Synthesis of a Novel Polymer Cholesterol-Poly(ethylene glycol) 2000-Glycyrrhetinic Acid (Chol-PEG-GA) and Its Application in Brucine Liposome

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ABSTRACT: In this research, a novel polymer cholesterol-poly(ethylene glycol) 2000-glycyrrhetinic acid (Chol-PEG-GA) was synthesized with four steps of chemical modification and elucidated by FTIR and ^{1}H-NMR spectra. To demonstrate the application of this Chol-PEG-GA in preparation of liposomes (CPGL), conventional liposome (CL) composed of PC and Chol was prepared and the effects of the quantity of Chol-PEG-GA on the physicochemical properties (entrapment efficiency, particle size, stability of storage, and so on) of CPGL were also evaluated. The ability of the sustained release and the liver targeting ability of CPGL were further studied *in vivo* in rats and mice. The results show that, the AUC and MRT of CPGL were increased 2.31 and 2.11 times when compared with CL, respectively. The CPGL delivered about seven times higher drug into liver as compared with CL. From the targeting parameters of CPGL and CL, we can also conclude that the CPGL is able to improve the liver targeting of brucine. All these results suggested that, the Chol-PEG-GA modified liposomes were potential as the sustained and liver targeting drug delivery. © 2011 Wiley Periodicals, Inc. J Appl Polym Sci 124: 4554–4563, 2012

Key words: liposome; brucine; glycyrrhetinic acid; targeting parameters

INTRODUCTION

Nowadays, hepatoma which has a higher prevalence in clinical practice is very harmful to human beings. However, the common delivery system, such as liposome, emulsion, etc., is recognized as foreign bodies and phagocytized by the body defense system. So the effect of the drug was very poor. In recent years, to change this situation, the hepatic targeted drug delivery systems (HTDDS) get a rapid progress with the development of TDDS, which can enhance the targeting efficiency and also reduce its side effects.

Among them, receptor-modified polymer has been the focus of research in gene and drug delivery. As we know, some natural products, such as glycyrrhetinic acid (GA), glycyrrhizic acid (GL, glycyrrhizin), and bile acid could be collected in liver. Bile acid is a biological compound, which was synthesized originally in the liver. To achieve liver-selective drug targeting, the hepatotropism of bile acid can be used.[1] GA and GL, representing the main constituents of licorice root, induce oxidative stress in liver mitochondria responsible for the induction of membrane permeability transition. They could accumulate in liver because the hepatic cell membrane has the receptor for GA.[2–4] These compounds are widely used in medicine for treatment of liver pathologies and also constituents of many therapeutic agents.[5–7] The lipid was proved to have a target for hepatocytes after being conjugated with GA.[8,9]

Although, the liposome modified by GA can be successfully delivered to the liver, they may unnecessarily be effective from the pharmacokinetic point of view. Since, the recognition of GA liposome by the receptors was highly efficient, the liposome could be rapidly delivered to the target cells. Consequently, the concentration-time profile in the target was rapidly switched to the elimination phase, which in turn, would result in a short duration of

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Zhi-peng Chen and Lu Xiao contributed equally to the project and are considered co-first authors

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drug exposure.[10] To avoid frequent repeated administration, the rate of drug delivery to the target needs to be reduced by some means.

In recent years, many anticancer drugs were derived from natural products such as paclitaxel, camptothecin, etc. It played an important role in improving the quality of patient's life, prolonging the patient's life, and controlling the development of the diseases. Strychnos nux-vomica L. (Loganiaceae) is grown extensively in Southern Asian countries. Historically, this plant had been widely used in treating diseases such as tumor and rheumatic arthritis.[11–13] As summarized in many reviews that it could significantly inhibit the proliferation of various tumor cells *in vitro* and the growth of tumor cells *in vivo*. Phytochemical analysis revealed that alkaloids including brucine and strychnine were the major components of the species.[14] In our previous studies, the total alkaloid fraction from Nux Vomica was shown to remarkably suppress tumor cells growth. And the main components of the fraction were revealed to be brucine, strychnine, isostrychnine, and brucine *N*-oxide, which were confirmed having cytotoxic effects on HepG2 cells.[15] Further studies revealed that brucine exhibited the strongest inhibitory effect on HepG2 cells proliferation through Ca^{2+}- and Bcl^{2-}-mediated mitochondrial pathways, which were involved in brucine-induced HepG2 cell apoptosis.[16] Unfortunately, the potential use of brucine is severely limited because of its violent toxicity.[17] Until now, brucine has not been used clinically or been researched in clinical trials.

The aim of this work was to synthesize and characterize a novel polymer Chol-PEG-GA, which containing a liver-targeting and sustained groups in the main chain. Since, PEG is well known to reduce the interaction of liposome with biological components such as MPS,[18–20] we assumed that coating with PEG could reduce the MPS uptake of CPGL and also retard GA receptor-mediated uptake of CPGL. And we had developed a novel liposomes modified by Chol-PEG-GA to encapsulate brucine by the ammonium sulfate gradient loading method. The physicochemical characteristics of the brucine-loaded novel liposomes were also described in detail and compared with CL. And then we examine the feasibility of PEG coating on CPGL to achieve controlled delivery of the liposomes via a receptor-mediated process *in vitro*. At last, the ability of sustained release and liver targeting ability *in vivo* were evaluated in rats and mice.

EXPERIMENTAL

Materials and methods

Chemicals

N,*N*-dicyclohexylcarbodiimide (DCC) was obtained from Sinopharm Chemical Reagent. The chemical, 4-dimethylaminopyridine (DMAP), was purchased from the Zhejiang Xianju Pharmaceutical and Chemical Experimental Plant. Cholesterol (China Medicine Shanghai Chemical Reagent Corporation, China) and succinic anhydride (Shanghai Chemical Reagent, China) were used. GA was precipitated from Shanghai Winherb Medical S&T Development. CH_2Cl_2 was refluxed over P_2O_5 and then distilled. Brucine was obtained from Tokyo Chemical Industry, Tokyo, Japan. All the other reagents were of AR.

Structural analysis

IR spectra of GA, ethyl ester, PEG_{2000}, and the reaction products were recorded on a Nicolet Avatar 370 DTGS spectrophotometer (KBr disk). ^{1}H-NMR was performed on a Bruker (AVACE) AV-500 using $CDCl_3$ as a solvent.

Animals

Wistar rats (male, 180–220 g) and Kunming mice (male, 8 weeks old, weighing 20 ± 2 g) were purchased from Shanghai Slac Laboratory Animal (Shanghai, China). The rats and mice were acclimatized for at least 1 week in a 12 h light/dark cycle with free access to standard chow and water. The protocols of animal experiments were approved by the Animals Ethics Committee of Nanjing University of Chinese Medicine.

Synthesis of Chol-PEG-GA

The synthesis of Chol-PEG-GA involved four steps of chemical modification on GA by esterification, the first step is to obtain acetyl glycyrrhetinic acid with acetylchloride, and the second is to obtain compound **3** with PEG, on the other hand, esterify cholesterin with butanedioic anhydride to get compound **6**, finally, condense **3** and **6** to obtain the desired product according to Figure 1.

Synthesis of Glycyrrhetinic acid ethyl ester. Acetylchloride (2.2 mL, 30 mmol) was added dropwise over 10 min to a stirred solution of GA (9.4 g, 20 mmol) and CH_2Cl_2 (100 mL) at 0°C (ice bath). After stirring over 10 h at room temperature, ice water (100 mL) was added, and the organic layer was washed with water (100 mL), brine (100 mL), and dried over magnesium sulfate. The solvent was removed *in vacuo* below 40°C to provide an off-white solid acetyl glycyrrhetinic acid (9.7 g), which was pure enough for further reaction. ^{1}H-NMR ($CDCl_3$, ppm) was shown in Figure 2(a). IR (γ/cm) was shown in Figure 3(a) and Electrospray ionization (ESI) (−)/70 eV: 511.4 $[M-H]^-$.

Synthesis of GA-PEG conjugates. PEG_{2000} (22 g, 11 mmol), Glycyrrhetinic acid ethyl ester (5.13 g, 10

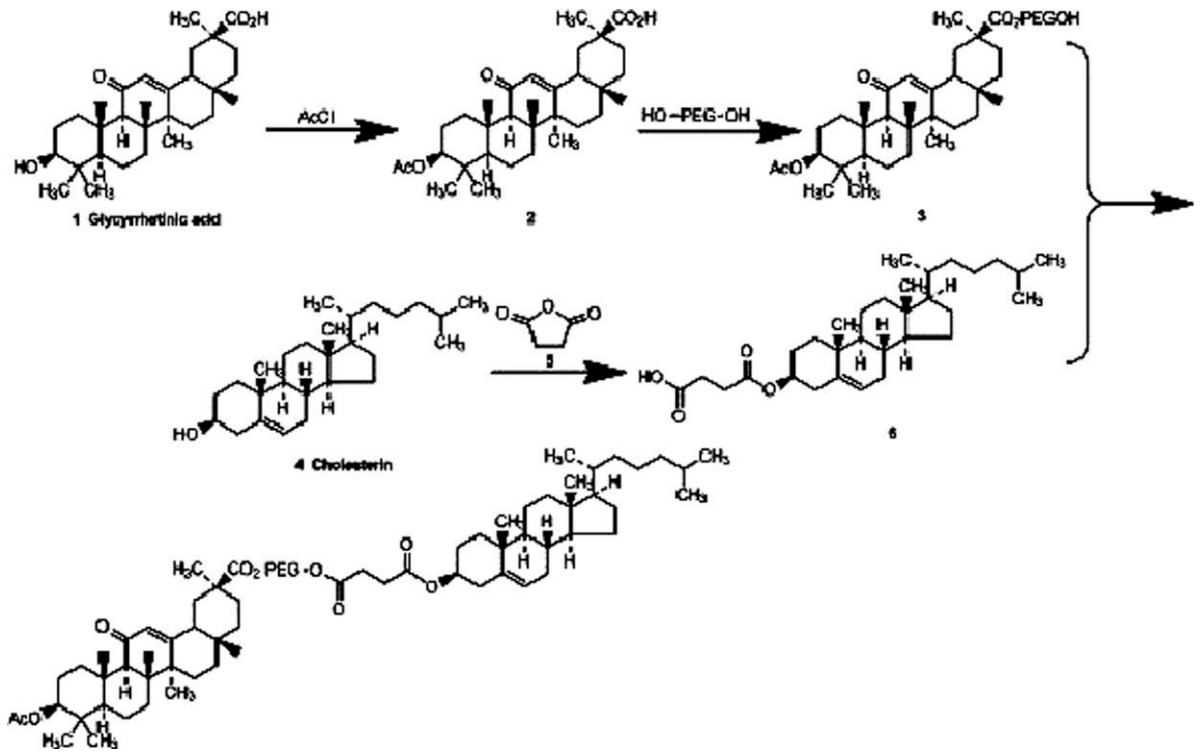

Figure 1 Chemical reactions scheme for preparing the Chol-PEG-GA conjugate.

mmol), and toluene (400 mL) were charged into a round-bottom flask, and the mixture was refluxed with water bath apparatus until no water dripped. Then the solvent was removed *in vacuo* below 45°C and redissolved in anhydrous CH_2Cl_2 (250 mL). Then, DCC (2.06 g, 10 mmol) and DMAP (0.1 g) were added into the solution and stirred at room temperature for 48 h. After filtered, the filtrate was washed with saturated aqueous solution of sodium bicarbonate (250 mL × 3), brine (200 mL × 3), dried with magnesium sulfate and concentrated under reduced pressure. The product was further purified by column chromatography on silica gel H using dichloromethane/methanol [50 : 1, (v/v)] as an eluant. A single spot by TLC analysis $Rf = 0.52$ (CH_2Cl_2: methanol: acetic acid = 10 : 1 : 0.05) was visualized with iodine vapor. The dried product can be used directly in the next step.

Synthesis of compound 6: Cholesterin (3.86 g, 10 mmol), pyridine (50 mL), and butanedioic anhydride (1.2 g, 12 mmol) were charged into a round-bottom flask. The mixture was stirred for 10 h at room temperature. The solvent was poured into ice-water (250 mL) with stirring. The mixture was adjusted to pH 2–3 by dropwise addition of concentrated hydrochloric acid, keep the temperature below 15°C. The mixture was then filtered and the off-white solid product was washed with water (50 mL × 3) and dried *in vacuo*. The product can be used directly in the next step. ^{1}H-NMR ($CDCl_3$, ppm) was shown in Figure 2(b), IR (γ/cm) was shown in Figure 3(b), and ESI (−)/70 eV: 485.4 $[M-H]^-$.

Synthesis of Chol-PEG-GA. Compound **3** (6.24 g, 2.5 mmol), compound **6** (1.22 g, 2.5 mmol), DCC (0.52 g, 2.5 mmol), DMAP (0.1 g), and anhydrous CH_2Cl_2 (250 mL) were charged into a round-bottom flask. The mixture was stirred for 48 h at room temperature. After filtered, the filtrate was washed with saturated aqueous solution of sodium bicarbonate (250 mL × 3), brine (200 mL × 3), dried with magnesium sulfate, and concentrated under reduced pressure. The product was further purified by column chromatography on silica gel H using dichloromethane/methanol [50 : 1, (v/v)] as an eluant. A single spot by TLC analysis $Rf = 0.65$ (CH_2Cl_2 : methanol : acetic acid = 10 : 1 : 0.05) was visualized with iodine vapor. 1H-NMR ($CDCl_3$, ppm) was shown in Figure 2(c), IR (γ/cm) was shown in Figure 3(c).

Liposome preparation

The CL was prepared by the ammonium sulfate gradient loading method as described previously.[21] Briefly, SPC and cholesterol were dissolved in anhydrous ethanol until clarification and then injected into the ammonium sulfate solution under magnetic stirring at 25°C. Subsequently, the suspension was transferred into a round-bottom flask and the ethanol was evaporated odorless under vacuum followed by probe sonicating for 50 times to obtain small

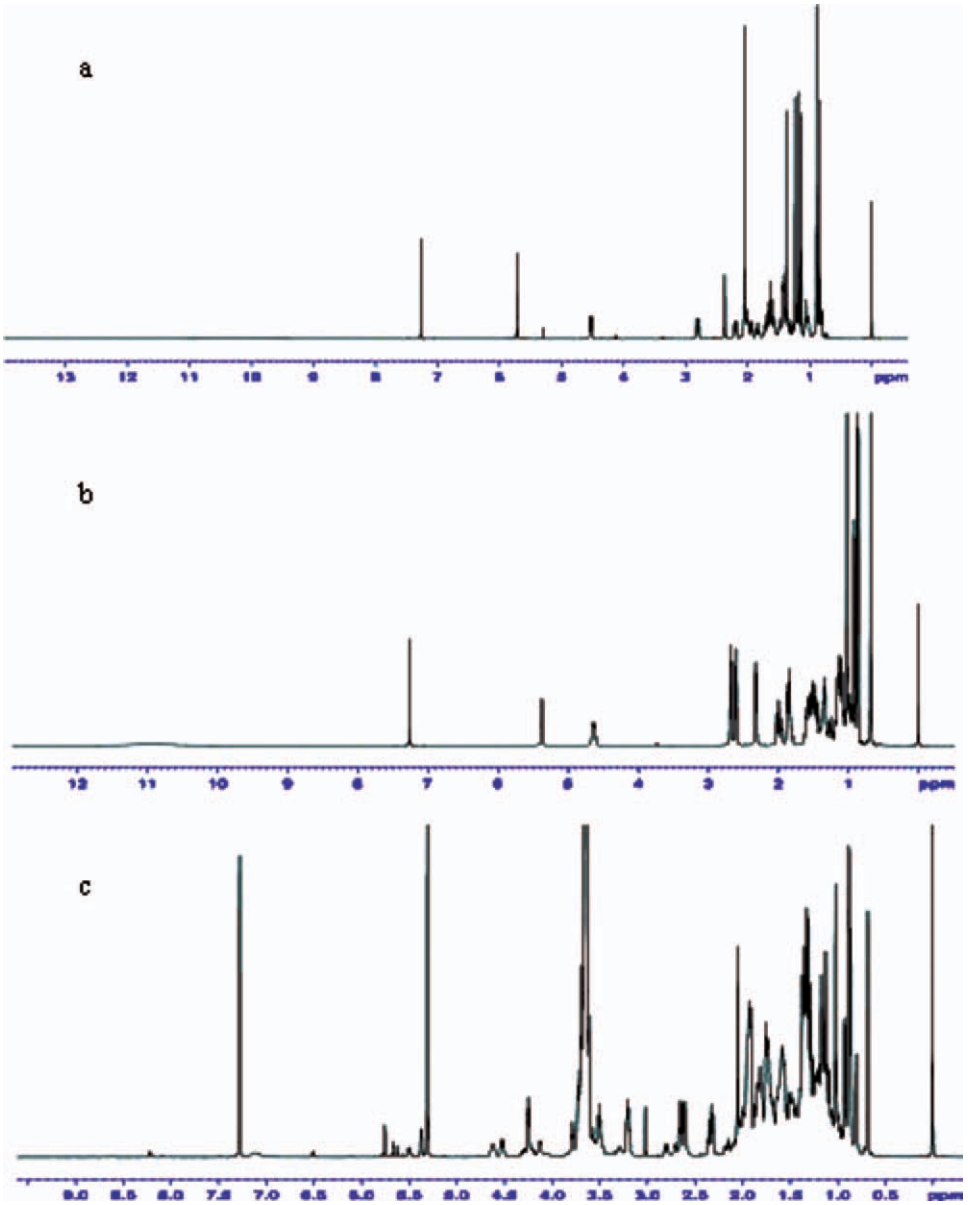

Figure 2 ^{1}H-NMR spectrogram of (a) Glycyrrhetinic acid ethyl ester, (b) compound 6, and (c) Chol-PEG-GA.

unilamellar vesicles. After that, the suspension was dialyzed with an aliquot of phosphate buffer solution (PH 7.4) for 8 h [1 : 10, (v/v)] in dialysis bag and need to be changed the solution every 2 h. The brucine was dissolved in chloroform and the solution was evaporated under vacuum to remove the solvent. The transmembrane ammonium sulfate gradient was created by four consecutive dialysis exchanges (2 h at a time) against 20 volumes of PBS under room temperature. Brucine in powder form was added to liposome at 25°C for 20 min. The preparation of CPGL was according to the CL, and the Chol-PEG-GA was added along with the SPC and cholesterol.

The determination of entrapment efficiency

The method to determine the entrapment efficiency (EE) of CL and CPGL was according to the report of Chen et al.[22] Briefly, the total amount of brucine in liposome suspension was determined as following: 0.5 mL liposome was disrupted by the addition of 2 mL ethanol–isopropanol [1 : 4, (v/v)] to form a clear solution. To completely remove unentrapped drug, CL or CPGL was passed through a Sephadex G-50 column. The liposome was collected and the encapsulated brucine concentration was determined.

HPLC conditions

The analysis of pharmacokinetics was performed using a Shimadzu HPLC-UV system (Shimadzu Company, Japan).[23] The sample separation was performed on a Hanbang C_{18} analytical column (4.6 × 250 mm^2, 5 μm, Hanbang, China) coupled with a C_{18} guard cartridge (10 × 4.6 mm^2, 5 μm, Hanbang, China), maintained at

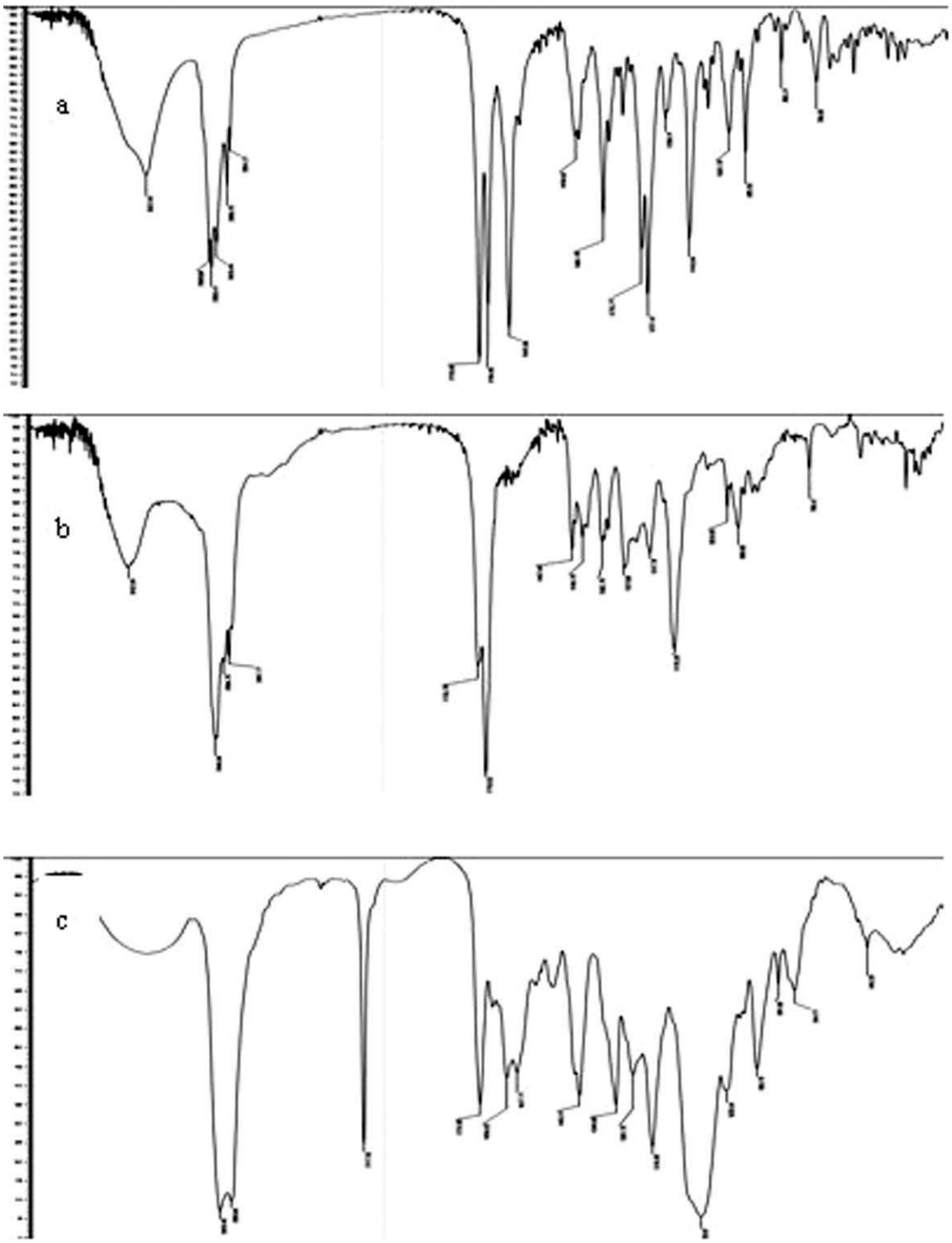

Figure 3 IR spectrogram of (a) Glycyrrhetinic acid ethyl ester, (b) compound 6, and (c) Chol-PEG-GA.

37°C. The mobile phase was mixture of 24% acetonitrile and 76% buffer. The flow rate was 1.0 mL/min. The detection wavelength was set at 264 nm.

HPLC-MS conditions

The analysis of tissue distribution was performed using a Shimadzu LC-MS system. The mobile phase was composed of methanol, 20 m*M* ammonium of formate and formic acid [38 : 62 : 0.62, (v/v/v)] at a flow rate of 0.4 mL/min.

ESI source in positive mode was used for mass spectrometric detection. The ionspray voltage was set at 4000 V. The sheath and auxiliary gas was nitrogen, with the pressure and flow rate of 35 psi and 6 L/min, respectively. The heated capillary

temperature was 300°C. The extra energy of 10 V was added for source collision-induced dissociation. Selected ion monitoring mode was used for the quantification at m/z 335 for strychnine, m/z 395 for brucine with a dwell time of 0.2 s. The detection wavelength was set at 254 and 264 nm.

The effect of the amount of Chol-PEG-GA

The effect of the amount of Chol-PEG-GA in the liposome on the physical properties and EE of CL and CPGL was studied. The CPGL which contained different mount of Chol-PEG-GA [PC/Chol-PEG-GA = 10 : 0.3, 10 : 0.5, 10 : 0.7, 10 : 1, 10 : 1.5, 10 : 3, (w/w)] were prepared according to the description. The EE and physical properties were also determined.

Physical properties of CL and CPGL

The analysis of size and zeta-potential of CPGL and CL were performed by dynamic light scattering (Zetasizer 3000HSA, Malven Instruments, UK). The suspension of liposomes was filtered with a 0.45 μm filter and each batch was analyzed in triplicate.

The shape of the liposome was observed by the transmission electron microscope (TEM) (H-7000 Hitachi, Japan) at an accelerating voltage of 75 kV. One drop of liposome suspension was placed on a copper grid and stained with 2% phosphotungstic acid solution for 2 min. The grid was allowed to dry at room temperature and was examined with TEM.

Storage stability

The storage stability assays of CL and CPGL were carried out according to a modified method of Ishida et al.[24] In brief, CPGL or CL was mixed with isotonic PBS (pH 7.4) at a ratio of 1 : 9 (v/v), and then stored at 4°C. At various time points, aliquots were withdrawn for the determination of EE. The concentration of liposomal brucine was assayed as described earlier.

In vitro release experiments of CL and CPGL

The release of brucine from CL and CPGL was evaluated by dialysis. Each sample (2 mL) was placed in a dialysis pocket, and then the pocket was immersed in 200 mL of release medium, PBS (pH 7.4, NaCl 10.4 g, 37°C), to maintain sinking condition. When stirred the release medium using the magnetic stirrer at 400 rpm, sample (1 mL) was taken at predetermined time intervals from the release medium at preset intervals over a period of 24 h and refilled with the same amount of the fresh medium. Concentration of solution was analyzed by HPLC. The release percentage was calculated according to the following equation:

$$\text{Drug release}\,(\%) = \left(\frac{M_{\text{release}}}{M_{\text{total}}}\right) \times 100\%$$

where M_{release} is the amount of brucine released from brucine liposomes into dialysis medium at scheduled intervals, M_{total} is the total amount of brucine in brucine liposomes.

To evaluate the effect of serum albumin on drug release from liposome, CPGL, and CL suspension combined with isometric fetal calf serum (FCS) was placed in the dialysis bag.

Pharmacokinetics studies in rats

Eighteen Wistar rats (male, 180–220 g) were randomly divided into three groups. Solution CL or CPGL were respectively injected into rats (5 mg/kg) via tail vein at a single dose. After intravenous injection, the rats were anaesthetized with ether and a heparinized capillary was then inserted into the eye ground veins to get 0.5 mL blood into a plastic centrifuge tube at the time intervals of 10, 30, 60, 120, 180, 360, 600, and 1200 min, respectively. And then the blood was centrifuged at 1000 × *g* for 5 min at 4°C to get the plasma. All samples were immediately frozen at −20°C until analysis.

Tissue distribution studies in mice

Fifty-six mice (18–20 g) were randomly divided into two groups, CL or CPGL were respectively injected into mice (5 mg/kg) via tail vein at a single dose. After intravenous injection, according to the preexperiment, four mice were sacrificed at predetermined time point, and the tissues (brain, liver, kidney, lung, spleen, and heart) and blood samples were obtained, respectively. Tissue samples were put into physiological saline solution to remove the blood or consent, blotted on filter paper, and then weight for wet weight. Plasma samples were prepared from blood by centrifugation at 4000 rpm for 5 min. The obtain tissue or plasma samples were immediately stored at −20°C until analysis.

Sample preparation

An 80 μL of plasma sample or homogenate tissue were transferred to centrifuge tube, 20 μL internal standard and 500 μL of saturated Na_2CO_3 were added. The mixture was vortexed for 30 s and extracted with 4 mL of *n*-hexane-dichloromethane-isopropanol [65 : 30 : 5, (v/v/v)] by thoroughly vortexing for 3 min. After centrifugation, the upper organic layer was taken and evaporated to dryness

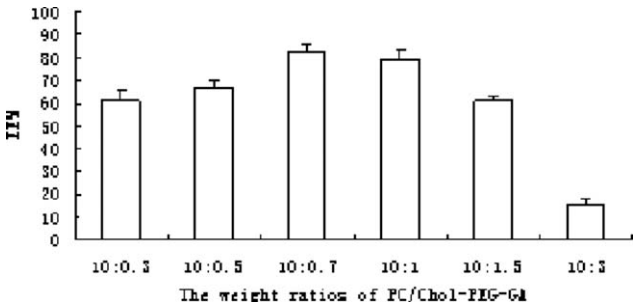

Figure 4 The effect of the amount of Chol-PEG-GA on the EE of CPGL.

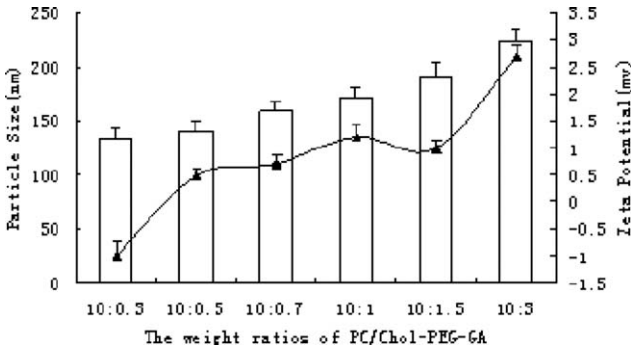

Figure 5 The effect of the amount of Chol-PEG-GA on the physical properties of CPGL.

under a mild stream of N_2 at 60°C. The residue was reconstituted in 100 μL of mobile phase. After being centrifuged for 3 min, an aliquot of 10 μL was injected for LC or LC-MS analysis, respectively.

RESULTS AND DISCUSSION

Synthesis of Chol-PEG-GA

The main objective of this study was to examine the feasibility of PEG coating on the HTDDS to achieve controlled delivery of the liposome via receptor-mediated process. So according to the structure of Chol, PEG and GA, we designed the process of the Chol-PEG-GA synthesis as Figure 1 shown.

To detect whether esterification occurred, the FTIR and the ^{1}H-NMR analysis were performed and the spectra of acetyl-glycyrrhetinic acid, cholesterol ester succinate, PEG_{2000} and Chol-PEG-GA were recorded, respectively. In the FTIR spectra, the carbonyl stretching vibrations of ester linkage for acetyl-glycyrrhetinic acid, cholesterol ester succinate, and Chol-PEG-GA were observed at 1730, 1734, and 1732 cm^{-1}, respectively, which were absent in the original PEG. So we could confirm that the ester was formed by the reaction with the hydroxyl group in PEG and the carboxylic group of acetyl-glycyrrhetinic acid and cholesterol ester succinate. The peaks in the fingerprint region, the bands at 1108 and 1109 cm^{-1} were assigned to —C—O—C— stretching vibration in PEG_{2000} and Chol-PEG-GA, respectively. All these results confirmed that GA and cholesterol ester succinate had been placed on the PEG backbone. In the ^{1}H-NMR spectrum of Chol-PEG-GA, the signals at 5.71 ppm were attributed to the fragment of acetyl-glycyrrhetinic acid. The dual peaks at 2.60 and 2.66 ppm were assigned to the fragment of cholesterol ester succinate. The signals at 3.49–3.79 ppm were attributed to the repeating units in PEG. These results further indicated that the esterification had definitely occurred.

The effect of the amount of Chol-PEG-GA

The effect of PC/Chol-PEG-GA weight ratios on the physical properties of CPGL were shown in Figures

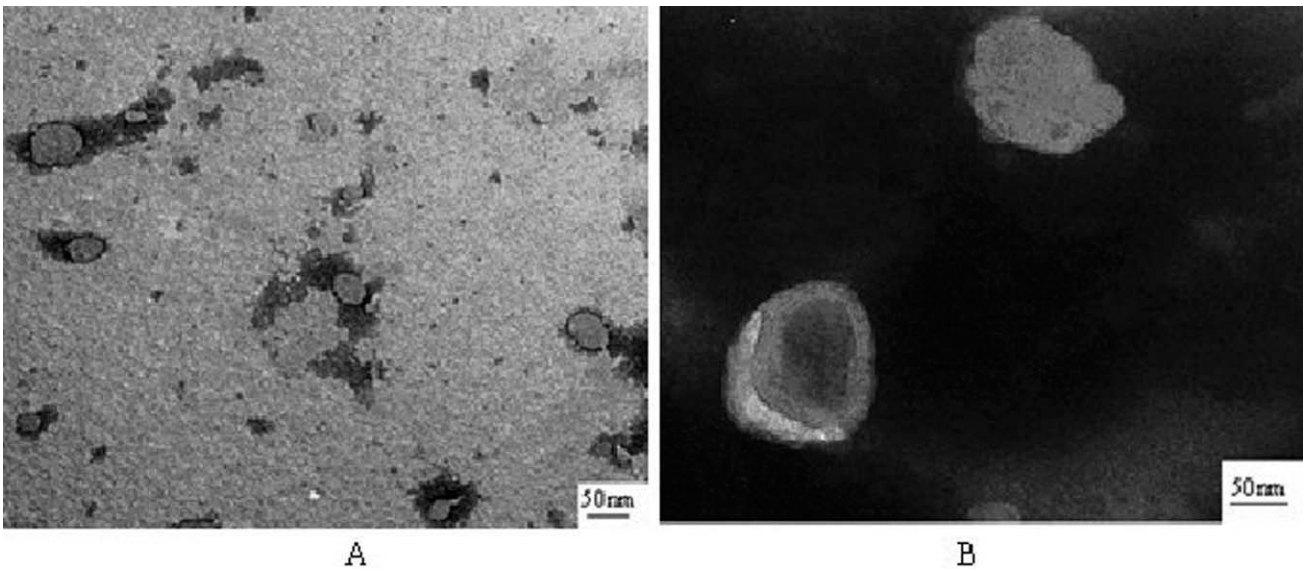

Figure 6 Transmission electron microscope photographs of CL (a, ×10,000) and CPGL (b, ×25,000).

TABLE I
The EE and Physical Properties of CL and CPGL ($n = 3$)

	EE (%)	Zeta-potential(mv)	Particle size (nm)
CL	87.55 ± 4.64	−10.93 ± 2.86	121.6 ± 13.55
CPGL	82.45 ± 3.46	0.7 ± 0.18**	158.9 ± 15.93*

Compared with CL, * $p < 0.05$, ** $p < 0.001$.

4 and 5. In Figure 4, the EE was significantly affected by the mount of Chol-PEG-GA. As the PC/Chol-PEG-GA weight ratios increased, the EE of CPGL was increased rapidly when the PC/Chol-PEG-GA weight ratios below 10 : 0.7. Especially, the EE increased to 82.45 ± 3.46%, when the PC/Chol-PEG-GA weight ratio was 10 : 0.7. However, when the PC/Chol-PEG-GA weight ratio was over 10 : 1, the EE of CPGL was decreased rapidly along with the PC/Chol-PEG-GA weight ratios increased. The EE was only 15.63 ± 2.46% when the PC/Chol-PEG-GA weight ratio was 10 : 3. On the other hand, the results of the mount of PC/Chol-PEG-GA effect on the particle size and zeta-potential of CPGL were shown in Figure 5. These interesting results proposed that the particle size and zeta-potential of CPGL match with the increase of PC/Chol-PEG-GA. Therefore, we finally selected 10 : 0.7 as the PC/Chol-PEG-GA weight ratio.

Physicochemical properties of CL and CPGL

According to the optimal formulation, we prepared three batches of CPGL. The physicochemical properties of the optimal CL and CPGL formulation were studied. As shown in Figure 6, the shape of liposomes was discovered to be round or oval by the TEM. The EE of CL and CPGL were 87.55 ± 4.64% and 82.45 ± 3.46%, respectively. As shown in Table I, the average vesicle diameters and zeta-potentials of CL and CPGL were 121.6 ± 13.5 and 158.9 ± 15.93 nm, −10.93 ± 2.86 and 0.7 ± 0.18 mV, respectively. We could conclude that when compared with CL, there was no significant difference in the EE of CPGL while the particle size ($p < 0.05$) and zeta-potential ($p < 0.01$) were changed greatly.

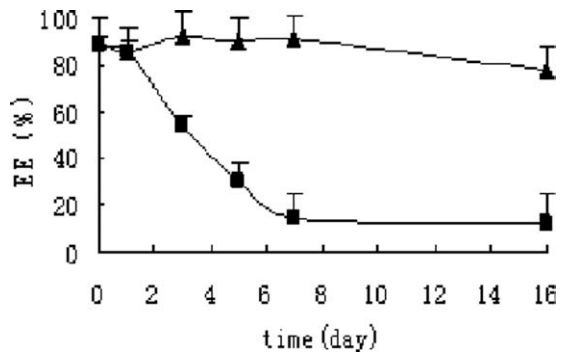

Figure 7 The storage stability of CL (■) and CPGL(▲) ($n = 3$).

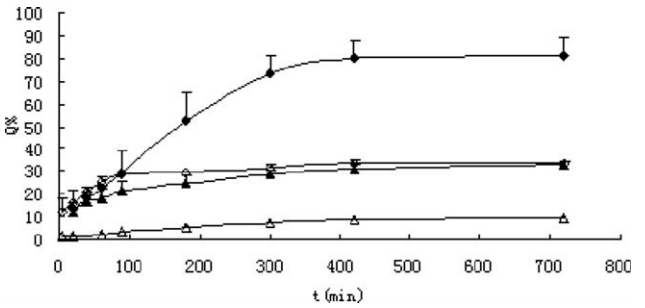

Figure 8 The *in vitro* drug release profiles of the CL and CPGL ($n = 3$). [with the presence of FCS: CL(■), CPGL(▲); without the presence of FCS: CL (◇), CPGL (△)].

Storage stability

The storage stability assays of CL and CPGL were shown in Figure 7. The EE of CL was decreased from 91% (0 days) to 11% (7 days) in 7 days. And the rapid EE decreasing was detected between 3 and 7 days and then the decrease of EE was hardly observed. The EE of CL was no longer decreased after 7 days. It is possibly because of the drug adsorbed on the surface of the CL that made it unable to be released from liposome as time went by. As to CPGL, the decrease of EE was very slow. The EE remained to be about 90% for 7 days, which means that the CPGL were more stable than CL. These evidences suggested that the leakage of liposomal brucine from CPGL was markedly slower than that of CL. A possible explanation could be that the long chain of Chol-PEG-GA around the liposomes can inhibit the drug release from liposomes and improve the stability of CPGL.

In vitro release experiments

The *in vitro* drug release profiles were shown in Figure 8. Without the absence of FCS, the release of

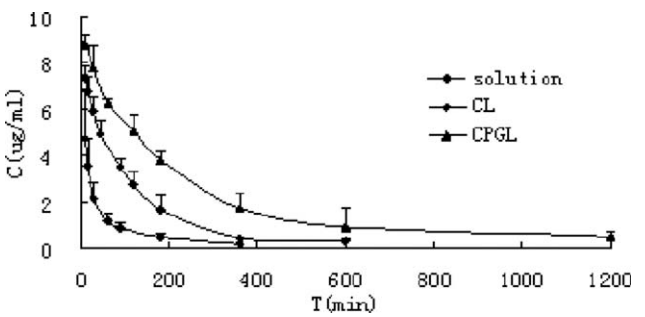

Figure 9 The concentration-time curves of brucine in plasma of rats after i.v. administration of brucine in different formulations ($n = 6$, mean ± SD).

TABLE II
Pharmacokinetic Parameters of Brucine After Intravenous Injection of Solution, CL or CPGL in Rats ($n = 6$)

Compartmental parameters	Parameters values ± SD		
	Solution	CL	CPGL
MRT_{0-T} (min)	99.19 ± 19.28	132.77 ± 3.92	280.76 ± 71.06*
AUC_{0-T} (min/ng/mL)	323.99 ± 89.98**	1001.71 ± 93.54	2309.89 ± 387.99*
Cl [mg/min/(ng/mL)]	0.015 ± 0.0048**	0.0033 ± 0.00021	0.0014 ± 0.00012*
k_{10} (min^{-1})	0.021 ± 10.23**	0.0054 ± 0.00034	0.0029 ± 0.00026*
C_{max} (ng/mL)	4.78 ± 2.05*	7.41 ± 0.47	8.82 ± 0.43

Values are expressed as mean ± SD, compared with CL, * $p < 0.05$, ** $p < 0.01$.

brucine from CPGL was less than 10% in 12 h, while about 30% of brucine was released from CL. However, with the existence of FCS, the release of brucine was accelerated dramatically and a large extent of drug release was observed both in CL and CPGL. The results indicated that the retention time of the loaded brucine in the liposome was significantly increased through being modified by Chol-PEG-GA. Compared with CL, the CPGL was proved to improve drug retention significantly. Only 32.97 ± 1.22% encapsulated brucine had been released from the CPGL and 81.31 ± 7.99% released from the CL in 12 h. In short, the Chol-PEG-GA could form a hydration shell around the liposome and inhibit the release of encapsulated brucine.

Pharmacokinetics studies in rats

The mean plasma concentration-time profiles of solution, CL and CPGL following i.v. were shown in Figure 9. And the major pharmacokinetic parameters were listed in Table II. From Table II, we could know that the AUC_{0-t} and MRT_{0-t} of CPGL were increased 2.31 times and 2.11 times compared with that of CL. The Cl and k_{10} of CPGL were highly decreased when compared with that of CL and solution. All these results shown that a signification change in drug pharmacokinetic parameters through modifying the liposome by Chol-PEG-GA. And these results also indicated a longer elimination half-life for brucine in modified liposome formulation. It appeared that brucine in CPGL could be given intravenously with a long duration of action because of high stability.

Tissue distribution studies in mice

A comparative tissue distribution studies were conducted between CL and CPGL to understand the magnitude of change in redistribution profile of brucine. The AUC of different deliveries in each tissue or plasma and the targeting parameters were calculated and shown in Table III.

The intake rate (Re) was defined as $Re = AUC_{CPGL}/AUC_{CL}$. If the value of Re is greater than one, the tissue is exposed to drug to a greater extent by CPGL. In our studies, the value of Re for liver was 7.11 in the case of CPGL, indicating that the exposure of brucine to liver was significantly increased by entrapment in CPGL. Therefore, it could be concluded that CPGL was more specific delivery for carrying brucine to liver compared with CL.

Re provides a good indication about the relative efficacy of two delivery systems in reference to one tissue. But it does not provide any information about regarding the efficacy of a given delivery

TABLE III
Targeting Parameters of Solution, CL and CPGA After Intravenous Administration in Mice

Sample	AUC [(ng/mL) min]		Re	Te		Ratio of Te	Ce
	CL	CPGA		CL	CPGA		
Plasma	25,884.85	75,403.59	2.91	0.22	0.53	2.44	2.51
Heart	16,751.70	64,880.86	3.87	1.67	3.06	1.84	2.88
Spleen	40,234.68	57,335.15	1.42	0.69	3.47	5.00	1.58
Lung	30,937.37	138,283.33	4.46	0.90	1.44	1.59	6.37
Kidney	92,656.60	207,570.92	2.24	0.30	0.96	3.18	2.48
Brain	19,757.52	20,577.52	1.04	1.41	9.66	6.84	4.02
Liver	27,923.12	198,787.36	7.11	1.00	1.00	1.00	16.68

$Re = (AUC)_{CPGA}/(AUC)_{CL;}$ $Te = AUC_{targeted}/AUC_{untargeted}$; Ratio of Te = $(Te)_{CPGA}/(Te)_{CL}$; $Ce = (C_{max})_{CPGA}/(C_{max})_{CL}$.

system through the ratio of target to all tissue distribution of drug. To farther demonstrated the efficiency of a delivery system against the whole tissue, we defined the values of targeting efficacy (Te) which determined using the following equation, $Te = AUC_{targeted}/AUC_{untargeted}$. From the result we could conclude that the Te of liver to other tissues increased significantly. And the ratios of Te_{CPGL}/Te_{CL} were also suggesting the same result.

The peak concentration ration (Ce) which demonstrated the efficiency of a delivery system on the biodistribution was defined as $Ce = (C_{max})_{CPGL}/(C_{max})_{CL}$, where the numerator and the denominator denoted peak concentration in tissue or plasma after administration of the CPGL and CL, respectively. The Ce in liver increased by a factor of 16.68 compared with CL in the case of CPGL. Therefore, it could be concluded that CPGL had a preference to accumulate brucine in the liver. It is also known that, CPGL send drug to the brain was decreased greatly as compared with CL, which mean that the CPGL could reduce the central nervous toxic.

All these results indicated that, CPGL had the significant liver targeting characteristics when compared with CL. The high distribution of brucine in liver with CPGL may be explained by the receptor-ligand binding of GA and GA receptors. These indicated that the liposome, which modified by Chol-PEG-GA in this study was preferable for targeting to liver rather than other tissues compared with CL.

CONCLUSIONS

In this study, we have synthesized a novel polymer Chol-PEG-GA, which contains a liver-targeted and sustained group in the main chain and successfully modified liposomes of brucine. The TEM images indicated that all the liposome was spherical in shape and homogeneous in size. As the amount of Chol-PEG-GA in the CPGL increased, the particle size and zeta-potential increased, which mean that the radius of the hydration shell might be increased. And the hydration shell around the liposome could inhibit the release of encapsulated brucine and improve the liposome stability.

The studies of pharmacokinetics in blood of solution, CL, and CPGL had shown that the values of AUC, C_{max}, and MRT of CPGL were increased greatly and the Cl and k_{10} of CPGL were decreased when compared with that of CL and solution which mean that the CPGL could sustain release *in vivo* through modifying the liposome by using Chol-PEG-GA. The liver targetability of CPGL was increased as compared with CL.

All these results showed that, the incorporation of Chol-PEG-GA into liposomes could be a great potential as drug delivery carriers for liver targeting that has targeted and sustained drug delivery. And the novel carrier also decreased the drug in the brain which can reduce central nervous toxicity.

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