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# In vitro characterization, and in vivo studies of crosslinked lactosaminated carboxymethyl chitosan nanoparticles

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ABSTRACT

The liver targeting and controlled release nanoparticles based on carboxymethyl chitosan derivatives were prepared: firstly, novel thiolated lactosaminated carboxymethyl chitosan (LAC-CMC) was synthesized, its chemical structure was characterized by $^1$H NMR spectroscopy. Then, glycyrrhizic acid was chosen as model drug and encapsulated within thiolated LAC-CMC nanoparticles through ionic gelification. The crosslinked glycyrrhizic acid-loaded nanoparticles dissociated to release drug in the presence of glutathione (GSH) at a concentration comparable to the intracellular environment, featuring the potential ability of this system for intracellular delivery. Crosslinked nanoparticles modify the tissue distribution profile of the glycyrrhizic acid solution, the kidney excretion rate is reduced and the drug accumulation in the liver is increased. According to these results, the nanoparticles have the potential to be used as drug delivery system with hepatic targeting and controlled release properties.



## 1. Introduction

Recently, there have been many studies of targeting liver systems using methods such as passive trapping of nanoparticles or active targeting based on hepatic receptor recognition (Kato, Onishi, & Machida, 2001a, 2001b; Kim et al., 2005; Wang et al., 2010). Nanoparticles with moderate size can be delivered to specific sites by size-dependent passive targeting. It has been reported that when the diameter is less than 200 nm, the nanoparticles can be captured easily by Kupffer cells in the liver (Allémann, Gurny, & Doelker, 1993; Wang et al., 2010). Active targeting systems of receptor recognition can be attained by using the molecules with receptor trapping specific ligands. It is well known that hepatocytes can recognize the asialoglycoprotein receptor (ASGP-R) among the liver-associated cell surface receptors (Ashwell & Harford, 1982; Fall & Schwartz, 1988). The receptor shows high binding capacity and efficient cellular uptake of galactosylated ligands. For this reason, the ASGP-R has been exploited as a hepatocyte-specific marker for drug and gene delivery (Park et al., 2003, 2001, 2000). Considerable effort has been made to utilize galactose or lactose as ligand of hepatocyte-specific targeted agents. Kato et al. (2001a, 2001b) 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. Recently, Yang et al. (2009) prepared lactose-conjugated poly(ethylene glycol) (PEG)-graft-chitosan for liver-targeted delivery of diammonium glycyrrhizinate (DG). The lactose conjugated polyion complex micelles (PIC micelles) delivered more DG to the liver than conventional PIC micelles, indicating that LAC-PIC micelles were promising liver-targeted nanocarriers for DG.

Among the polymers generally used in the pharmaceutical field, the cationic polymer chitosan is biocompatible, biodegradable and nontoxic (Qi, Xu, Li, Jiang, & Han, 2005). Owing to these properties, it has many applications such as biomaterials for tissue engineering, in wound healing and as excipients for drug and gene delivery (Ganji & Abdekhodaie, 2008; Heras, Rodriguez, Ramos, & Agullo, 2001; Inez, Lubben, Verhoef, Borchard, & Junginger, 2001; Yoksan & Akashi, 2009). However, due to its water insolubility, the use of chitosan in biomedical field is restricted. To eliminate this drawback, versatile modifications were performed on the hydroxyl and amino groups on glucosamine unites of chitosan. O-carboxymethyl-chitosan (CMC) has successfully overcome the limited water solubility and has been proved to have favorable biocompatibility as chitosan. CMC is often used as a pharmaceutical excipient (Lu et al., 2007; Palmberger, Hombach, & Bernkop-Schnürch, 2008; Prabaharan & Gong, 2008; Prabaharan & Mano, 2007; Zhao et al., 2010). In spite of considerable research focused on grafting galactose or lactose onto chitosan for hepatic targeting, relatively few reported studies are about grafting galactose or lactose onto CMC.

A number of thiolated chitosan have been studied thus far (Jayakumar, Nwe, Tokura, & Tamura, 2007; Jayakumar, Reis, & Mano, 2007). Covalently bonded disulfides can be formed spontaneously by autoxidation of sulfhydryls, primarily via oxidation

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upon exposure to air, which can reversibly be cleaved in the presence of reducing agents. The advantage of the nanoparticles with a disulfide crosslinkage in drug delivery is that the disulfide bond is stable in the blood, but cleaved inside the cell. Because the concentrations of glutathione, the most abundant reducing agent in most cells including mammals, are in a millimolar range inside the cells, whereas those in blood plasma are in a micromolar range (Meister & Anderson, 1983). The reversible nature of the disulfide bond is exploited in drug and gene delivery (Kakizawa, Harada, & Kataoka, 1999, 2001; Miyata et al., 2005; Vachutinsky et al., 2011). Consequently, a hepatic targeting and controlled release drug carriers with thiolated LAC-CMC can be provided.

The objective of this study was to synthesize and characterize novel thiolated LAC-CMC as hepatic targeting and controlled drug release carriers for glycyrrhizic acid. It is expected that dissociation of nanoparticles in the blood is suppressed through the covalent link between thiolated LAC-CMC. Once inside the cell, the disulfide should be cleaved, leading to the release of the entrapped glycyrrhizic acid. To study its delivery property, thiolated LAC-CMC/glycyrrhizic acid nanoparticle were prepared by gelification with calcium ions, and were characterized by shape, particle size, zeta potential, glycyrrhizic acid encapsulation efficiency (EE), loading capacity (LC) and in vitro release. The pharmacokinetic parameters in rabbits and tissue distribution in mice were evaluated.

## 2. Experimental

### 2.1. Materials

CMC (viscosity average molecular weight $8.6 \times 10^4$ and substitution degree 85%) was purchased from Qingdao Xunbo Biotechnology Co. Ltd. (China). Glycyrrhizic acid was purchased from Nanjing Zelang Medicine Co. Ltd. (China). 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), and N-hydroxysuccinimide (NHS) were obtained from Sigma–Aldrich and used without further purification. The mice, weighing 18–22 g, and the rabbits, weighing 1.5 kg, 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, then the mixed solution 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.

### 2.3. Synthesis of thiolated LAC-CMC

A solution of LAC-CMC (1.5 g in 50 ml of distilled water) was treated with 0.192 g (1 mmol) of EDC and 0.115 g (1 mmol) of NHS for 30 min in order to activate the carboxyl groups of LAC-CMC. 0.15 g of cysteamine hydrochloride was then added. The pH of the reaction mixture was adjusted using NaOH and HCl. After a reaction period of 12 h at room temperature under constant stirring, the reaction mixtures were dialyzed for 3 days in total at 4 °C in the dark. Thereafter, samples were lyophilized. The product was stored at 4 °C until further use.

### 2.4. Characterization

$^1$H NMR spectrum of the samples was recorded on a Bruker DPX 300 spectrometer using tetramethylsilane as internal standard and $D_2O$ as a solvent at 25 °C.

### 2.5. Determination of thiol groups

The amount of thiol groups present on thiolated LAC-CMC was determined spectrophotometrically with Ellman's reagent (Riddles, Blakeley, & Zerner, 1979). First, 0.5 mg of thiolated LAC-CMC were hydrated in 250 μl of 0.5 M phosphate buffer (PBS, pH 8.0) and 500 μl Ellman's reagent (3 mg of 5,5′-dithiobis (2-nitrobenzoic acid) in 10 ml of 0.5 M PBS (pH 8.0)) was added. The samples were incubated for 3 h at room temperature. The supernatant was separated from the precipitated polymer by centrifugation (18,000 rpm, 5 min). Thereafter, the absorbance was measured at wavelength of 450 nm. The quantity of free thiol groups was calculated from a standard curve obtained by solutions with increasing concentrations of L-cysteine hydrochloride hydrate.

### 2.6. Preparation of thiolated LAC-CMC nanoparticles

Thiolated LAC-CMC (20 mg) was dissolved in 10 ml PBS (pH 7.4) and glycyrrhizic acid (10 mg) was added. Then $CaCl_2$ solution (1 mg/ml) was added to the 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 transmission electron microscope (TEM). As a control, unmodified LAC-CMC was used for nanoparticles formulation.

### 2.7. Physicochemical characterization

The average size and size distribution of nanoparticles were measured by Nano-ZS ZEN3600 (MALVERN Instrument). All measurements were carried out at 25 °C. The data were analyzed by the cumulant method to obtain the size and the size distribution, respectively.

Zeta potential measurement of the nanoparticles (both non-crosslinked and crosslinked system) dispersion in dialyzed water were performed at 25 °C. The nanoparticles were dispersed in dialyzed water, and the zeta potential was measured at the default parameters of dielectric constant, refractive index, and viscosity of water. The sampling time was set to automatic.

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

To determinate the encapsulation efficiency (EE), the glycyrrhizic acid loading nanoparticles were separated from the aqueous suspension medium by ultracentrifugation with 20,000 rpm (KDC-160HR) at 4 °C for 30 min. The amount of free glycyrrhizic acid in the clear supernatant was determined using Shimadzu high performance liquid chromatography (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.

EE of glycyrrhizic acid loaded and loading capacity (LC) were calculated from the following equation:

$$\text{EE}\ (\%) = \text{total amount of drug} - \text{free drug} \times \frac{100}{\text{total amount of drug}}$$

$$\text{LC}\ (\%) = \text{total amount of drug} - \text{free drug} \times \frac{100}{\text{nanoparticles dry weight}}$$

### 2.8. Release experiment in vitro

Lyophilized nanoparticles dissolved in 3 ml PBS was tested in the glutathione (GSH) solution with various concentrations (0, 10 μM, 100 μM, 1 mM) in 30 ml PBS (pH 7.4). At predetermination time intervals, 1.0 ml sample was drawn from the dialysis medium and analyzed for glycyrrhizic acid with HPLC as described above.

### 2.9. Pharmacokinetic and distribution studies

Fifteen rabbits were divided randomly into three groups and were fasted for 12 h with free access of water. The glycyrrhizic acid solution, unmodified LAC-CMC nanoparticles (the added initial glycyrrhizic acid concentration was 1.0 mg/ml, the glycyrrhizic acid concentration in nanoparticle suspension was 0.627 mg/ml) and crosslinked thiolated LAC-CMC (the added initial glycyrrhizic acid concentration was 1.0 mg/ml, the glycyrrhizic acid concentration in nanoparticle suspension was 0.619 mg/ml) nanoparticles equivalent to 50 mg/kg of glycyrrhizic acid were i.v. injected into the ear vein of each group of rabbits.

After i.v. injection, 0.5 ml of blood was taken from the ear vein of each rabbit at the same intervals of 15, 30, and 60 min and 2, 4, 8, 12, 24, 48, and 72 h, respectively. 100 μl supernatant plasma samples were collected after centrifugation at 3000 rpm for 10 min. 400 μl methanol was added to the supernatant, after agitating 3 min and centrifuging for 15 min (12,000 rpm), 20 μl of supernatant was injected into the HPLC system for analysis.

One hundred and fifty mice were divided randomly into three groups and were fasted for 12 h with free access to water. The glycyrrhizic acid solution, non-crosslinked thiolated LAC-CMC nanoparticles and crosslinked thiolated LAC-CMC nanoparticles equivalent to 50 mg/kg of glycyrrhizic acid were i.v. injected into the tail vein of each group of mice, respectively.

The heart, liver, spleen, lungs and kidneys of each mouse was rapidly excised following sacrifice at the time intervals of 15, 30, and 60 min and 2, 4, 8, 12, 24, 48 and 72 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 (12,000 rpm), 20 μl of supernatant was injected into the HPLC system for analysis.

## 3. Results and discussion

### 3.1. Preparation and characterization of thiolated LAC-CMC

Thiolated LAC-CMC was synthesized using a two-step procedure. The first step involves a reductive amination reaction, carboxymethyl chitosan was coupled with lactose bearing galactose group for liver specificity. In the second step, carboxyl groups of LAC-CMC were conjugated with the amino groups of cysteamine hydrochloride (Fig. 1). It was reported that the pH-value during the thiolation reactions has a great impact on the amount of thiol groups bounded onto the polymer back bone (Kast & Bernkop-Schnurch, 2001). In this work, the preparation of thiolated LAC-CMC was performed at pH 3, 5, 6, 7, and 9 in order to determine the optimum pH-value for the maximum covalent attachment of thiol groups. The results showed that at pH 3 and pH 5, the amount of covalently attached thiol groups was low due to the poor efficiency of EDC coupling reaction and poor solubility of LAC-CMC at the two

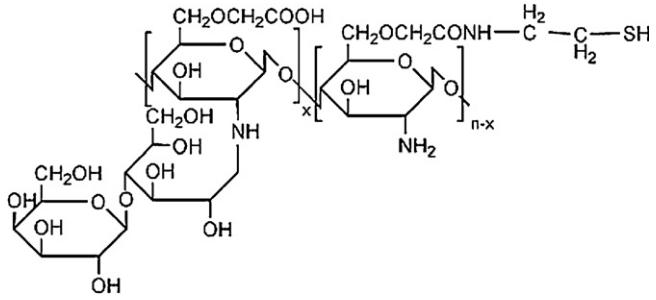


**Fig. 1.** Chemical structure of thiolated LAC-CMC.

pH-value. The grafting reactions, which were performed at pH 6, led to the highest yield in the thiolated polymer. At this pH, the efficient EDC-mediated reaction between cysteamine and LAC-CMC may be responsible for this observation. In contrast, at pH 7 and pH 9 the yield of polymer-bound thiol groups decreased again. The reason for this observation may be the oxidation of the thiol groups during the reaction (Bernkop-Schnurch, Leitner, & Moser, 2004). Table 1 shows the types of thiolated polymers prepared under different pH value and their thiol groups contents.

Fig. 2 shows the $^1$H NMR spectra of CMC, LAC-CMC and thiolated LAC-CMC. The spectrum of CMC shows the protons of 2-amino-2-deoxy-b-D-glucopyranosyl residues at 3.0, 3.3–4.0 ppm. The chemical shift at 4.4 ppm was the protons of carboxymethyl groups at $C_6$ position of the CMC. The spectrum of thiolated LAC-CMC shows signals at 2.65–2.8, which were originated from methylene protons of cysteamine. Also, new peaks at 4.32 ppm and 3.27–4.13 ppm were observed due to the presence of lactose. These results clearly confirmed that CMC has been successfully grafted with both lactose and cysteamine.

### 3.2. Physicochemical characterization of nanoparticles

Particle size, zeta potential, entrapment efficiency and loading capacity of non-crosslinked LAC-CMC and crosslinked thiolated LAC-CMC nanoparticles are shown in Table 2. The morphology of thiolated LAC-CMC nanoparticles was examined by TEM in Fig. 3. The nanoparticles take near spherical shape.

### 3.3. The release behavior in vitro of the crosslinked nanoparticles

It is reported that the intracellular environment is more reductive than the extracellular fluid and the concentrations of glutathione (GSH) in mammalian cells are in the millimolar range (0.5–10 mM), whereas micromolar concentrations are found in blood plasma (Meister & Anderson, 1983). The considerable gap

**Table 1**
Types of thiolated polymers and their reaction pH and thiol group contents ($n = 3$).

| Polymer type | pH | Thiol groups (μmol/g polymer ± SD) |
|---|---|---|
| Thiolated LAC-CMC 1 | 3 | 81.09 ± 10.45 |
| Thiolated LAC-CMC 2 | 5 | 129.54 ± 14.97 |
| Thiolated LAC-CMC 3 | 6 | 412.39 ± 31.52 |
| Thiolated LAC-CMC 4 | 7 | 380.17 ± 30.86 |
| Thiolated LAC-CMC 5 | 9 | 192.48 ± 25.33 |

**Table 2**
Size, zeta potential, EE and LC of non-crosslinked and crosslinked nanoparticles ($n = 3$).

| Nanoparticles | Diameter (nm) | PDI | Zeta potential | EE (%) | LC (%) |
|---|---|---|---|---|---|
| Unmodified LAC-CMC | 117.3 ± 14.9 | 0.068 | −16.2 | 62.7 | 17.3 |
| Crosslinked LAC-CMC | 123.9 ± 16.7 | 0.15 | −6.8 | 61.9 | 17.1 |

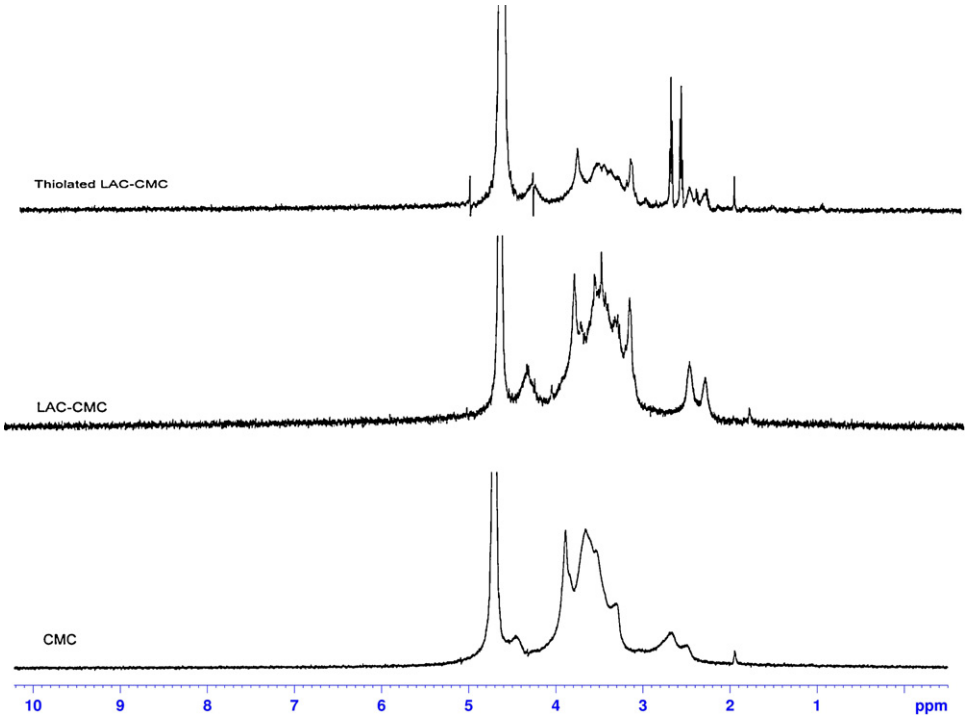


**Fig. 2.** $^{1}$H NMR spectrum of thiolated LAC-CMC, LAC-CMC and CMC.

in GSH concentrations would result in a difference in the cleavage rate of disulfide bonds between the intracellular and extracellular compartments.

For efficient care effect, the crosslinked nanoparticles have to dissociate to release glycyrrhizic acid inside the cell in response to the intracellular reductive environment. We examined the possibility of the dissociation using a physiologically relevant reductive reagent, GSH. Crosslinked nanoparticles were treated with various concentrations of GSH (0, 10 μM, 100 μM, 1 mM) for 72 h. The released glycyrrhizic acid was analyzed by HPLC. As seen in Fig. 4, the release of glycyrrhizic acid was dependent on the reaction time and the GSH concentration. As the concentration of GSH increased, the amount of glycyrrhizic acid release from nanoparticles increased. About 60% of glycyrrhizic acid entrapped in crosslinked nanoparticles were released after 72 h in PBS containing 1 mM GSH.

### 3.4. Pharmacokinetics and tissue distribution study

Plasma pharmacokinetic parameters of crosslinked thiolated LAC-CMC nanoparticles were compared to those of unmodified LAC-CMC nanoparticles and glycyrrhizic acid solution formulation in rabbits. The pharmacokinetic parameters and the compartment model were analyzed by software program 3p87. The results show that all nanoparticles and solution plasma concentration-time curves can be fitted into the open two-compartment model. The relevant pharmacokinetic parameters are listed in Table 3. The areas under the curve (AUC) for crosslinked nanoparticles were sig-

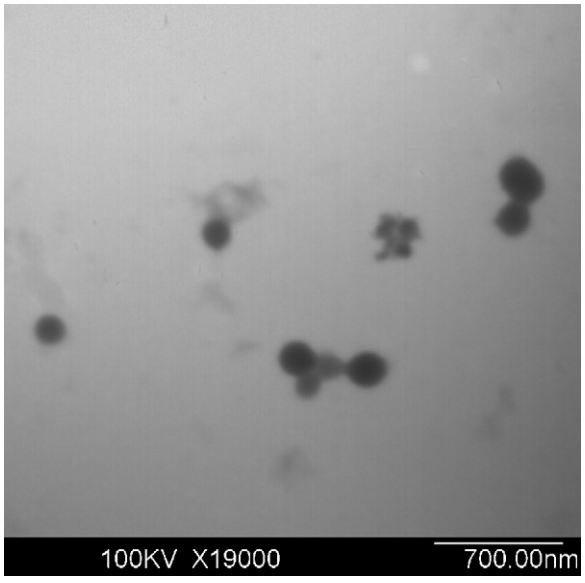


**Fig. 3.** TEM of thiolated LAC-CMC nanoparticles.

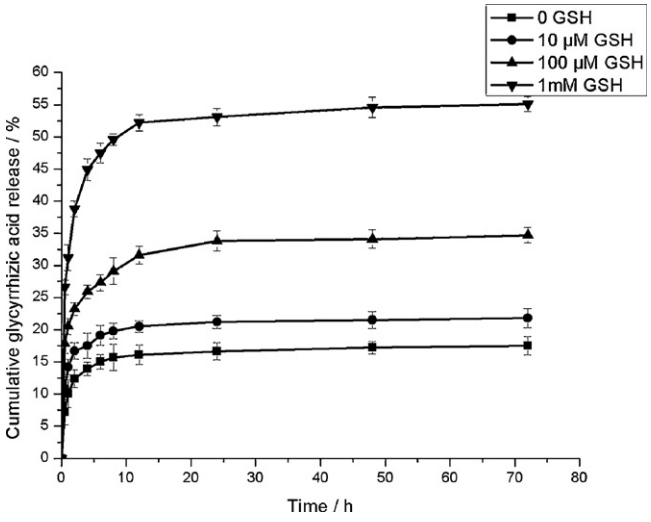


**Fig. 4.** The thiolated LAC-CMC nanoparticles release behavior ($n = 3$).

**Table 3**
Pharmacokinetic parameters of glycyrrhizic solution, non-crosslinked LAC-CMC nanoparticles and crosslinked thiolated LAC-CMC nanoparticles ($n = 5$).

| Parameters | Definitions | Units | Glycyrrhizic solution | Non-crosslinked | Crosslinked |
|---|---|---|---|---|---|
| A | Hybrid parameter | μg/ml | 143.402 | 144.477 | 154.489 |
| B | Hybrid parameter | μg/ml | 2.582 | 3.752 | 7.144 |
| T1/2 alpha | Plasma half-life for the distribution phase | h | 0.482 | 0.569 | 0.630 |
| T1/2 beta | Plasma half-life for the elimination phase | h | 9.583 | 12.911 | 13.254 |
| AUC | Area under the curve | μg/ml h | 135.471 | 188.502 | 277.012 |

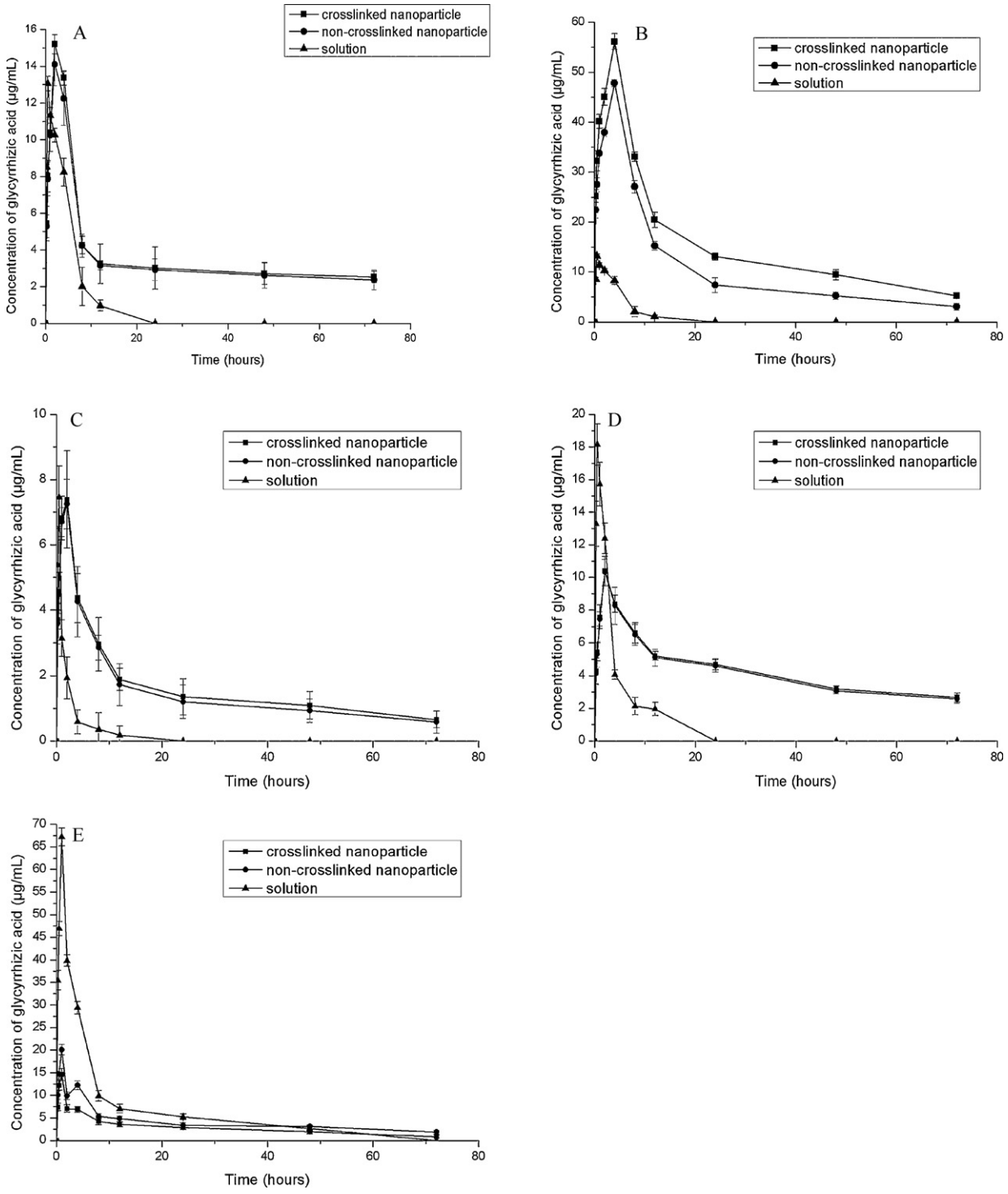


**Fig. 5.** Tissue distribution profile after intravenous injection of cross-linked thiolated LAC-CMC, unmodified LAC-CMC nanoparticles and glycyrrhizic acid solution in mice organs: (A) heart; (B) liver; (C) spleen; (D) lungs; (E) kidneys ($n = 5$).

nificantly higher than non-crosslinked nanoparticles, indicating a higher amount of glycyrrhizic acid available for tissue uptake. The slower clearance of the crosslinked nanoparticles from the circulation could be ascribed to both a longer alpha phase ($T_{1/2\ alpha}$ increased from 0.569 h to 0.630 h) as well as a longer beta phase ($T_{1/2\ beta}$ increase from 12.911 h to 13.254 h).

Fig. 5 represents the glycyrrhizic acid level in the heart, liver, spleen, lungs, and kidneys at different time points after i.v. administration of crosslinked thiolated LAC-CMC, unmodified LAC-CMC nanoparticles and glycyrrhizic acid solution. Glycyrrhizic acid solution was quickly degraded. Crosslinking of the nanoparticles was observed to enhance the accumulation in the liver and reduce the accumulation in the kidney, which demonstrates that crosslinked nanoparticles could enhance targeting effect and reduce nephrotoxicity.

## 4. Conclusions

The aim of this study was to design controlled release and live targeting delivery system using sensitive disulfide bonds to control drug release and galactose moiety to achieve targeting. The structure of thiolated LAC-CMC was characterized by $^1$H NMR techniques. Thiolated 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 drug release properties of the crosslinked nanoparticles were sensitive to reducing conditions similar to the intracellular environment. In vivo investigations in rabbits and mice revealed that blood concentrations were increased and organ accumulation was altered depending on crosslinked nanoparticles. The thiolated LAC-CMC may be used as a potential drug carrier with hepatic targeting and controlled release.

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