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Novel Galactosylated Poly(ethylene glycol)-Cholesterol for Liposomes as a Drug Carrier for Hepatocyte-Targeting

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In this study, three types of galactosylated cholesterol (i.e., gal-PEG194-chol, gal-PEG1000-chol and gal-PEG2000-chol) were synthesized with one terminal of polyethylene glycol of various chain lengths conjugated to the galactoside moiety, and the other terminal conjugated to the cholesterol. The galactose-modified liposomes were prepared by thin film-hydration method and doxorubicin (DOX) was loaded to the liposomes by using a ammonium sulfate gradient procedure. The liposomal formulations with galactosylated cholesterol were characterized. Flow cytometry and laser confocal scanning microscopy analyses showed that the galactose-modified liposomes facilitated the intracellular uptake of liposomes into HepG2 via asialoglycoprotein receptor (ASGP-R) mediated endocytosis. Cytotoxicity assay showed that the cell proliferation inhibition effect of galactose-modified liposomes was higher than that of the unmodified liposomes. Additionally, the study on frozen section of liver showed that the galactose-modified liposomes enhanced the intracellular uptake of liposomes into hepatocytes. Taken together, these results suggested that liposomes containing such galactosylated cholesterol (i.e., gal-PEG-chol), had a great potential as drug delivery carriers for hepatocyte-selective targeting.

Keywords: Hepatocyte-Targeting, Galactose, Doxorubicin, Liposomes, Asialoglycoprotein Receptor.

1. INTRODUCTION

Human hepatocellular carcinoma (HCC) is one of the most deadly and most rapidly increasing cancer in the world, it is frequently a terminal complication of chronic inflammatory and fibrotic liver diseases.[1,2]

The asialoglycoprotein receptor (ASGP-R) is expressed by liver parenchymal cells, which contain $1–5 \times 10^5$ binding sites per cell.[3–5] It can bind desialylated proteins from the serum and internalize them in the cell interior.[6,7] The ASGP-R can recognize a wide variety of desialylated glycoproteins and neoglycoproteins that contain terminal β-D-galactose or N-acetylgalactosamine residues.[8,9] The affinity of the ASGP-R increases for mono-, di-, tri-, and tetra-antennary oligosaccharides with correspondingly decreasing Kd values of $\sim 10^{-3}$, $\sim 10^{-6}$, $\sim 5 \times 10^{-9}$, and $\sim 10^{-9}$ M, respectively.[5,10]

Liposomes can alter the distribution and bioavailability of encapsulated drugs, which represents a good approach for treatment of several diseases.[14–16] Liposomes are commonly cleared by the reticular endothelial system (RES) when administered intravenously.[13,17] Kupffer cells in the liver are part of the RES. Relatively high accumulation of administered liposomes is usually observed in the liver, mostly in non-parenchymal cells.[13,18]

Polyethylene glycol (PEG)-coated liposomes can remain in the circulating blood for long periods and have passive targeting effect to tumor sites, which improve the therapeutic effects of encapsulated drugs. In addition to passive targeting, active targeting of liposomes is a promising approach to improving the pharmacological effect of drugs and reducing their side-effects during treatment.[19,20] Receptor-mediated drug targeting is a promising approach

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to obtain selective drug delivery to diseased tissues *in vivo*, leading to a reduction in drug toxicity and improved therapeutic outcomes. Uptake of ligand-incorporated liposomes is significantly higher than that of liposomes without ligands. Delivery of drugs using liposomes bound to ASGP-R in a specific manner would provide significant therapeutic benefits in hepatic diseases. Galactose-terminated compounds, such as lactosylceramide,[21] asialofetuin (AF)[22] or synthetic glycolipids[23] have been used to modify liposomes for selective accumulation in the liver, i.e., targeting hepatocytes.[13] Since the recognition of galactosylated liposomes by the receptors is highly efficient, the liposomes can be rapidly delivered to the target cells.

In recent decades, different types of glycolipids with lipophilic anchor moieties have been synthesized for incorporation into liposomes.[24, 25] Cholesterol, one of the lipid components used to form liposomes, is usually selected as the lipophilic anchor moiety for stably introducing the galactosyl moiety into liposomes.[9, 20, 24, 26]

In this study, we synthesized a novel multi-functional galactosylated cholesterol derivative (Gal-PEG2000-chol) containing
(i) a lipophilic anchor moiety (cholesterol) for stable incorporation into liposomes,
(ii) a polyethylene glycol for steric stabilization and long-circulating effect, and
(iii) a galactose moiety for the cell surface receptors in hepatocytes.[27]

It is well known that DOX can be efficiently encapsulated in liposomes via transmembrane sulfate ammonium gradients and form a stable drug-sulfate gel in the liposomes interior, which results in the greater stability of DOX liposomes in plasma and during storage.[9, 28] Therefore, DOX was chosen as the model compound for the demonstration of contents delivery because of its appeal as a cancer chemotherapeutic agent and its fluorescence allows it to be identified within tissues and cells.

The goal of this study is to develop a galactose modified liposomal formulation for doxorubicin (DOX) delivery and evaluate its hepatocyte targeting effect *in vitro* and *in vivo*. Gal-modified liposomes are composed of 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), Cholesterol (Chol) and Gal-PEG2000-chol. *In vitro* cellular uptake of Gal-modified liposomes was investigated in human hepatoma HepG2 cells which are known to express asialoglycoprotein receptors. Flow cytometry and laser scanning confocal microscope were used to evaluate the cellular uptake of DOX liposomes in HepG2 cells. *In vivo* liver-targeting effects of a variety of DOX liposomal formulations were studied in mice.

2. MATERIALS AND METHODS

2.1. Materials

Cholesterol was purchased from Guangzhou Pharmaceuticals Corporation. PEG2000 was purchased from Sigma Chemical Co. (St. Louis, MO, USA). PEG194 and PEG1000 were purchased from Aladdin Chemistry Co., Ltd.; Galactose was purchased from Aladdin Chemistry Co., Ltd. and Shanghai Baoman Biological technology Co., Ltd.; DSPC was purchased from Genzyme Pharmaceuticals. DSPE-PEG2000 was purchased from Avanti Polar Lipids, Inc.

HepG2 cells and HeLa cells were purchased from the laboratory animal center of Sun Yat-Sen University. Cells were cultured in DMEM medium supplemented with 10% fetal bovine serum and antibiotics (streptomycin 100 lg/mL, penicillin 100 U/mL) at 37 °C in humidified air with 5% CO_2.

2.2. Experimental Animals

Male KM mice aged 6–8 weeks (18–20 g) were purchased from the laboratory animal center of Sun Yat-Sen University.

2.3. Synthesis of Galactose-PEG-Cholesterol Conjugates

The route of synthesis of galactose-PEG-cholesterol conjugates is shown in Figure 1.

(2S,3R,4S,5S,6R)-6-(acetoxymethyl)tetrahydro-2 H-pyran-2,3,4,5-tetrayl tetraacetate (1). Anhydrous sodium acetate (2 g) was slowly dissolved in 20 mL of acetic anhydride under reflux. D-galactose (2 g) was then added to the solution and the mixture was left to react under reflux for 1.5 h. The reaction mixture was poured into ice, and stirred to attain a yellow powder, recrystallizated with ethanol to obtain compound 1 (85% yield).

(2R,3S,4S,5R,6R)-2-(acetoxymethyl)-6-hydroxytetrahydro-2 H-pyran-3,4,5-triyl triacetate (2). To the solution of ethylenediamine (195 μl) in anhydrous DCM (30 mL), glacial acetic acid (185 μl) was added dropwise. Then, compound 1 (908.7 mg, 2.33 mmol) was added. After the completion of the reaction monitored by TLC, the reaction mixture was diluted with water (50 ml) and extracted with DCM (3×50 ml). The combined organic layer was washed with 1 N HCl (2×50 ml) and sat. $NaHCO_3$ (2×50 ml), and dried over anhydrous Na_2SO_4. Then, organic layer was filtered and condensed under reduced pressure without further purification to provide desired product 2 as a white oil (462 mg, 57%).

(2R,3S,4S,5R,6S)-2-(acetoxymethyl)-6-(2,2,2-trichloro-1-iminoethoxy)tetrahydro-2 H-pyran-3,4,5-triyl triacetate (3). To a stirred solution of compound 2 (462 mg, 1.33 mmol) in dry CH_2Cl_2 (3 mL), powdered K_2CO_3 (309.2 mg, 2.24 mmol) and trichloroacetonitrile (98%, 0.39 mL, 3.8 mmol) were added at room temperature. The reaction mixture was stirred for 22 h and filtered and the filtrate was evaporated in vacuo to dryness. The residue was purified by column chromatography (petroleum ether–EtOAc, 3:1). Compound 3 was obtained as a white powder (320 mg, 49%). 1 H NMR (400 MHz, $CDCl_3$),

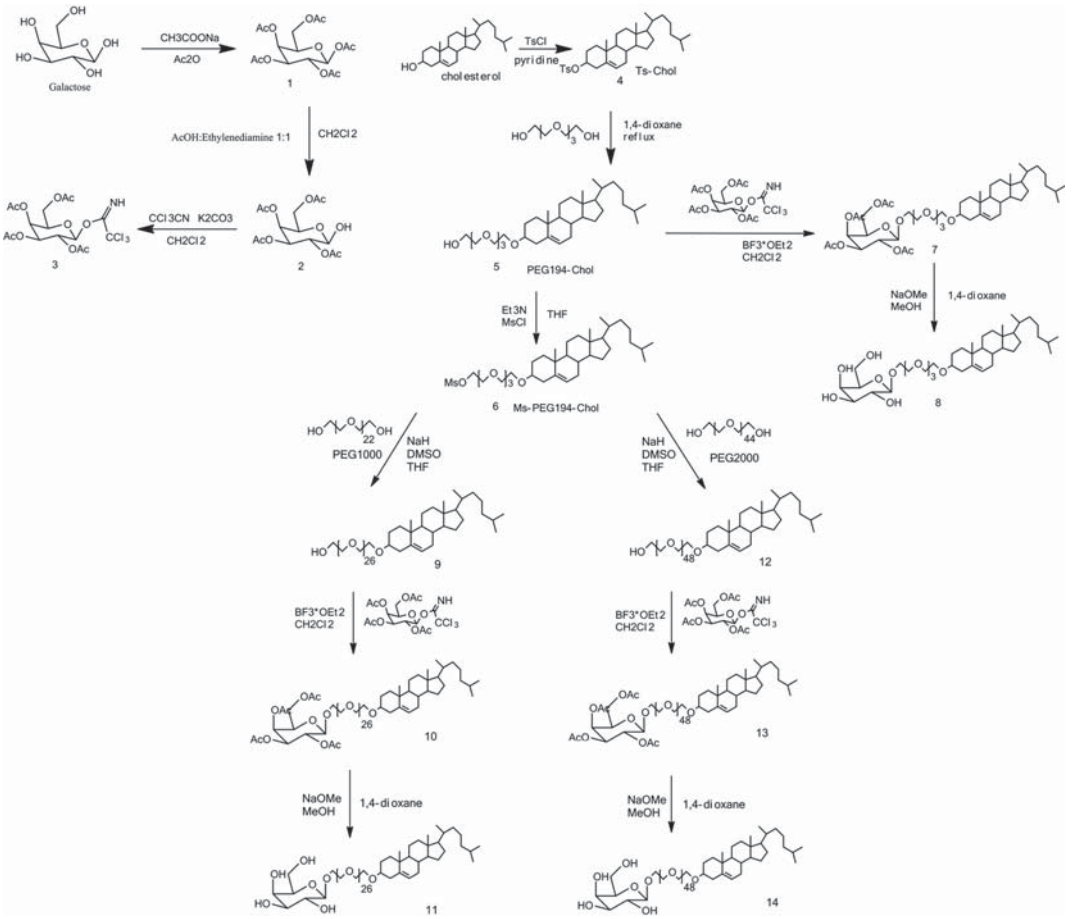

Figure 1. Synthesis of galactose-PEG-cholesterol conjugates.

δ/ppm: 8.67 (*s*, 1 H), 6.61 (*d*, 1 H), 5.57 (*d*, 1 H), 5.42 (*m*, 2 H), 4.46 (*m*, 1 H), 4.20 (*m*, 1 H), 4.08 (*m*, 1 H), 2.17 (*s*, 3 H), 2.02 (*d*, 9 H).

Cholest-5-en-3β-ol-*p*-toluenesulfonate (4). A solution of cholest-5-en-3b-ol (10 g, 25.86 mmol) and *p*-toluenesulfonylchloride (10 g, 52.47 mmol) in dry pyridine (100 ml) was stirred for 16 h at room temperature. After adding water (25 ml) to the reaction, the mixture was extracted with chloroform (50 ml, three times). The organic phase was dried over anhydrous sodium sulfate and concentrated in vacuo. The residue was purified by flash SiO_2 column chromatography with elution of petroleum ether/ethyl acetate (20:1). Compound 4 was obtained as a white powder (80% yield). 1 H NMR (400 MHz, $CDCl_3$), δ/ppm: 7.81 (*d*, 2 H), 7.34 (*d*, 2 H), 5.31 (*d*, 1 H), 4.32 (*m*, 1 H), 0.96 (*s*, 3 H), 0.91 (*s*, 3 H), 0.89 (*d*, 6 H), 0.65 (*s*, 3 H).

11-(cholest-5-en-3b-yloxy)-3,6,9-trioxaundecan-1-ol (5). Tetraethyleneglycol (6 mL) was added to a solution of cholest-5-ene-3β-tosylate (3 g, 5.4 mmol) in anhydrous 1,4-dioxane (54 mL), and the mixture was stirred under reflux for 24 h in an inert atmosphere. The solution was cooled and the solvent removed in vacuo. The residue was partitioned between $CHCl_3$ (100 mL) and water (100 mL), washed sequentially with saturated $NaHCO_3$ (2×50 mL), water (50 mL), and saturated brine (50 mL), and dried over anhydrous Na_2SO_4, and the solvent was removed in vacuo. The residue was purified by column chromatography on silica gel (200–300 mesh) by using petroleum ether/ethyl acetate (10:1) $\rightarrow$ CH_2Cl_2:MeOH (35:1) to obtain compound 5 (54.2% yield). 1 H NMR (400 MHz, $CDCl_3$), δ/ppm: 5.34 (*d*, 1 H), 3.66 (*m*, 16 H), 3.17 (*m*, 1 H), 0.65 (*s*, 3 H).

Ms-PEG194-chol (6). To a solution of 5 (1.65 g, 2.93 mmol) and triethylamine (2.0 ml, 14.6 mmol) in anhydrous THF (146 ml) in ice bath, methanesulfonyl chloride (340 μl, 4.4 mmol) was added. After 10 min, the solution was allowed to attain room temperature to stirred overnight. The reaction mixture was concentrated. The resulting precipitation was partitioned between EtOAc and H_2O (1:1, v/v). The aqueous layer was then extracted with EtOAc. The combined organic layer was dried on

anhydrous Na_2SO_4 and concentrated. The residue was purified by flash SiO_2 column chromatography with elution of petroleum ether/ethyl acetate (5:6) to obtain compound 6 (37.9% yield). 1 H NMR (400 MHz, $CDCl_3$), δ/ppm: 5.34 (*d*, 1 H), 3.76 (*m*, 16 H), 3.08 (*s*, 3 H), 0.68 (*s*, 3 H).

11-(Cholest-5-en-3b-yloxy)-3,6,9-trioxaundecanyl-2,3,4,6-tetra-O-acetyl-b-D-galactopyranoside (7). A mixture of compound 5 (704 mg, 1.25 mmol), compound 3 (741.6 mg, 1.51 mmol) and freshly activated molecular sieves (4 Å, 3.6 g) in dry CH_2Cl_2 (36 ml) was stirred at room temperature for 30 min in an N_2 atmosphere and then cooled to 0 °C. The promoter $BF_3 \cdot OEt_2$ (100 μL) in dry CH_2Cl_2 (8 mL) was added dropwise, and the mixture was stirred for 3.5 h. Additional $BF_3 \cdot OEt_2$ (500 μL) in CH_2Cl_2 (8 mL) was then added, and the mixture was allowed to be warmed to room temperature and stirred for 6 h. The reaction mixture was filtered and washed with CH_2Cl_2 (30 mL) and the filtrate was evaporated in vacuo to dryness. The residue was purified by column chromatography (petroleum ether–EtOAc, 1:1) to obtain compound 7 (227 mg, 20.3%).

11-(cholest-5-en-3b-yloxy)-3,6,9-trioxaundecanyl-b-D-galactopyranoside(gal-PEG194-chol) (8). To a solution of 7 (227 mg, 0.254 mmol) in 1,4-dioxide (15 ml), sodium methoxide (0.1 M in methanol, 6 ml) was added and the mixture was stirred for 4 h at room temperature in an N_2 atmosphere. After neutralization with HCl, the mixture was concentrated in vacuo and purified by silica gel chromatography (CH_2Cl_2/MeOH 10:1) to obtain compound 8 (80% yield). 1 H NMR (400 MHz, $CDCl_3$), δ/ppm: 5.34 (*d*, 1 H), 4.32 (*d*, 1 H), 4.02 (*m*, 2 H), 3.85 (*m*, 3 H), 3.65 (*m*, 18 H), 0.68 (*s*, 3 H). 13C NMR (400 MHz, DMSO-D6), δ/ppm: 163.12 (–O—C═C–), 125.95 (–C═C–, chol), 103.38 (—O—C—O—, gala). MS: [M + Na] 747.5. IR (cm^{-1}): $\upsilon_{O—H}$ 3432, $\upsilon_{C—H}$ 2930, $\upsilon_{C—O}$ 1100.

PEG1000-chol (9). A mixture of PEG1000 (10 g, 10 mmol) and NaH (60%, 430 mg, 10.75 mmol) in dry THF (50 ml) and DMSO (50 ml), was stirred at room temperature for 30 min in an N_2 atmosphere. Then the solution of compound 6 (1.708 g, 2.66 mmol) in THF (20 ml) was added, and the mixture was stirred at 60 °C for 8 h. The reaction mixture was cooled to room temperature and poured into 50 ml ice water. The mixture was then extracted with CH_2Cl_2, (3 × 100 ml). The combined organic layer were dried on anhydrous Na_2SO_4 and concentrated. The residue was purified by SiO_2 column chromatography with elution of CH_2Cl_2/MeOH 35:1 to obtain compound 9 (48% yield). 1 H NMR (400 MHz, $CDCl_3$), δ/ppm: 5.34 (*d*, 1 H), 3.64 (*m*, 90 H), 3.17 (*m*, 1 H), 0.68 (*s*, 3 H).

gal-PEG1000-chol (11). A mixture of compound 9 (689 mg, 0.44 mmol), compound 3 (282.7 mg, 0.57 mmol) and freshly activated molecular sieves (4 Å, 1.2 g) in dry CH_2Cl_2 (13 ml) was stirred at room temperature for 30 min in an N_2 atmosphere and then cooled to 0 °C. The promoter $BF_3 \cdot OEt_2$ (100 μL) in dry CH_2Cl_2 (2.6 mL) was added dropwise, and the mixture was stirred for 3.5 h. Additional $BF_3 \cdot OEt_2$ (200 μL) in CH_2Cl_2 (2.6 mL) was then added, and the mixture was allowed to be warmed to room temperature and stirred for 6 h. The reaction mixture was filtered and washed with CH_2Cl_2 (30 mL) and the filtrate was evaporated in vacuo to dryness. The residue was purified by column chromatography (CH_2Cl_2/MeOH 15:1) to give a mixture (this step was not to get a purity of compound 10).

To a solution of the mixture (433 mg) in 1,4-dioxide (15 ml), sodium methoxide (0.1 M in methanol, 5.5 ml) was added, and the mixture was stirred for 4 h at room temperature in an N_2 atmosphere. After neutralization with HCl, the mixture was concentrated in vacuo and purified by silica gel chromatography (CH_2Cl_2/MeOH 8:1) to obtain compound 11 (16.5% yield). 1 H NMR (400 MHz, DMSO-D6), δ/ppm: 5.31 (*d*, 1 H), 4.78 (*d*, 1 H), 4.64 (*d*, 1 H), 4.54 (*m*, 1 H), 4.30 (*d*, 1 H), 4.10 (*d*, 1 H), 3.55 (*m*, 115 H), 0.65 (*s*, 3 H). 13C NMR (400 MHz, DMSO-D6), δ/ppm: 140.50 (–O—C═C–), 120.99 (–C—C–, chol), 103.55 (–O—C—O–, gala). IR (cm^{-1}): $\upsilon_{O—H}$ 3436, $\upsilon_{C—H}$ 2944, $\upsilon_{C—O}$ 1069.

PEG2000-chol (12). A mixture of PEG2000 (8.9 g, 4.45 mmol) and NaH (60%, 182.1 mg, 4.55 mmol) in dry THF (20 ml) and DMSO (20 ml) was stirred at room temperature for 30 min in an N_2 atmosphere. Then the solution of compound 6 (711 mg, 1.11 mmol) in THF (10 ml) was added, and the mixture was stirred at 60 °C for 8 h. The reaction mixture was cooled to room temperature and poured into 50 ml ice water. The mixture was then extracted with CH_2Cl_2 (3 × 100 ml). The combined organic layer was dried on anhydrous Na_2SO_4 and concentrated. The residue was purified by SiO_2 column chromatography with elution of CH_2Cl_2/MeOH 25:1 to give compound 12 (45% yield). 1 H NMR (400 MHz, $CDCl_3$), δ/ppm: 5.34 (*d*, 1 H), 3.64 (*m*, 182 H), 0.68 (*s*, 3 H).

gal-PEG2000-chol (14). A mixture of compound 12 (756.5 mg, 0.3 mmol), compound 3 (239.2 mg, 0.49 mmol) and freshly activated molecular sieves (4 Å, 1.3 g) in dry CH_2Cl_2 (9 ml) was stirred at room temperature for 30 min in an N_2 atmosphere and then cooled to 0 °C. The promoter $BF_3 \cdot OEt_2$ (40 μL) in dry CH_2Cl_2 (1.8 mL) was added dropwise, and the mixture was stirred for 3.5 h. Additional $BF_3 \cdot OEt_2$ (115 μL) in CH_2Cl_2 (1.8 mL) was then added, and the mixture was allowed to be warmed to room temperature and stirred for 6 h. The reaction mixture was filtered and washed with CH_2Cl_2 (30 mL) and the filtrate was evaporated in vacuo to dryness. The residue was purified by column chromatography (CH_2Cl_2/MeOH 10:1) to give a mixture (this step was not to get a purity of compound 13).

To a solution of the mixture (710 mg) in 1,4-dioxide (13 ml), sodium methoxide (0.1 M in methanol, 5.25 ml)

was added, and the mixture was stirred for 4 h at room temperature in an N_2 atmosphere. After neutralization with HCl, the mixture was concentrated in vacuo and purified by silica gel chromatography (CH_2Cl_2/MeOH 5:1) to obtain compound 14 (15.8% yield). 1 H NMR (400 MHz, DMSO-D6), δ/ppm: 5.31 (*d*, 1 H), 4.75 (*d*, 1 H), 4.61 (*d*, 1 H), 4.50 (*m*, 1 H), 4.28 (*d*, 1 H), 4.10 (*d*, 1 H), 3.83 (*d*, 1 H), 3.51 (*m*, 192 H), 0.65 (*s*, 3 H). 13C NMR (400 MHz, DMSO-D6), δ/ppm: 140.51 (–O—C═C–), 121.00 (–C—C–, chol), 103.56 (–O—C—O–, gala). IR (cm^{-1}): $\upsilon_{O—H}$ 3443, $\upsilon_{C—H}$ 2913, $\upsilon_{C—O}$ 1100. ^{1}HNMR, 13C NMR spectrum and IR spectra were seen in supplementary information.

2.4. Preparation of Liposomes

The galactose-modified liposomes were prepared by thin-film hydration, followed by remote loading of DOX with ammonium sulfate gradient. Lipids were dissolved in $CHCl_3$ (gal-PEG-chol was dissolved in $CHCl_3$, MeOH (1:2)) and dried under a N_2 stream. A trace amount of chloroform was removed by keeping the lipid film under a vacuum. The lipid film was hydrated with 250 mM $(NH_4)_2SO_4$ to obtain a blank liposomes suspension. The liposomes suspension was then sequentially extruded through polycarbonate membranes with pore size of 200 nm and 100 nm. The resulting liposomes were dialyzed (molecular weight cutoff size 14,000) against PBS (pH 7.4) at 37 °C For drug loading, DOX was dissolved in a small volume of deionized water and added to the liposomes to achieve a drug/lipid ratio of 1/10 (mol/mol). The loading process was carried out at 65 °C for 30 min and DOX liposomes was obtained. The lipids composition of the DOX liposomes was shown in Table I. Gal-194-liposomes (2%), Gal-194-liposomes (4%), Gal-1000-liposomes (2%), Gal-1000-liposomes (4%), Gal-2000-liposomes (2%) and Gal-2000-liposomes (4%) were prepared to investigate the effect of the liver targeting of galactosylated cholesterol with various PEG chain lengths (i.e., gal-PEG194-chol, gal-PEG1000-chol and gal-PEG2000-chol).

2.5. Characterization of DOX Liposomes

The particle size and zeta-potential of the DOX-liposomes were analyzed by using a Malvern Zetasizer Nano ZS90 (Malvern Instruments Ltd., UK). To determine the encapsulation efficacy, unencapsulated DOX was separated from liposomes by size exclusion chromatography using a Sephadex G-50 column. PBS (pH 7.4) was used as the eluent. The eluted liposomes were collected and lysed with Triton X-100 (1% v/v). The DOX concentration was determined by UV spectrophotometry (480 nm). The envelopment efficiency (EE%) of DOX was calculated based on the ratio of liposomal drug to total drug.

2.6. Intracellular Distribution of Liposomes by Laser Confocal Microscopy Analysis

Two different cell lines were used in this study. HepG2 cells expressing ASGP-R were derived from a human hepatocellular carcinoma. Hela cells without ASGP-R were used as a control. Cells were seeded on cover glass in a 24-well culture plate at a density of 7×10^4 cells/well. The cells were incubated for 24 h to 50% confluence and then treated with free-DOX and a variety of liposomal DOX formulations for 2 h. The cells were then washed three times with cold PBS, fixed by 4% paraformaldehyde at room temperature, and permeabilized with 0.5% Triton X-100 in PBS. The cells were stained with DAPI (1 μg/ml) to visualize the nuclei. Zeiss LSM710 laser confocal microscope was used to investigate the intracellular uptake and subcellular distribution of DOX (excitation/emission wavelength: 488 nm/560 nm).

2.7. Cellular Uptake of Liposomes by Flow Cytometry Analysis

Cells suspension (8×10^5 cells/well) were seeded in a 24-well culture plate and incubated for 24 h at 37 °C in 5% CO_2 until 80% confluence. The cells were then treated with free DOX and a variety of liposomal DOX formulations for 2 h. The cells were harvested and washed three times with cold PBS. The cellular uptake of DOX was measured by using a flow cytometer EPICS XL (Beckman Coulter). The intracellular DOX was excited with an argon

Table I. The lipids composition of liposomes.

Mol	DSPC	Cholesterol	DSPE-PEG2000	Gal-PEG 194-chol	Gal-PEG 1000-chol	Gal-PEG 2000-chol
Common-liposomes	55	45				
PEG-liposomes	50	45	5			
Gal-2000-liposomes (5%)	55	40				5
Gal-194-liposomes (5%)	55	40		5		
Gal-1000-liposomes (5%)	55	40			5	
Gal-194-liposomes (2%)	52	43	3	2		
Gal-194-liposomes (4%)	54	41	1	4		
Gal-1000-liposomes (2%)	52	43	3		2	
Gal-1000-liposomes (4%)	54	41	1		4	
Gal-2000-liposomes (2%)	52	43	3			2
Gal-2000-liposomes (4%)	54	41	1			4

laser at the wavelength of 488 nm and the fluorescence was detected at 575 nm. Files were collected for 10,000 gated events and analyzed with the FlowJo software.

2.8. Competitive Inhibition Experiments of Galactose

Galactose was used as a competitive inhibitor, to study whether the cellular uptake of the Gal-2000-liposomes was via ASGP-R, HepG2 cells were seeded in 24-well plates and incubated for 24 h, to which 100 mM galactose solution was added and then Gal-2000-liposomes was added to incubate for 2 h. The treatment of the confocal and flow cytometry samples was the same as that in 2.6 and 2.7.

2.9. *In Vitro* Cytotoxicity Assay

The cytotoxicities of various liposomes were tested on HepG2 cells and Hela cells, respectively. Briefly, each well of 96-well plates was seeded with 4000 cells and incubated for 24 h. The cells were then exposed to serial concentrations of free DOX or a variety of liposomal DOX formulations. After for a 48 h period of incubation, 10 μl of MTT solution (5 mg/mL) was added to each well, followed by incubation for another 4 h. Then the medium was removed and 150 μl of DMSO was added to each well for to dissolve the blue formazan crystals. The absorbance was read on a microplate reader at a wavelength of 570 nm. The experiment was carried out in triplicate. The data reported represented the mean of triplicate measurement.

2.10. Study on Frozen Section of Liver

All *in vivo* protocols were reviewed and approved by Animal Ethical and Welfare Committee of Sun Yat-sen University. Free-DOX and a variety of liposomal DOX formulations were administrated via tail vein at a dose of 5 mg/ml into mice. Mice were sacrificed at 1 h after injection. The liver was excised and frozen rapidly in dry ice, allowing the generation of ten-μm-thick cryosections. The tissue sections were fixed in cold acetone, washed with PBS, stained with FITC-Phalloidin (sigma), and mounted with the DAPI containing medium (VECTASHIELD® with DAPI). Images were captured by using Zeiss LSM710 laser confocal microscope.

3. RESULTS AND DISCUSSION

3.1. Synthesis of Galactose-PEG-Cholesterol Conjugates

Cholesterol is an important component of the liposomes membrane and can improve the stability of the liposomes. Cholesterol is more stable than phospholipids, which can resist harsh reaction conditions. Thus we used galactose, polyethylene glycol (PEG) and cholesterol as the starting materials to synthesize galactosylated cholesterol of gal-PEG-chol. The conjugates can be inserted into the lipid bilayer membranes (lipophilic groups), and functionalize the liposomes with a liver targeting moiety (i.e., galactose).

Three types of galactosylated cholesterol (i.e., gal-PEG194-chol, gal-PEG1000-chol and gal-PEG2000-chol) were successfully synthesized. The 1 HNMR, 13CHMR and IR identification results showed that the structures of the three types of galactosylated cholesterol were correct. 1 HNMR, 13CNMR spectrum and IR spectra were seen in supplementary information.

3.2. Characteristics of the Liposomes

Liposomes were prepared by thin film hydration followed by the extrusion as described above. The liposomes had a mean diameter of approximately 150 nm and relatively narrow distribution. The Zeta potential of the liposomes ranged from -36 to -18 mV. The negative zeta potentials might be due to the presence of the negatively charged lipid mPEG-DSPE in the formulation. Entrapment efficiency of DOX into liposomes was $>93\%$ at the drug/lipid ratio of 1:10. The results were summarized in Table II.

After loading the DOX, the characteristics of the liposomes did not change. DOX was encapsulated into liposomes by transmembrane sulfate ammonium gradients with high encapsulation efficiency. DOX and the sulfate formed a stable drug-sulfate gel in the liposomes interior, which resulted in greater stability of DOX liposomes during storage. The drug loading efficiencies were similar for liposomes with and without galactose modification.

3.3. Intracellular Uptake of the Liposomes

Intracellular uptake of the various liposomal formulations was evaluated by using confocal microscopy and flow cytometry analysis. Figure 2, showed the intracellular uptake of liposomes containing DOX (excitation/emission: 488/560 nm). The distribution pattern of fluorescent signals in HepG2 cells were significantly different among non-modified-liposomes (Fig. 2(A1)), PEG-liposomes (Fig. 2(B1)) and Gal-2000-liposomes (Fig. 2(C1)). Strong DOX fluorescence intensity was observed in the nuclei of HepG2 cells treated with galactose-modified liposomes.

To confirm the intracellular uptake of DOX-loaded liposomes, the fluorescence intensity of DOX against HepG2 cells was evaluated by using flow cytometry. As shown in Figure 3, the fluorescence intensity of DOX delivered by the Gal-liposomes (including Gal-2000-liposomes (5%), Gal-194-liposomes (5%), and Gal-1000-liposomes (5%)) in HepG2 cells was higher than that of other liposomes, indicating that galactose on liposomes surface could bind with ASGP-R, which is frequently expressed on the surface of hepatocytes.

Gal-2000-liposomes (5%) was more efficiently taken up by the cells compared to non-modified liposomes. The uptake could be blocked by 100 mM free galactose (Fig. 2(D)). Similarly, flow cytometry results showed that the cellular uptake of galactose modified liposomes was higher than that of non-modified liposomes and could be blocked by free galactose (Fig. 3). Taken together,

Table II. The characteristic of the DOX liposomes ($n = 3$).

DOX-liposomes	Mean diameter (nm)	PDI	*Z*-potential (mV)	EE (%)
Common-liposomes	189.3 ± 4.3	0.080 ± 0.020	− 24.2 ± 1.2	96.55
PEG-liposomes	173.6 ± 5.6	0.030 ± 0.027	− 28.9 ± 1.5	96.52
Gal-2000-liposomes (5%)	162.2 ± 3.2	0.099 ± 0.028	− 18.2 ± 2.6	94.53
Gal-194-liposomes (5%)	164.6 ± 5.1	0.177 ± 0.008	− 23.2 ± 2.3	93.17
Gal-1000-liposomes (5%)	161.9 ± 5.4	0.145 ± 0.030	− 17.4 ± 2.0	95.19
Gal-194-liposomes (2%)	180.1 ± 1.3	0.093 ± 0.035	− 36.7 ± 0.3	94.39
Gal-194-liposomes (4%)	184.5 ± 1.4	0.039 ± 0.013	− 35.0 ± 1.2	95.70
Gal-1000-liposomes (2%)	158.5 ± 0.6	0.086 ± 0.036	− 34.6 ± 1.0	97.32
Gal-1000-liposomes (4%)	156.8 ± 0.7	0.050 ± 0.034	− 31.0 ± 1.8	98.52
Gal-2000-liposomes (2%)	146.8 ± 0.9	0.107 ± 0.003	− 26.6 ± 1.2	96.36
Gal-2000-liposomes (4%)	164.0 ± 1.9	0.080 ± 0.020	− 24.2 ± 1.2	93.22

these results indicated that liposomes which contained Gal-PEG2000-chol could effectively target the HepG2 cells via the ASGP-R.

The mechanism of drug cellular uptake for drug-loaded liposomes was explained by diffusion, endocytosis or membrane fusion, depending on the characteristics of liposomes and cells. HepG2 cells treated with PEG-modified liposomes did not show any DOX fluorescence in cytoplasm (Fig. 2(B1)) suggesting that non-specific endocytosis was not involved in the DOX uptake of liposomal formulations. Therefore, the DOX uptake for PEG-modified liposomes might be mainly through the mechanism of diffusion.

The enhanced cellular uptake of DOX by Gal-modified liposomes is due to receptor-mediated endocytosis. The nuclei of the cells treated with galactose-modified liposomes (Fig. 2(C1)) showed stronger DOX fluorescence than that with PEG-modified liposomes (Fig. 2(B1)) within 2 h of incubation, indicating that process of cellular uptake was rapid and that DOX was quickly released from the endosomes and accumulated in the nuclei. In agreement with confocal microscopy observation, cellular uptake efficiency measured by flow cytometry revealed a remarkable difference in DOX level in cancer cells treated with PEG-liposomes and Gal-modified liposomes. As shown in Figure 2, the red fluorescence intensity of Gal-modified

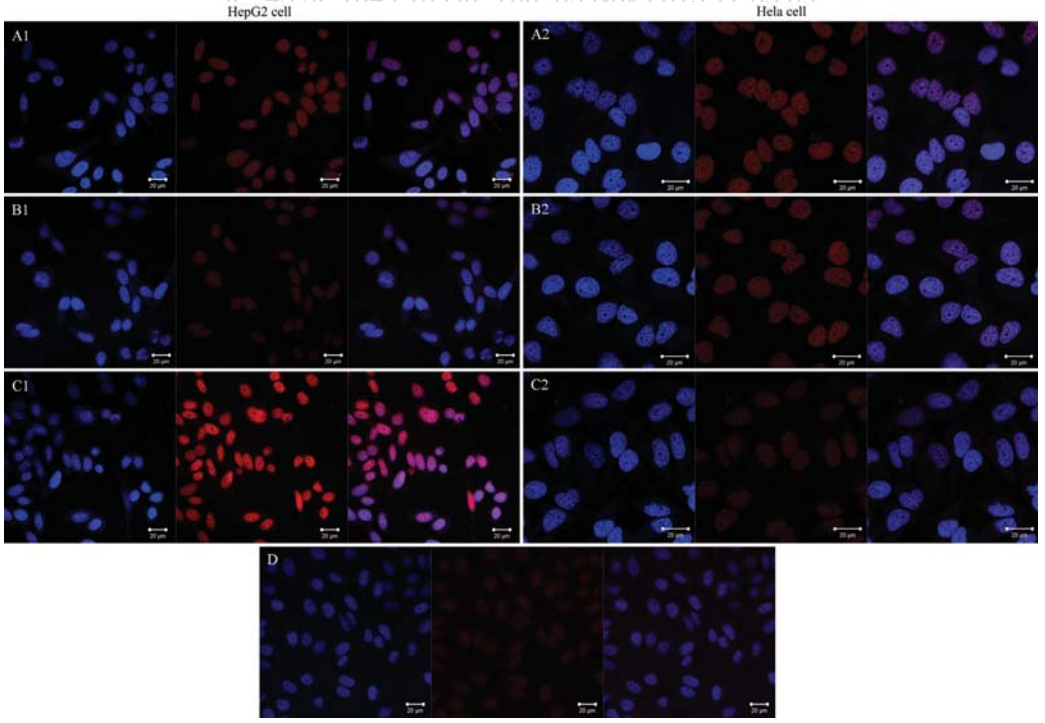

Figure 2. Confocal microscopy images of HepG2 cells and Hela cells incubated with common-liposomes (A1), (A2), PEG-liposomes (B1), (B2) and Gal-2000-liposomes (5%) (C1), (C2), 100 mM galactose + Gal-2000-liposomes (5%) (D) for 2 h at 37 °C. Cells were fixed and then treated with DAPI (blue) for nuclei staining. Red: Fluorescence of Dox. Blue: fluorescence of DAPI. Pink: The merger fluorescence of blue and red.

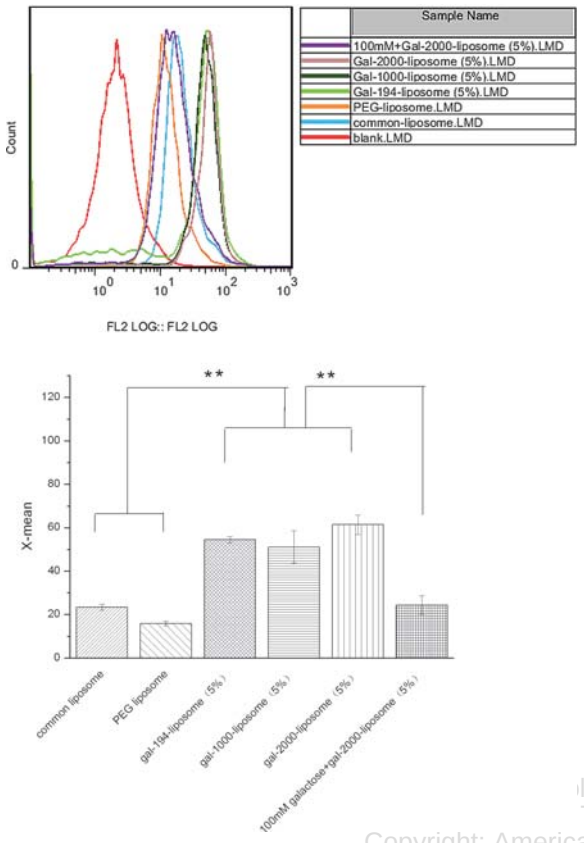

Figure 3. Flow cytometry analysis of HepG2 cells after incubated with PEG-liposomes and Gal-modified liposomes for 2 h with 10%FBS medium ($n = 3$).

liposomes in HepG2 cells was much higher than that of PEG-liposomes, suggesting that receptor-mediated endocytosis was responsible for the improved cellular uptake of DOX for Gal-modified liposomes but not for PEG-liposomes because terminal galactoside residue was able to bind with surface ASGP-R of HepG2 cells.

Galactosylated cholesterol was conjugated to PEG with various chain lengths and their effects on cellular uptake were investigated. To prepare Gal-194-liposomes (2%), Gal-194-liposomes (4%), Gal-1000-liposomes (2%), Gal-1000-liposomes (4%), Gal-2000-liposomes (2%) and Gal-2000-liposomes (4%), galactosylated cholesterol conjugated with various PEG chain lengths was mixed with DSPE-PEG2000 at different concentrations. We speculated that due to the various length of the PEG chain of the galactosylated cholesterol, the galactose ligand on the liposomes exposure to varying degrees when DSPE-PEG2000 was incorporated into the liposomes, thus influenced the cellular uptake of the galactose-modified liposomes.

The confocal images of cellular uptake in HepG2 cells were shown in supplementary information. As expected, there was no significant difference among cells treated with Gal-194-liposomes (2%), Gal-194-liposomes (4%), Gal-1000-liposomes (2%) and Gal-1000-liposomes (4%), which showed weaker DOX fluorescence than Gal-2000-liposomes (2%) and Gal-2000-liposomes (4%). Greater DOX fluorescence intensity was observed in HepG2 cells treated with Gal-2000-liposomes (4%) compared to that treated with Gal-2000-liposomes (2%). The flow cytometry data shown in Figure 4 agreed with the confocal microscopy imaging results.

Although Gal-194 (2%), Gal-194 (4%), Gal-1000 (2%) and Gal-1000 (4%) liposomes contained galactosylated cholesterol (gal-PEG194-chol and gal-PEG1000-chol), DSPE-PEG2000 with a longer PEG chain was also added to the liposomes. It is likely that the galactose ligand could not be exposed on the surface of the liposomes, and therefore the galactose ligand could not be interacted with the ASGP-R which was expressed on HepG2 cells. There was no significant difference between the fluorescence intensity of DOX in HepG2 cells treated with Gal-194 (2%), Gal-194 (4%), Gal-1000 (2%) and Gal-1000 (4%) liposomes and that of PEG-liposomes.

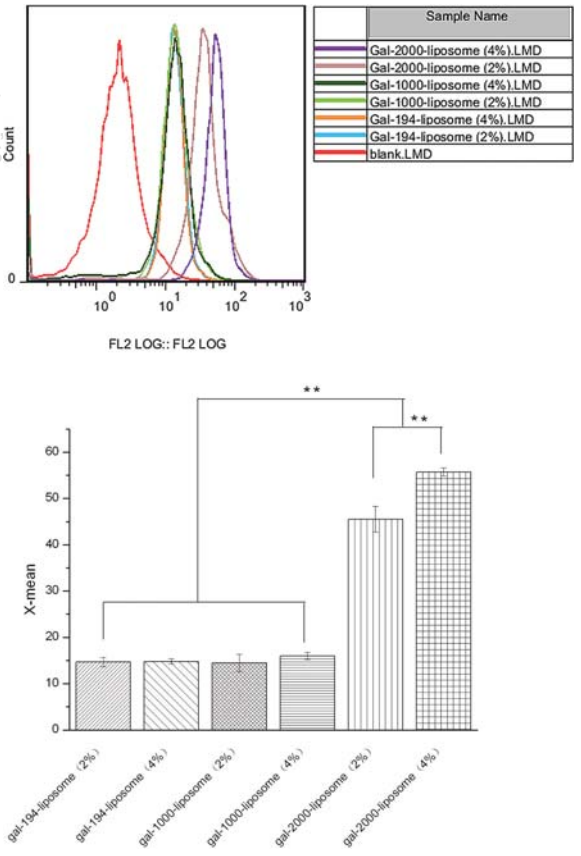

Figure 4. Flow cytometry analysis of HepG2 cells after incubated with different PEG chain length of galactose modified DOX liposomes for 2 h with 10%FBS medium ($n = 3$).

Gal-2000 (2%) and Gal-2000-liposomes (4%) contained a certain amount of gal-PEG2000-chol. Although a certain amount of DSPE-PEG200 was added, gal-PEG2000-chol had longer PEG chains, therefore galactose ligand could be exposed on the liposomes surface and could be interacted with ASGP-R on the HepG2 cells. Hence, the fluorescence intensity of DOX in HepG2 cells treated with Gal-2000 (2%) and Gal-2000-liposomes (4%) was higher than that of the liposomes which contained galactosylated cholesterol with a shorter PEG chains.

In this study, we choosed Hela cell lines which lacked ASGP-R to further confirm that cellular uptake of the galactose-modified liposomes was via the ASGP-R interaction. We could speculate that there was no significant difference among the DOX fluorescence intensity was observed in Hela cells treated with galactose-modified liposomes, common liposomes and PEG-modified liposomes.

As expected, Gal-modified liposomes (Gal-2000-liposomes (5%)) (Fig. 2(C2)) showed a minor tendency to be internalized by Hela cells, and there was no significant difference between PEG-liposomes (Fig. 2(B2)) and Gal-modified liposomes (Fig. 2(C2)). The fluorescence intensity of Gal-modified liposomes in Hela cells was weaker than that in HepG2 cells. And the flow cytometry results (shown in supplementary information) agreed with the result of confocal micrograph images.

Hela cells did not express the ASGP-R, thus Gal-modifed liposomes did not have the effect of increasing cellular uptake via receptor-mediated endocytosis. On the other hand, HepG2 cells expressed the ASGP-R, and the galactose ligand on the surface of Gal-modified liposomes could be interacted with the ASGP-R and increase the HepG2 cells uptake.

3.4. Cell Cytotoxicity Assay

Figure 5 showed the cell proliferation inhibition of HepG2 cells with increasing concentration of DOX solution and DOX-liposomes. Inhibition of cells proliferation after exposure to Gal-modifed liposomes was much more significant than that of non-modifed liposomes. The cytotoxicity of Gal-2000-liposomes (5%) and Gal-2000-liposomes (4%) was greater than that of other liposomes. These results indicated that Gal-2000-liposomes (5%) and Gal-2000-liposomes (4%) could enter HepG2 cells efficiently. These results were consistent with the observation that liposomes which contained gal-PEG2000-chol exhibited higher intracellular uptake in HepG2 cells than that of non-modified liposomes. Free DOX showed the highest inhibition. Doxorubicin is a small molecule drugs with highly nucleophilic nature. It could rapidly enter the cells by diffusion and inhibit the synthesis of RNA and DNA, leading to significantly higher inhibition.

The cell proliferation inhibition of free DOX and DOX liposomes in Hela cells after incubation of 48 h were shown in supplementary information. Free DOX showed the highest inhibition effect. No significantly difference in the inhibition of Hela cells was shown among non-modified liposomes and gal-modified liposomes (i.e., gal-PEG194-chol, gal-PEG1000-chol or gal-PEG2000-chol). Hela cells do not express the ASGP-R, therefore the galactose modified liposomes did not have the effect of increasing the inhibition of Hela cells.

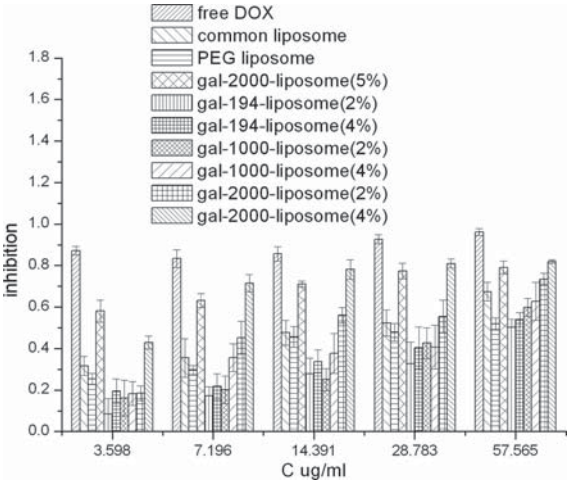

Figure 5. Cell proliferation inhibition of free DOX and DOX liposomes in HepG2 cells ($n = 3$).

3.5. Study on Frozen Section of Liver

Confocal microscopy was used to evaluate the hepatocyte uptake of free-DOX and DOX-liposomes *in vivo*. Figure 6 showed the images of liver frozen sections of KM mice harvested 1 h after intravenous injection of free-DOX and DOX-liposomes. The nuclei of the cells were stained with DAPI, the F-actin outlining the cell membrane was stained green with FITC Phalloidin, and the DOX was shown in red. Figure 6(A) showed the image of blank control. And the other images demonstrated that virtually all cell nuclei (as detected by DAPI staining) were also labeled by doxorubicin.

Examination of the images in Figure 6 revealed that virtually all the cell nuclei, irrespective of shape, which were stained by DAPI were also labeled by doxorubicin. Some labeled nuclei were large and round (presumed hepatocyte) and brightly stained, while other nuclei were oblong, oval (presumed non-parenchymal), or in some cases indented.[29, 30] Thus, the non-parenchymal cells and hepatocytes can be distinguished by their distinct morphologies as indicated by the arrow.

Distribution of relatively strong DOX fluorescence to nuclei could be observed in the hepatocytes treated with galactose-modified liposomes, indicating that the liposomes incorporated with the Gal-PEG2000-chol showed a remarkably specific targeting effect to the liver

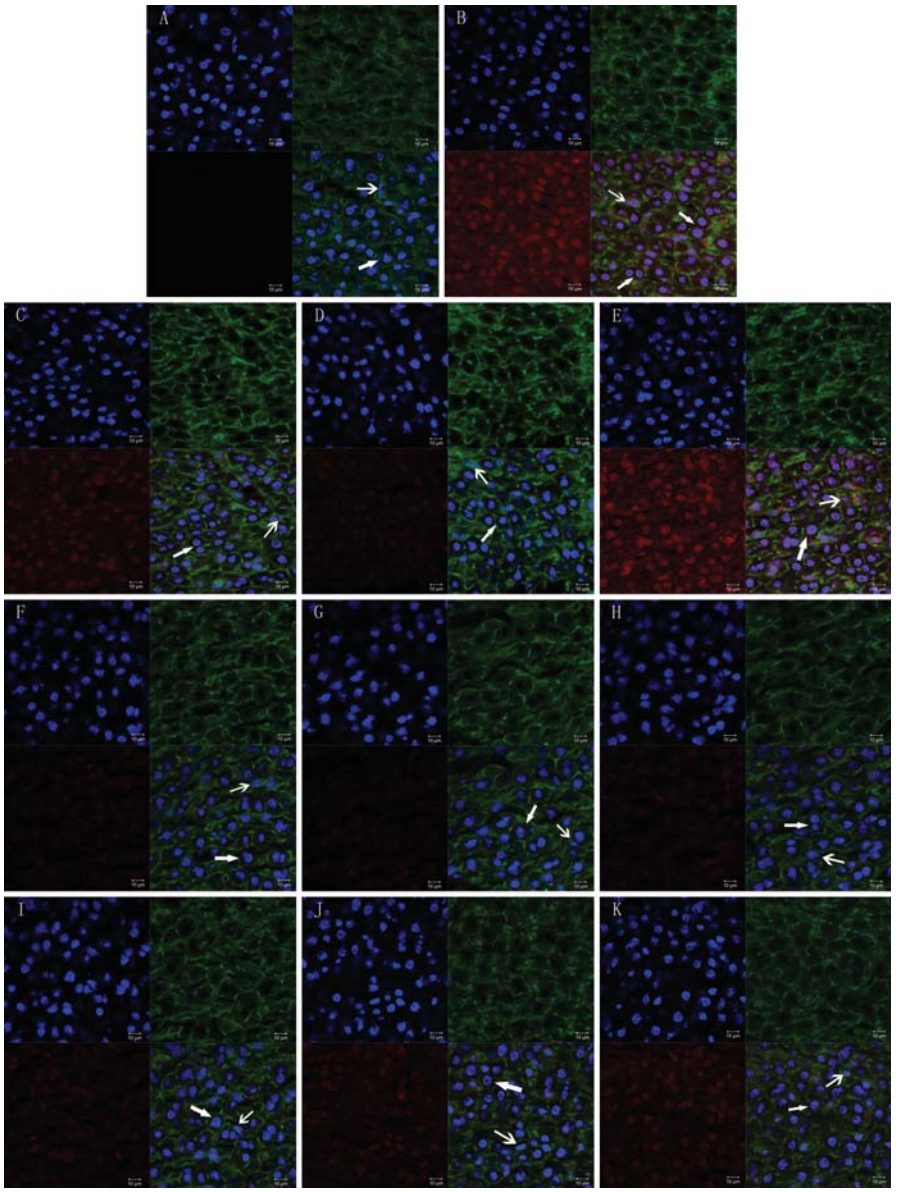

Figure 6. Confocal microscopy images of liver sections of free DOX and DOX liposomes, Blank (A), free DOX (B), common-liposomes (C), PEG-liposomes (D), Gal-2000-liposomes (5%) (E), Gal-194-liposomes (2%) (F), Gal-194-liposomes (4%) (G), Gal-1000-liposomes (2%) (H) , Gal-1000-liposomes (4%) (I), Gal-2000-liposomes (2%) (J), Gal-2000-liposomes (4%) (K). Nuclei were stained blue with DAPI, FITC was shown as green fluorescence, doxorubicin was shown with red fluorescence, and the merger image was on the bottom right.

tissue. The DOX fluorescence was observed in the following order of intensity: Gal-2000-liposomes (5%) > common-liposomes > PEG-liposomes. There was no significant difference in the detected fluorescent intensity between Gal-2000-liposomes (4%) (Fig. 6(K)) and Gal-2000-liposomes (5%) (Fig. 6(E)), but they were relatively stronger than that of Gal-2000-liposomes (2%). Weak fluorescence intensity was observed in the hepatocytes treated with PEG-liposomes (Fig. 6(D)), Gal-194-liposomes (2%) (Fig. 6(F)), Gal-194-liposomes (4%) (Fig. 6(G)), Gal-1000-liposomes (2%) (Fig. 6(H)) and Gal-1000-liposomes (4%) (Fig. 6(I)).

The kinetics of DOX entering the cell nuclei was also investigated by detecting the fluorescence at various time points after intravenous injection. Figure 7 presented the images showing doxorubicin labeling in sections of liver tissue from animals sacrificed at different time point following intravenous injection of Gal-2000-liposomes (5%) containing doxorubicin. Figure 7(A) demonstrated that after 10 min following intravenous injection, liver

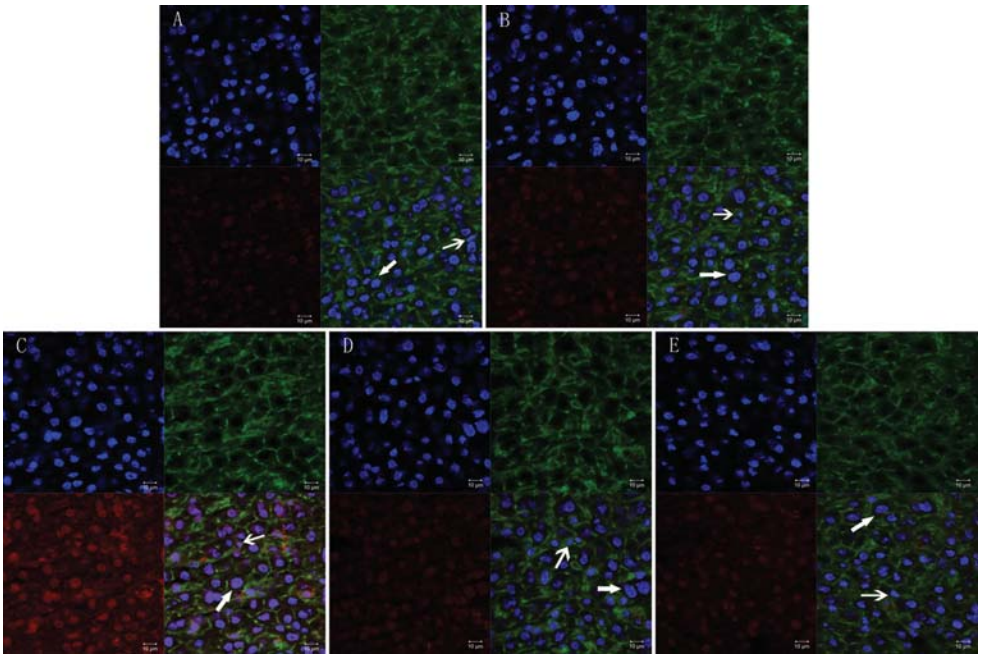

Figure 7. Confocal microscopy images of liver sections of Gal-2000-liposomes (5%), 10 min (A), 30 min (B), 1 h (C), 4 h (D), 6 h (E). Nuclei were stained blue with DAPI, FITC was shown as green fluorescence, doxorubicin was shown as red fluorescence, and the merger image is on the bottom right.

cell nuclei were already clearly labeled. As shown in Figure 7(C), cell nuclei were clearly and intensely labeled 1 h after administration. Intensity of doxorubicin labeling appeared to decline between 4 h (Fig. 7(D)) and 6 h (Fig. 7(E)).

Within the liver, histologic analysis indicated doxorubicin was present in the nuclei of all identifiable cell types (Figs. 6 and 7). The release of doxorubicin from the Gal-2000-liposomes (5%) was apparently quite rapid, as nuclei were labeled with doxorubicin fluorescence within 10 min after systemic administration (Fig. 7(A)).

The release of doxorubicin and its appearance within cell nuclei was remarkably rapid (within 10 min), and did not require an externally applied stimulus such as ultrasound. At present, the mechanism of this apparent triggered release is not known. One hypothesis, which remains to be tested, is that the interaction of the liposomes with the ASGP-R is sufficiently strong so that the liposomes bilayer is destabilized, resulting in release of entrapped contents.[30]

Doxorubicin proved to be an excellent compound for the demonstration of targeted liposomes content delivery. It could be conveniently loaded into liposomes at high concentration. The purpose of this investigation was to test whether content delivery of doxorubicin could be targeted to normal liver. The next step of this study will be to explore the targeted delivery characteristics of this formulation in liver tumor animal models.

4. CONCLUSION

In this study, three types of novel galactosylated cholesterol with a mono-galactoside moiety have been synthesized, including gal-PEG194-chol, gal-PEG1000-chol and gal-PEG2000-chol. The incorporation of gal-PEG2000-chol (4% and 5% mol/mol) into liposomes enhanced the liver target ability of liposomal DOX. Moreover, the results of intracellular uptake study, cell cytotoxicity assay *in vitro* and liver frozen sectioned study *in vivo* provided evidence that the recognition of galactosylated residues of gal-PEG2000-chol by the ASGP-R on the surface of parenchymal cells accounted for the liver accumulation of Gal-2000-liposomes. These results suggested that liposomes containing gal-PEG2000-chol could be a useful drug carrier system for hepatocyte selective targeting.

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