrrhizic acid

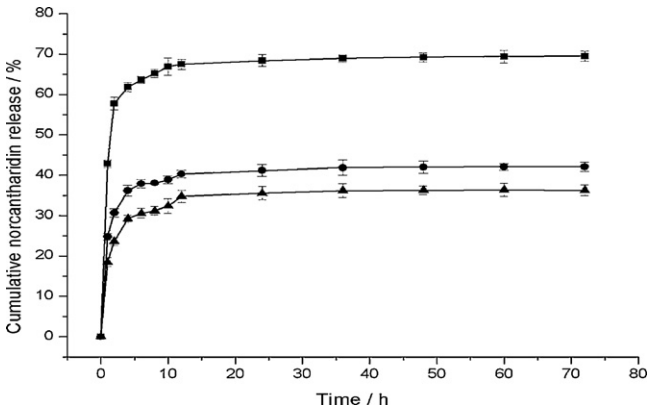

Fig. 7. The influence of MW on glycyrrhizic acid release behavior: (■) MW = 4.7×10^4; (●) MW = 8.6×10^4; (▲) MW = 17.5×10^4.

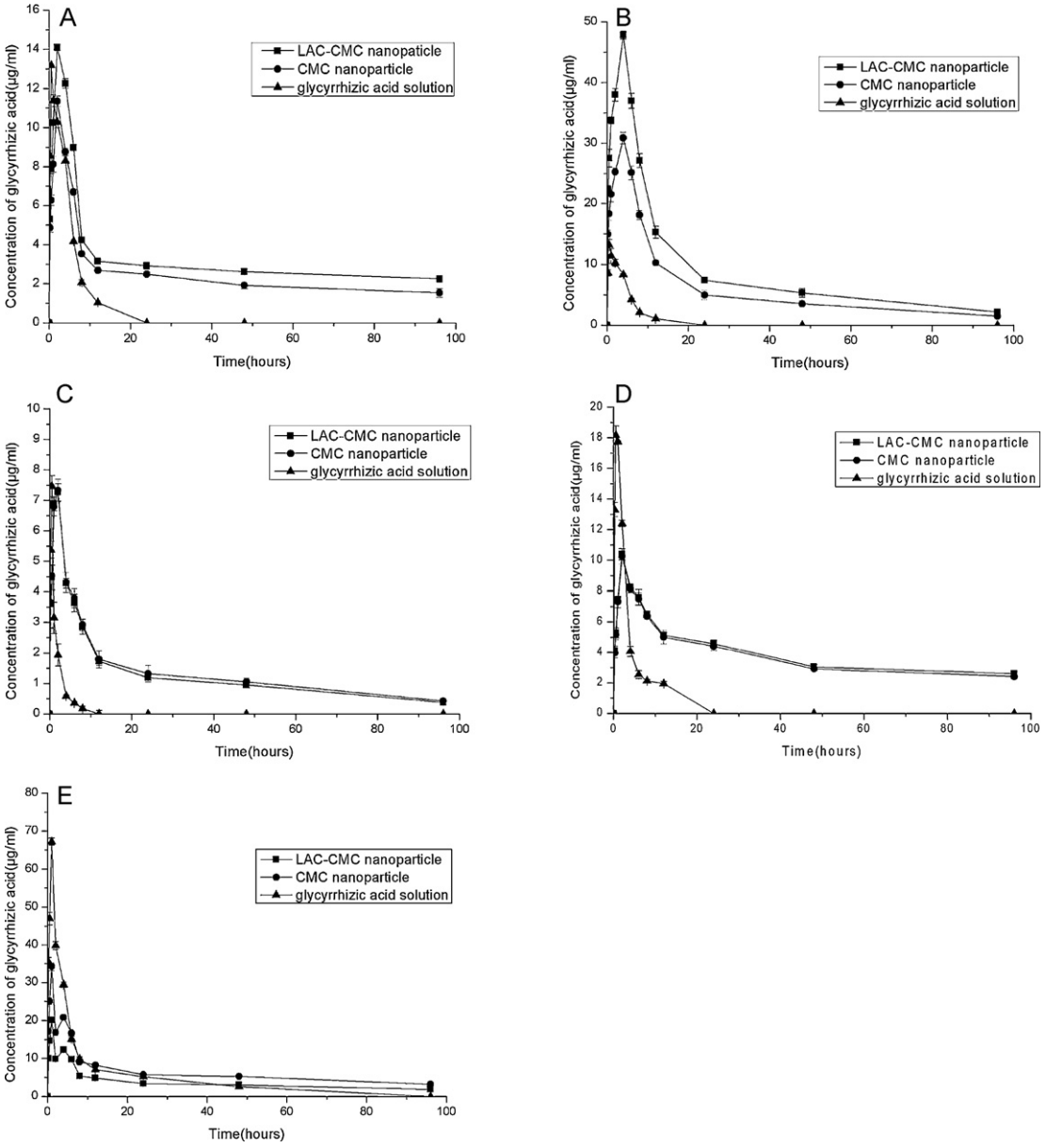

Fig. 8. Tissue distribution profile after intravenous injection of glycyrrhizic acid nanoparticles and glycyrrhizic acid solution in mice organs: (A) heart; (B) liver; (C) spleen; (D) lungs; (E) kidneys.

from LAC-CMC nanoparticles in the liver reached the maximum of 47.82 μg/ml at 4 h post-injection, the distribution of glycyrrhizic acid from CMC nanoparticles reached the maximum of 30.88 μg/ml at 4 h post-injection, and that from the glycyrrhizic acid solutions reached the maximum of 13.19 μg/ml at 0.5 h post-injection. The liver uptake of LAC-CMC nanoparticles was significantly higher than that of CMC nanoparticles and glycyrrhizic acid solutions. However, the glycyrrhizic acid from solutions reached the maximum in the kidney tissue. Liver tissue, the major site of activity for glycyrrhizic acid, presented much higher glycyrrhizic acid concentration after nanoparticles administration. Kidney tissue, the major site of toxicity for glycyrrhizic acid, presented much higher glycyrrhizic acid concentration after solutions administration.

4. Conclusions

CMC derivative containing galactose moiety, which was recognized specifically by the ASGR, was synthesized by introduction of the galactose group into the amino group of CMC. The structure of LAC-CMC was characterized by FT-IR, ^{1}H NMR techniques. LAC-CMC nanoparticles loaded glycyrrhizic acid could be satisfactorily prepared by the ionic gelification with calcium ions. TEM demonstrate they exhibit a shape of near sphere with a smooth surface. The nanoparticle preparation was affected by LAC-CMC molecular weight, glycyrrhizic acid concentration and LAC-CMC concentration. The drug release from the nanoparticles exhibits a biphasic pattern, initial burst release and consequently sustained release. The administration in mice model showed that this delivery system can be an efficient strategy to improve the bioavailability of glycyrrhizic acid in the liver. The LAC-CMC may be used as a potential drug carrier with hepatic targeting.

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