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Preparation, characterization and uptake by primary cultured rat hepatocytes of liposomes surface-modified with glycyrrhetinic acid

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3-Succinyl-30-stearyl glycyrrhetinic acid (Suc-GLAOSt) was synthesized as a targeting molecule, and incorporated in ordinary to liposomes (LP) to prepare a liposome surface-modified with glycyrrhetinic acid (LP-GLA), which could bind to the hepatocyte through the specific binding site of glycyrrhetinic acid (GLA) on the surface of rat cellular membrane. The maximal molar ratio of Suc-GLAOSt to total lipids in LP-GLA was 1 : 10. Calcein loaded liposome (Cal-LP) and calcein loaded LP-GLA (Cal-LP-GLA) were prepared by an ethanol injection method. The average diameter of Cal-LP and Cal-LP-GLA was 65 nm $\pm$ 16 nm and 68 nm $\pm$ 21 nm, respectively. The characteristics of cellular uptake of the two types of liposome were investigated through cellular uptake and competitive inhibition experiments. The uptake of Cal-LP-GLA by rat hepatocytes was markedly higher (3.3-fold) than that of Cal-LP ($P < 0.01$). The uptake of Cal-LP-GLA was inhibited, but the uptake of Cal-LP was not influenced by adding extraneous GLA. LP-GLA may be internalized by hepatocytes via the specific binding site, and can be used as a novel and promising carrier for targeting drug delivery to hepatocytes.

1. Introduction

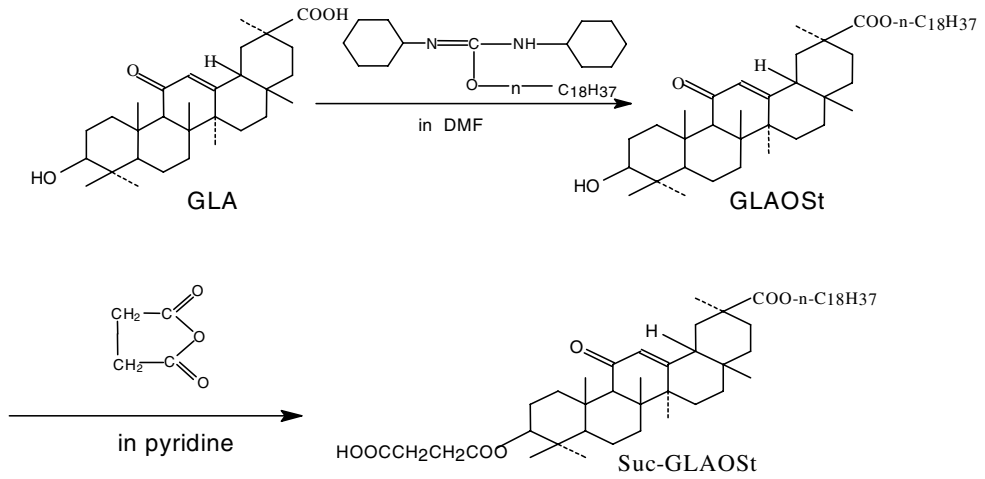
The application of liposomes in drug delivery systems (DDS) is of particular interest because it provides possibilities for drug targeting and has potential for drug carriers for selective delivery to specific tissues or cells. Receptor-mediated drug targeting is a promising approach to selective drug delivery. For example, a mannose receptor-mediated liposome was designed for targeting delivery of bioactive compounds to nonparenchymal liver cells (Opanasopit et al. 2002) and peptides have been used to guide liposomes to the desired receptors for selective cell targeting in cardiovascular drug delivery (Lestini et al. 2002). In addition, folic acid and anti-HER2 have been used as ligands for targeting liposomes to tumor cells (Sudimack et al. 2000; Park et al. 2001; Shi et al. 2002; Saul et al. 2003; Turk et al. 2004). Many fatal diseases occur in hepatocytes, such as chronic hepatitis, cirrhosis, enzyme deficiency and hepatoma, but because of the poor uptake efficiency of current drugs by hepatocytes, there are few effective therapeutic methods for hepatic diseases. Hence, it is important to target delivery of drugs to the hepatocytes.

Among the various types of cells in the body, only hepatocytes have high affinity cell-surface receptors that can bind to asialoglycoproteins and subsequently internalize them to the cell interior. Delivery of drugs using liposomes bound to asialoglycoprotein in a specific manner would provide significant therapeutic benefits in hepatic disease. Extensive studies on chemical modification of liposomes with asialoglycoproteins or low-molecular weight glycolipids have been carried out to achieve effective targeting to hepatocytes (Spanjer and Scherphof, 1983; Tsuchiya et al. 1986; Ishihara et al. 1990; Ghosh and Bachhawat 1991; Hirabayashi et al. 1996; Wu et al. 1998; Kawakami et al. 1998; Yoshiyuki et al. 2000; Kawakami et al. 2000). It has been reported that galactose-bearing liposomes are also taken up by Kupffer cells, but the distribution of these liposomes in hepatocytes is not very high (Spanjer et al. 1984). Therefore, investigation of new ligand binding to hepatocytes, and developing novel drug delivery system targeting to hepatocytes is needed.

GLA, an aglycone of glycyrrhizin (GL), is one of the main compounds extracted from the root of *Glycyrrhiza glabra* L. (licorice). It has been shown that there is a specific GLA binding site on the cellular membrane of rat hepatocytes *in vitro* (Negishi et al. 1991). Shiro et al. found that a carrier-mediated transport system participates in the uptake of GL into isolated rat hepatocytes and the affinity site of the transport carrier commonly binds GLA (Ishida et al. 1993). Furthermore, GL has been confirmed to be an inhibitor and, therefore, potentially a transport substrate, of liver-specific organic anion transporting polypeptides (OATPs) in rat and man (Ismair et al. 2003). These results imply that GLA may also be a transport substrate of liver-specific OATPs and it may be used as a novel ligand for targeting liposomes to hepatocytes.

We have previously prepared liposomes surface-modified with GLA and studied their pharmaceutical characteristics (Mao et al. 2003). In this study, Cal-LP-GLA was pre-

Scheme



pared and the uptake of Cal-LP-GLA by *in vitro* rat hepatocytes was investigated. The interaction between Cal-LP-GLA and isolated rat hepatocytes and the factors influencing the interaction were investigated through *in vitro* cell culture experiments.

2. Investigations and results

2.1. Synthesis of targeting molecule

In order to modify the surface of a standard liposome, a new compound named Suc-GLAOSt which has a GLA moiety, was synthesized as a targeting molecule. The synthetic route of Suc-GLAOSt is shown in the Scheme, and spectral data of ^{1}H NMR and ^{13}C NMR for GLAOSt and Suc-GLAOSt are shown in the Table 1.

2.2. Determination of incorporation ratio of the targeting molecule

An appropriate amount of Suc-GLAOSt was added to an ethanol solution of phospholipids and cholesterol (2 : 1, w/w), and a series of solutions with different molar ratios (targeting molecules : total lipids) were obtained. The solutions were prepared following the previously mentioned procedure for the preparation of LP-GLA. The content of Suc-GLAOSt was determined by HPLC and the incorporation ratio of Suc-GLAOSt was calculated, with the result

Table 1: ^{1}H NMR and ^{13}C NMR spectral data for GLAOSt and Suc-GLAOSt

Compound	GLAOSt	Suc-GLAOSt
^{1}H NMR		
H-1′(1 H, d)	3.827	4.448
CH3(3 H, s)	0.828	0.768
H-2′(1 H, s)	2.324	
H-2″(1 H, s)		11.032
H-1″(1 H, s)	5.632	5.441
Stearyl-H	4.068 (−CH_2−OCO−, 2 H, dd)	4.056 (−CH_2−OCO−, 2 H, dd)
	1.278 (−(CH_2)n−, brs)	1.238 (−(CH_2)n−, brs)
Suc-H		2.490 (2 H, m)
		2.530 (2 H, m)
^{13}C NMR		
CH_3	17.3, 17.8, 18.3, 19.1, 20.7, 22.6	17.3, 17.8, 18.3, 19.3, 20.8, 22.5
CH_2	18.1, 25.3, 27.5, 28.5, 29.7, 30.3, 30.6, 31.2, 36.6	17.8, 22.0, 27.7, 28.5, 29.7, 30.3, 30.6, 31.2, 36.6
CH	48.7, 63.0, 79.6, 119.8	48.9, 63.0, 83.3, 119.8
C	23.8, 29.6, 32.5, 34.6, 37.1, 39.7, 44.6, 162.6, 176.0, 200.7	23.5, 29.6, 31.3, 32.5, 37.1, 39.7, 44.5, 162.5, 176.1, 200.5
Stearyl-C	14.0 (CH_3)	14.0 (CH_3)
	23.1, 26.5, 29.9, 30.0,	23.1, 26.5, 29.9, 30.0,
	30.3 (overlapped), 32.5, 67.3 (CH_2)	30.3 (overlapped), 32.5, 67.3 (CH_2)
Suc-C		28.7, 30.9 (CH_2)
		172.0, 177.0 (C)

Table 2: Incorporation ratios of targeting molecules

Suc-GLAOSt : Total lipids (mol : mol)	1 : 60	1 : 40	1 : 20	1 : 10	1 : 5
Incorporation ratio (%)*	93.3 ± 2.3	94.7 ± 1.5	95.2 ± 1.9	95.6 ± 2.0	/

Total lipids: lecithin and cholesterol; * Incorporation ratio data are expressed as mean ± S.D. of three experiments

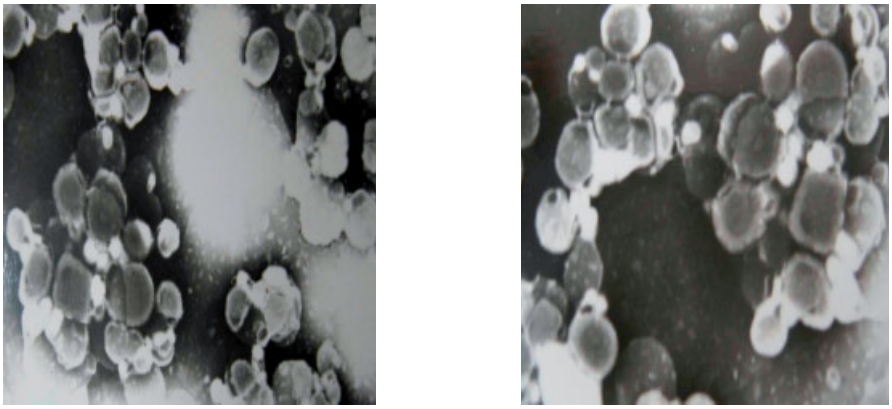


Fig. 1:
Transmission electron microscopy of Cal-LP (A) and Cal-LP-GLA (B) (× 15000)

shown in Table 2. When the molar ratio of targeting molecules to total lipids increased from 1 : 60 to 1 : 10, the maximum incorporation ratio was reached. When the molar ratio of targeting molecules increased to 1 : 5, white turbid solutions were obtained, and a large amount of floccule formed quickly in the solution. The maximum molar ratio of targeting molecules to total lipids is thus 1 : 10.

2.3. Drug loading, morphology, and particle size of Cal-LP and Cal-LP-GLA

The drug loading of Cal-LP and Cal-LP-GLA was 1.40 μg/mg lipids (total of lecithin and cholesterol) and 1.37 μg/mg lipids respectively. Transmission electron microscopy (Fig. 1) demonstrated that both Cal-LP (Fig. 1A) and Cal-LP-GLA (Fig. 1B) had a regular spherical surface. Table 3 shows that the average diameter of Cal-LP and Cal-LP-GLA was 65 nm ± 16 nm and 68 nm ± 21 nm, respectively.

2.4. Dose dependency of the uptake of Cal-LP and Cal-LP-GLA by primary cultured rat hepatocytes

0.1 ml, 0.2 ml, 0.4 ml and 0.8 ml of Cal-LP and Cal-LP-GLA solutions at the indicated concentration were added respectively to 1 ml of previously incubated hepatocyte suspension, and RPMI-1640 culture solution was added to each mixture up to 2 ml. The amount of calcein added to each culture dish was 0.41 μg, 0.82 μg, 1.64 μg and 3.30 μg respectively. The mixtures were incubated in 50 ml/L CO_2 at 37 °C for 2 h. The dose dependency of the uptake of Cal-LP and Cal-LP-GLA by primary cultured rat hepatocytes was examined, and the results are shown in Fig. 2. Cal-LP-GLA was taken up by rat hepatocytes to a much greater extent than was the control Cal-LP. The uptake of Cal-LP-GLA increased dose-dependently and was saturated at a dose of 1.2 mg lipids/dish.

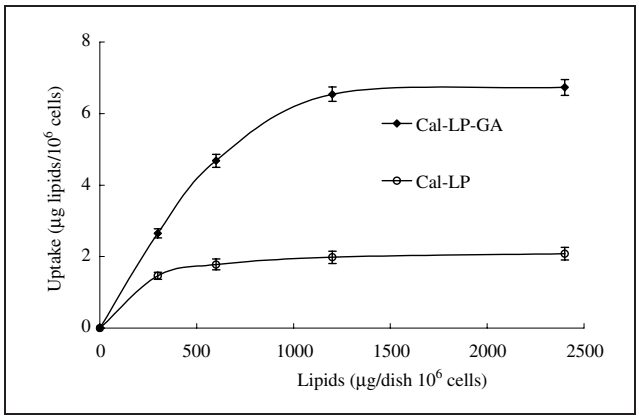


Fig. 2: Dose response curve of uptake of Cal-LP and Cal-LP-GLA by primary cultured hepatocytes. Each value is expressed as mean ± S.D. of three experiments

However, the uptake of Cal-LP increased only slightly and linearly.

2.5. Time course of the uptake of Cal-LP and Cal-LP-GLA by primary cultured rat hepatocytes

The concentration of total lipids in each culture dish was 1.2 mg and the mixtures were incubated in 50 ml/L CO_2 at 37°C for 0, 1, 2, 3 and 4 h respectively, and the incubation was stopped at the indicated time. The incubated mixtures were dealt with following the method process in section 4.9. The time course of the uptake of Cal-LP and Cal-LP-GLA by hepatocytes is shown in Fig. 3. It may be seen that Cal-LP-GLA had a high affinity to hepatocytes and that the time profile obtained for the uptake of Cal-LP-GLA was biphasic. The uptake of Cal-LP-GLA and Cal-LP by hepaocytes reached its maximum after the liposomes had been incubated for 2 h. The uptake of Cal-LP-GLA by rat hepatocytes was 3.3-fold higher than that of

Table 3: Particle size distribution of Cal-LP and Cal-LP-GLA

Drug loaded liposomes types	Particle size distribution*		
Cal-LP	d(0.1) = 36 nm ± 12 nm	d(0.5) = 65 nm ± 16 nm	d(0.9) = 113 nm ± 27 nm
Cal-LP-GLA	d(0.1) = 36 nm ± 14 nm	d(0.5) = 68 nm ± 21 nm	d(0.9) = 147 nm ± 35 nm

* Size data represents mean particle size ± S.D. (n = 3)

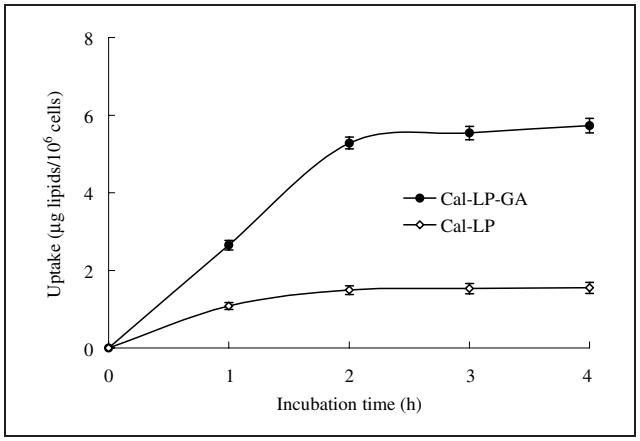


Fig. 3: Time course of uptake of Cal-LP and Cal-LP-GLA by primary cultured rat hepatocytes. Each value is expressed as mean ± S.D. of three experiments

Cal-LP. There was a statistically significant difference between the uptake of Cal-LP-GLA and Cal-LP ($P < 0.01$).

2.6. Inhibition experiment on Cal-LP-GLA uptake by hepatocytes

Based on the research of Negishi et al., the specific GLA binding sites on the surface of hepatocytes may contribute to the high affinity of Cal-LP-GLA for hepatocytes. To verify whether the uptake of Cal-LP-GLA was improved by specific GLA binding sites on the surface of hepatocytes, we examined whether the uptake of Cal-LP-GLA was inhibited by free GLA. GLA was dissolved and diluted to 0.4 mol/L with ethanol, and 0 μl, 50 μl, 100 μl, 200 μl and 250 μl of GLA solution were added to preincubated hepatocyte suspensions and the mixtures were incubated in 50 ml/L CO_2 at 37°C for 5 min. Then, 0.5 ml Cal-LP-GLA and Cal-LP solutions were added to the respective mixtures, and RPMI-1640 culture solution was added to each mixture to 2 ml (the amount of lipids in each culture dish was 1.2 mg). The culture plate was incubated in 50 ml/L CO_2 at 37 °C for 2 h. The incubated mixtures were dealt with following the method in section 4.9 and the intensity of fluorescence of calcein taken up by hepatocytes was measured. The results are shown in Table 4 and the corresponding inhibitory effect curve is shown in Fig. 4. The uptake of Cal-LP-GLA was inhibited in response to the concentration of GLA in the culture solution, and the inhibitory ratio was 70% at a concentration of 50 mM of GLA.

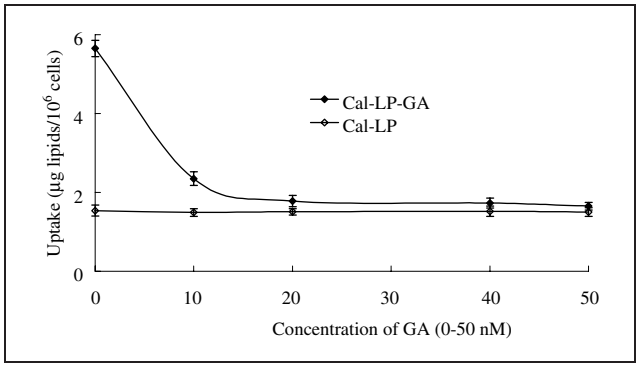


Fig. 4: Inhibitory effect of GL on the uptake of Cal-LP-GLA and Cal-LP by primary cultured rat hepatocytes. Each value is expressed as mean ± S.D. of five experiments

Table 4: Inhibitory effect of GLA on the uptake of Cal-LP-GLA and Cal-LP by primary cultured rat hepatocytes

Liposome	Concentration of GLA (mM)	Uptake (μg lipids/10^6 cells)	Inhibitory Ratio (%)
Cal-LP	0	1.54 ± 0.14	
	10	1.49 ± 0.10	–
	20	1.51 ± 0.08	–
	40	1.52 ± 0.13	–
	50	1.50 ± 0.11	–
Cal-LP-GLA	0	5.65 ± 0.21	
	10	2.35 ± 0.17	58
	20	1.78 ± 0.14	68
	40	1.73 ± 0.13	69
	50	1.65 ± 0.10	71

Each value of uptake represents the mean ± S.D. of five experiments. The inhibitory ratio was caculated as follows: Inhibitory ratio (%) $= \frac{\text{uptake (0)} - \text{uptake (c)}}{\text{uptake (0)}}$

Where uptake (0) and uptake (c) represent the uptake amount in the absence and the presence of c mM GLA (0–50 mM), respectively

3. Discussion

In order to modify the surface of liposomes with GLA, 3-succinyl-30-stearyl glycyrrhetinic acid (Suc-GLAOSt), an amphiphilic glycyrrhetinic acid derivative, was synthesized as a targeting molecule. In the structure of Suc-GLAOSt, the alkyl group of stearyl alcohol is hydrophobic and the residual moiety is hydrophilic. The amphiphilic molecules can be mixed into the lipid layer in a high proportion, which was verified by the results of Section 4.4 and Section 4.3.

When the molar ratio of targeting molecules to total lipids increased from 1 : 60 to 1 : 10, the maximum incorporation ratio was reached. When the molar ratio of targeting molecules increased to 1 : 5, white turbid solutions were obtained, and a large amount of floccule formed quickly in the solution. The maximum molar ratio of targeting molecules to total lipids is 1 : 10.

In *in vitro* hepatocyte uptake experiments, calcein was used as a model drug to investigate the uptake characterization of LP-GLA and LP. The uptake of Cal-LP-GLA depended on its dosage while the uptake of Cal-LP increased only slightly and linearly. The uptake of drug loaded LP-GLA by hepatocytes could be saturated when its dosage was large enough. Saturation of the uptake of LP-GLA indicates that the specific glycyrrhetinic acid binding site on the surface of hepatocytes is limited. The time course of the uptake of liposomes by hepatocytes is biphasic. During the first hour of incubation, LP-GLA was taken up rapidly by the cells, and subsequently the uptake rate of LP-GLA slowed down and increased in proportion to the incubation time. When the concentration of exogenous GLA increased, the uptake of drug-loaded LP-GLA by hepatocytes decreased, and the uptake finally approached that of unmodified liposomes. Under the same circumstances, the uptake of unmodified liposomes by hepatocytes increased slowly and linearly. Extraneous GLA did not influence the uptake of LP by hepatocytes, suggesting that GLA does not diminish the ability of hepatocytes to take up LP. If there are specific binding sites for GLA on the surface of hepatocytes, LP-GLA is likely to bind to this site with the GLA moiety. The excess uptake of LP-GLA by hepatocytes compared with LP is possibly caused by the internalization of LP-GLA mediated by specific GLA binding sites, which is partly confirmed by the results in Section 2.6. A previous study found that the rate

at which GL is taken up into the liver may depend upon the function and expression levels of hepatocellular OATPs (Ismair et al. 2003). This finding offers a rational explanation for the excess uptake of LP-GLA by hepatocytes compared with LP. The former was probably internalized by rat hepatocytes via an OATP pathway.

In our research, the uptake of Cal-LP-GLA by hepatocytes increased 3.3-fold compared with that of Cal-LP. To achieve optimal efficacy, the formulation of liposomes should take into account various aspects such as ligand affinity and drug incorporation (Kawakami et al. 2003). The amount of GLA on the surface of the hepatocyte membrane alone cannot determine the specific uptake of LP-GLA. The ability of GLA to combine with its specific binding sites and the characteristics of the surface of the liposomes also play an important role in the specific uptake of LP-GLA by hepatocytes.

In conculusion, there are specific binding sites of GLA on the surface of rat hepatocytes, LP-GLA may be internalized by hepatocytes via this site and LP-GLA can be used as a novel and promising drug carrier for active targeted delivery of drugs to hepatocytes.

4. Experimental

4.1. Chemicals and reagents

Lecithin was purchased from Taiwei Pharmacy Co.Ltd (Shanghai, China). Cholesterol was purchased from Bio Life Science & Technology Co.Ltd (Shanghai, China). Calcein was obtained from SSS Reagent (Shanghai, China). Glycyrrhetinic acid, desoxyribonuclease I and collagenase IV were purchased from Sigma (USA). PBS (12 mM NaH_2PO_4 : 51 mM Na_2HPO_4 : 77 mM NaCl, pH = 7.4, 280 mOsm · kg^{-1}), Hank's solution and RPMI-1640 culture medium were provided by the Department of Immunology, School of Preclinical Medicine, Sichuan University. All the other chemicals and reagents used were of analytical grade.

4.2. Synthesis of targeting molecules

Stearyl alcohol (2.7 g), 2.0 g DCC and an appropriate amount of CuCl were mixed and stirred at 80 °C for 4 h, 4.7 g GLA and 20 ml DMF were added to the solution with stirring at 80 °C. After continued stirring for 4 h, the solution was filtered and the filtrate was cooled in an ice-water bath, when a yellow precipitate was obtained. The precipitate was added to a silica column and eluted with a mixture of acetic ether and petroleum ether (4 : 1/v : v), and after the elutant was evaporated, 5 g of solid was obtained. The solid was recrystallized with petroleum ether, and 4 g white crystal solid were obtained, which was identified as GLAOSt by ^{1}H NMR and ^{13}C NMR.

GLAOSt (3.5 g) and 0.6 g succinic anhydride were dissolved in 3 ml pyridine, and the solution was reacted for 20 min in a microwave reactor. The product was added to a silica column and eluted with a mixture of acetic ether and petroleum ether (6 : 1/v : v), and after the elutant was evaporated, 3.5 g of white crystal line solid was obtained. The solid was identified as Suc-GLAOSt by ^{1}H NMR and ^{13}C NMR.

4.3. Assay of targeting molecules

The incorporation ratio of Suc-GLAOSt was calculated by measuring the content of Suc-GLAOSt in the liposomes by HPLC after gel filtration and the incorporation ratio was expressed as a percentage of the initial content before gel filtration. The analytical column was a Shim-pack CLC-ODS (150 mm × 4.6 mm i.d., 5 μm). The mobile phase consisted of acetonitrile and ethanol (10 : 90, v/v). The wavelength was set at 247 nm. The flow rate of mobile phase was set at 1.0 ml per minute. Chromatographic assay was performed at 20 °C. The regression equation of the calibration curve was $A = -20.78 + 774.64C$ ($r = 0.9997$), when the concentration of Suc-GLAOSt ranged from 10.8 to 538.5 μg/ml.

4.4. Preparation and characterization of LP and LP-GLA

Lecithin (200 mg) and 100 mg cholesterol were dissolved in 10 ml ethanol, and 3 ml of the solution was injected rapidly into 10 ml preheated (60 °C) PBS, and the solution was stirred (500 rpm) at 60 °C until all the ethanol was evaporated. Normal liposomes were thus obtained.

3-Succinyl-30-stearyl glycyrrhetinic acid (4 mg), 200 mg phospholipids and 100 mg cholesterol were dissolved in 10 ml ethanol, and the solution was prepared following the above-mentioned procedure, and the LP-GLA was obtained.

Polyoxyethylene nonylphenol ether and ethanol, 0.1 M HCl (1 : 20 : 30, v/v/v) were mixed thoroughly and an emulsifier OP solution was obtained. The emulsifier OP solution was used to disintegrate liposomes. LP or LP-GLA solutions (1 ml) were filtered with a G-50 Sephadex column and eluted with PBS, and the bluish eluted liposome solutions were collected and diluted to 10 ml with emulsifier OP solution. Suc-GLAOSt content of the solution was determined by HPLC and the incorporation ratio of Suc-GLAOSt in LP-GLA was calculated to be 95.6%. Suc-GLAOSt could thus be incorporated into the LP-GLA at a high molar ratio.

4.5. Preparation of Cal-LP and Cal-LP-GLA

Lecithin (200 mg) and 100 mg cholesterol were dissolved in 10 ml ethanol, and 3 ml of the solution was injected rapidly into 10 ml preheated (60 °C) Cal-PBS (1 mg Cal), and the solution was stirred (500 r/min) at 60 °C until all the ethanol was evaporated. Calcein loaded normal liposomes (Cal-LP) were thus obtained. 3-Succinyl-30-stearyl glycyrrhetinic acid (15 mg), 200 mg phospholipids and 100 mg cholesterol were dissolved in 10 ml ethanol, and the solution was prepared following the above-mentioned procedure, and calcein loaded liposomes surface-modified with glycyrrhetinic acid (Cal-LP-GLA) were obtained.

4.6. Determination of the drug loading of Cal-LP and Cal-LP-GLA

Cal-LP and Cal-LP-GLA colloidal solutions (5 ml) were ultracentrifuged (4×10^4 rpm) for 10 min, 2 ml of the supernatants were collected and diluted with deionized water, the fluorescence intensity (F) of the dilutions were measured and the drug loading of Cal-LP and Cal-LP-GLA were calculated.

4.7. Particle shapes and particle sizes of Cal-LP and Cal-LP-GLA

The particle shapes of Cal-LP and Cal-LP-GLA were observed under a transmission electron microscope (H-600, Hitachi, Japan) and the particle sizes of Cal-LP and Cal-LP-GLA were measured with a laser particle size analyzer (Malvern-2000, UK).

4.8. Primary culture of rat hepatocytes

Hepatocytes were isolated from normal livers of male Wistar rats (200 ± 20 g). The livers were purged with Hank's solution and scissored into tissue blocks (1 mm × 1 mm). The blocks were incubated with 1 mg/ml collagenase IV solution (37 °C, pH was adjusted to 7.4 using Hepes) for 120 min and filtered through a 200-mesh cell sieve, and a hepatocyte suspension was obtained. The hepatocyte suspension was washed 3 times with Hank's solution and once with by RPMI-1640 respectively. The concentration of the hepatocyte suspension was adjusted to 1×10^6 cells/ml. Hepatocyte suspension was plated into 6-well culture plates at a density of 1×10^6 cells/well, incubated in 50 ml/L CO_2 at 37 °C for 24 h, and used in the following experiments.

4.9. In vitro hepatocyte uptake experiments

Cal-LP and Cal-LP-GLA solutions were ultracentrifuged (4.0×10^4 rpm) for 30 min, the supernatants were discarded and the precipitate were dispersed with 2 ml PBS. Then, 0.1 ml, 0.2 ml, 0.4 ml and 0.8 ml Cal-LP and Cal-LP-GLA solutions were added to 1 ml preincubated hepatocyte suspension respectively, and RPMI-1640 culture solution was added up to 2 ml and mixed uniformly. Cal-LP and Cal-LP-GLA at the indicated concentrations were incubated with hepatocytes in culture solution. The culture solution was removed at the indicated time and the cells were washed 4 times with 12 ml ice-cold PBS. Distilled water (2 ml) was added to the washed cells and the mixture was stored at −10 °C for 2 h. The frozen mixture was defrosted at room temperature to disintegrate hepatocytes. The mixture obtained was centrifuged for 10 min (1×10^4 rpm) and the supernatants were diluted with PBS to 5 ml. The fluorescence intensity of the solutions was determined ($\lambda ex = 491.2$ nm, $\lambda em = 510.4$ nm, slit width = 10 nm, 10 nm). When the concentration of calcein ranged from 0.2 ng/ml to 6.4 ng/ml, the regression equation of calibration curve was $F = -1.51 + 97.58C$ (ng/ml), $r = 0.9997$. The uptake of the two types of liposome by hepatocytes was expressed as μg of total lipids per 10^6 cells. The data were calculated according to the drug loading and the determined fluorescence intensity.

In the inhibition experiment, the cells were preincubated with GLA solution (GLA dissolved in ethanol) at a concentration of 0–50 mM in culture solution. The uptake experiments were then carried out following the procedure described above.

In order to exclude the error caused by calcein absorption by the cell culture plate, parallel procedures were followed in all experiments. 0.1 ml, 0.2 ml, 0.4 ml and 0.8 ml Cal-LP and Cal-LP-GLA solutions were incubated, centrifuged, purged and treated as described above, their fluorescence intensity was determined and deducted from the fluorescence intensity of Cal-LP and Cal-LP-GLA solutions before the uptake of Cal-LP and Cal-LP-GLA by hepatocytes was calculated.

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