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Development of glycyrrhetinic acid-modified stealth cationic liposomes for gene delivery

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ABSTRACT

The glycyrrhetinic acid-modified stealth cationic liposomes (GA–PEG–CLs) loaded with pDNA (GA–PEG–CLPs) were developed and found to transfect human hepatocellular carcinoma cell line HepG2 with high efficiency. GA–PEG–CLs were comprised of DOTAP, cholesterol (Chol) and glycyrrhetinic acid–polyethyleneglycol–cholesterol conjugate (GA–PEG–Chol). Agarose gel electrophoresis revealed that 5% GA–PEG–CLs constituted by DOTAP/Chol/GA–PEG–Chol at molar ratio of 50:45:5 could completely entrap pDNA at a lower liposomes/pDNA weight ratios of 4:1 (N/P ratio: 1.14). Compared to ordinary cationic liposomes (CLs), steric cationic liposomes (PEG–CLs) and 1% GA–PEG–CLs made from DOTAP/Chol/MPEG2000-Chol/GA–PEG–Chol at molar ratio of 50:45:4:1, 5% GA–PEG–CLs were found to possess the highest transfection efficiency as gene vectors in serum-free or serum-containing medium in PKCα over-expressed HepG2 cells but no significance difference in human embryonic kidney cell line HEK 293. Additionally, 5% GA–PEG–CLs have the lowest cytotoxicity on human normal hepatocyte cell line L02. The competitive inhibition experiments mediated by GA were carried out in HepG2 cells, which demonstrated that GA–PEG–CLs could deliver selectively pDNA to hepatoma cells by the targeting moiety GA. In conclusion, GA–PEG–CLs containing 5% GA–PEG–Chol might be one of the most potential gene vectors as hepatoma targeting therapy.

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1. Introduction

Cationic liposomes, one of the promising and the most representative non-viral gene delivery vectors, have many advantages over viral ones such as less-immunogenicity, non-oncogenicity, large DNA capacity, non-restriction on the size of the DNA molecule, ability of evading random integration of vector DNA into host chromosome (avoiding any insertional mutagenesis) and large-scale production (Templeton, 2003; Lv et al., 2006; Karmali and Chaudhuri, 2007). Cationic liposomes could interact with negatively charged plasmids via electrostatic attractions at the physiological pH to form positively charged lipid–DNA complexes (lipoplexes) and get endocytosed by the negatively charged cell membrane (Karmali and Chaudhuri, 2007). Lipoplexes represent one kind of attractive alternative to viral vectors for cell transfection in vitro and in vivo but still suffer from relatively low efficiency (Duan et al., 2009) and certain toxicity. The non-specific electrostatic interactions between the lipoplexes and cells or tissues leaded to non-specific cell or tissue uptake, non-specific immune response (a toxic response), a shorter half-life in vivo (Lv et al., 2006; Karmali and Chaudhuri, 2007; Morille et al., 2008; Ditto et al., 2009). Therefore, many researchers focused on the development of novel, effective, long-circulating and targeted-delivery cationic liposomes.

According to literatures, cationic liposomes tagged with appropriate targeting ligands could function as an efficient targeted gene delivery system (Sudimack and Lee, 2000; Karmali and Chaudhuri, 2007; Morille et al., 2008; Yan and Qi, 2008; Pathak et al., 2009). The published ligands used to modify cationic liposomes included folate (Yan and Qi, 2008), galactosyl (Fumoto et al., 2003), integrins (Hood et al., 2002), sigma ligands (Mukherjee et al., 2005), estradiol (Reddy and Banerjee, 2005), transferrin (Penacho et al., 2008), TAT (MacKay et al., 2008), and so on. In this study, glycyrrhetinic acid (GA) was selected as the specific ligand of cationic liposomes. GA is one of the main compounds extracted from the root of *Glycyrrhiza glabra* L. (licorice) (Mao et al., 2007), which could inhibit liver carcinogenesis and cell proliferation of the human hepatocellular carcinoma (HCC) cell line HepG2 (Satomi et al., 2005). It has been proved that protein kinase C (PKC) α, the target binding sites of GA, expressed

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more highly in HCC cells than that in the adjacent non-tumor liver cells (O'Brian et al., 1990; Yang et al., 2003; Ying et al., 2008). Therefore, we hypothesized that GA-modified cationic liposomes could target selectively to HCC cells by the specific interaction of GA with PKCα over-expressed in HCC cells.

To increase the HCC cells-targeted efficiency in vivo probably by decreasing the clearance of reticuloendothelial system, poly(ethyleneglycol) (PEG) was selected as the linker between GA and cationic liposomes according to literatures (Green et al., 2007; Morille et al., 2009). PEG could dissimulate the positive charges by shielding cationic liposomes surface with hydrophilic chain, thereby weakening the toxic response caused by non-specific electrostatic interactions of lipoplexes with normal cells. Additionally, the introduction of PEG to targeted cationic liposomes would have many other advantages such as extending the half-life of lipoplexes by reducing opsonization and enhancing serum stabilization (Bombelli et al., 2007), avoiding the tendency of lipoplexes to form large aggregates (de Lima et al., 2001), improving their biocompatibility with biological fluids (Sudimack and Lee, 2000) and achieving the higher targeting efficiency (Green et al., 2007; Morille et al., 2009).

To our knowledge, no gene vectors modified with GA have been published so far. Thus, we aimed to develop GA-modified stealth cationic liposomes (GA–PEG–CLs) for gene delivery. In this paper, cholesterol (Chol) and PEG2000 were covalently linked to form PEG–Chol, and then GA was covalently conjugated to the distal end of PEG–Chol to produce GA–PEG–Chol. GA–PEG–CLs were made from GA–PEG–Chol, cholesterol and 1,2-dioleoyl-3-trimethylammonium-propane (chloride salt) (DOTAP). The toxicity of GA–PEG–CLs as gene vector was investigated. Additionally, in vitro cell transfection efficiency of gene loaded GA–PEG–CLs (GA–PEG–CLPs) was assessed on HCC HepG2 cell line and human embryonic kidney cell line HEK 293.

2. Materials and methods

2.1. Materials

1,2-Dioleoyl-3-trimethylammonium-propane (chloride salt) (DOTAP) was purchased from Avanti Polar Lipids Inc. (Alabaster, AL, USA). Cholesterol (Chol) was obtained from Shanghai Bio Life Science & Technology Co. Ltd. (Shanghai, China). 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDCI) and dimethylaminopyridine (DMAP) were purchased from Sigma. Green fluorescent protein plasmid DNA (pDNA) was kindly donated by Dr. Hong Xin Deng (Sichuan University). All reagents and solvents were of analytical grade and were used without further purification except chloroform.

2.2. Synthesis of GA–PEG–Chol conjugates

The synthesis process of GA–PEG–Chol conjugates was displayed in Scheme 1. Synthesis of 18β-glycyrrhetinic acid succinic anhydride ester (mGA-suc, compound 3) was carried out according to the literature (Tian et al., 2009). PEG–Chol (compound 6) was prepared as follows: Firstly, the mixture containing cholesterol, DMAP and succinic anhydride in DCM solution was stirred for 48 h, thereafter DMAP was removed by washing with 1 mol/l HCl solution and saturated $NaHCO_3$ solution. The combined organic layer was dehydrated with anhydrous Na_2SO_4 and then concentrated to yield cholesterol succinic anhydride ester (Chol-suc, compound 5). Secondly, Chol-suc, DMAP, EDCI and PEG2000 dissolved in chloroform were refluxed for 24 h. The mixture was concentrated under vacuum, and then re-dissolved with DCM. The DMAP and other by-products were removed as described in preparation process of Chol-suc. PEG–Chol was purified on a silica-gel column, eluting with a mixed solvent system of DCM and methanol. GA–PEG–Chol (compound 7) was prepared through the chemical reaction of mGA-suc and PEG–Chol. The purification process of GA–PEG–Chol was similar to that of PEG–Chol.

^{1}H NMR spectra of GA–PEG–Chol, mGA-suc and PEG–Chol were recorded on a JEOL JNM-a 400 instrument (Tokyo, Japan) at room temperature. All the samples were dissolved in $CDCl_3$.

2.3. Preparation of blank cationic liposomes

GA–PEG–CLs were prepared by a film dispersion method (Templeton et al., 1997). Briefly, DOTAP, Chol and GA–PEG–Chol at different molar ratios were dissolved in chloroform. The lipids solution was evaporated on a Büchi rotary evaporator to remove the solvent and then the formed thin film was further dried under high vacuum for 6 h. The dry lipid film was immediately hydrated in 5% (w/v) glucose solution. The suspension of lipids was sonicated by probe until a translucent lipid suspension was obtained. Thereafter, GA–PEG–CLs were passed through a 0.22 μm microporous membrane and stored at 4 °C until use.

The lipid compositions of ordinary cationic liposomes (CLs) and steric cationic liposomes (PEG–CLs) were DOTAP/Chol at molar ratio of 50:50 and DOTAP/Chol/MPEG2000-Chol at molar ratio of 50:45:5, respectively. CLs and PEG–CLs were prepared by the same method as GA–PEG–CLs. Experiments were performed in triplicate.

2.4. Preparation of lipoplexes

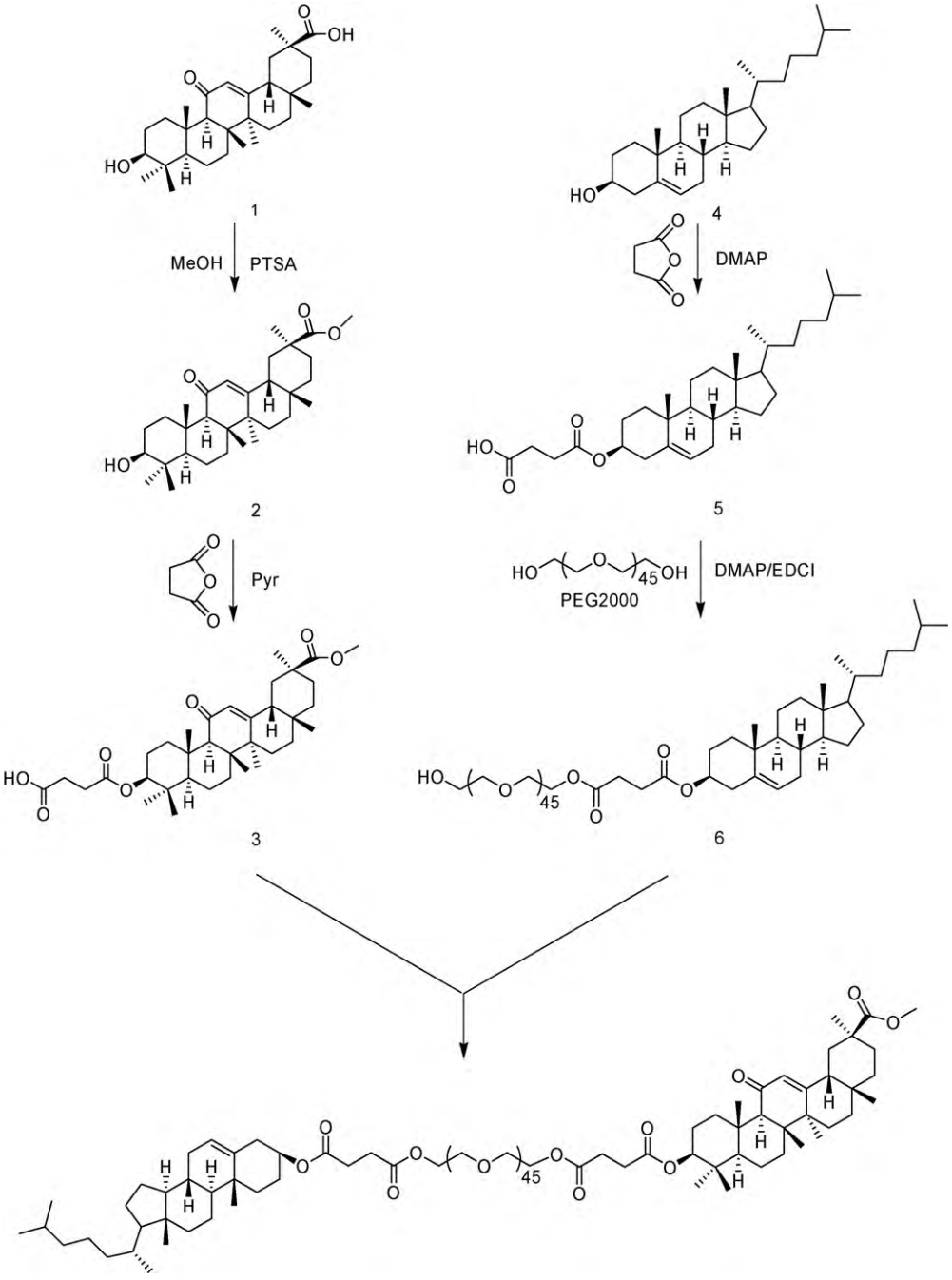
The 5% (w/v) glucose solution of pDNA was added into the stock suspensions of different blank cationic liposomes (CLs, PEG–CLs and GA–PEG–CLs) respectively, and then incubated at room temperature for 30 min to form different lipoplexes (CLPs, PEG–CLPs and GA–PEG–CLPs). The weight ratios of pDNA to blank cationic liposomes were optimized by electrophoresis experiments. Electrophoresis was conducted on 1% (w/v) agarose gel in TAE buffer (40 mM Tris/HCl, 1% acetic acid, 1 mM EDTA, pH 7.4) containing ethidium bromide as nucleic acid stain. 10 μL of lipoplexes mixed with 3 μl of loading buffer were applied to agarose at a constant voltage of 120 V for 20 min. The electrophoresis gel was visualized and digitally photographed by a gel documentation system (Gel Doc 1000, Bio-Rad Laboratories, Hercules, CA, USA).

2.5. Characterization of blank cationic liposomes and lipoplexes

The mean particle size and zeta potential were measured using a Zetasizer Nano ZS90 (Malvern Instruments, Ltd., Malvern, Worcestershire, UK). The samples were suitably diluted with 5% (w/v) isotonic glucose solution (pH 7). The mean particle size was measured by dynamic light scattering at a fixed angle of 90°. The zeta potential was automatically calculated from the electrophoretic mobility. Experiments were performed in triplicate.

2.6. Cell culture

Human hepatoma cell line HepG2, human normal hepatocyte cell line L02 and human embryonic kidney cell line HEK 293 were obtained from State Key Laboratory of Biotherapy, Sichuan University. The cells were cultured as a monolayer in Dulbecco's Modified Eagles's Medium (DMEM) supplemented with penicillin, streptomycin, and 10% fetal bovine serum and in a humidified atmosphere containing 5% CO_2 at 37 °C.



Scheme 1. Synthetic scheme for GA–PEG–Chol.

2.7. Cytotoxicity experiments

Cytotoxicity of various blank cationic liposomes was determined by MTT assay on L02 and HepG2 cell lines as described before (Song et al., 2009). Cells were seeded on 96-well plates (Corning Inc., Corning, NY, USA) at a density of 3×10^3 cells per well. Following attachment for 24 h, cells were treated by various blank cationic liposomes with different concentrations. These treatment agents were incubated with cells for an additional 48 h. Then 20 μl of MTT stock solution (5 mg/ml in saline) was added to each well and the cells were further incubated at 37 °C for 4 h. Finally, the culture medium was removed by aspiration and then 150 μl of dimethyl sulfoxide (DMSO) was added to each well to dissolve formazan crystals. The absorbance of each well was read at 490 nm on a Bio-Tek μQuant Universal Microplate Spectrophotometer (Bio-Tek Instruments Inc., Winooski, VT, USA). The relative cell viability compared to control was calculated as $(A_{sample}/A_{control}) \times 100\%$. All the experiments were repeated in triplicates.

2.8. Transfection experiments

HepG2 cells and HEK 293 cells were seeded on a Costar 6-well plate (Corning Incorporated, Corning, NY, USA) at 1.5×10^5 cells/well in 2 ml of culture medium. After 24 h of growth, the culture medium was replaced by 800 μl DMEM in each well. CLPs, PEG–CLPs and GA–PEG–CLPs in a final volume of 200 μl containing 1 μg pDNA were subsequently added to the wells. After 6 h incubation (in typical culture conditions), cell culture

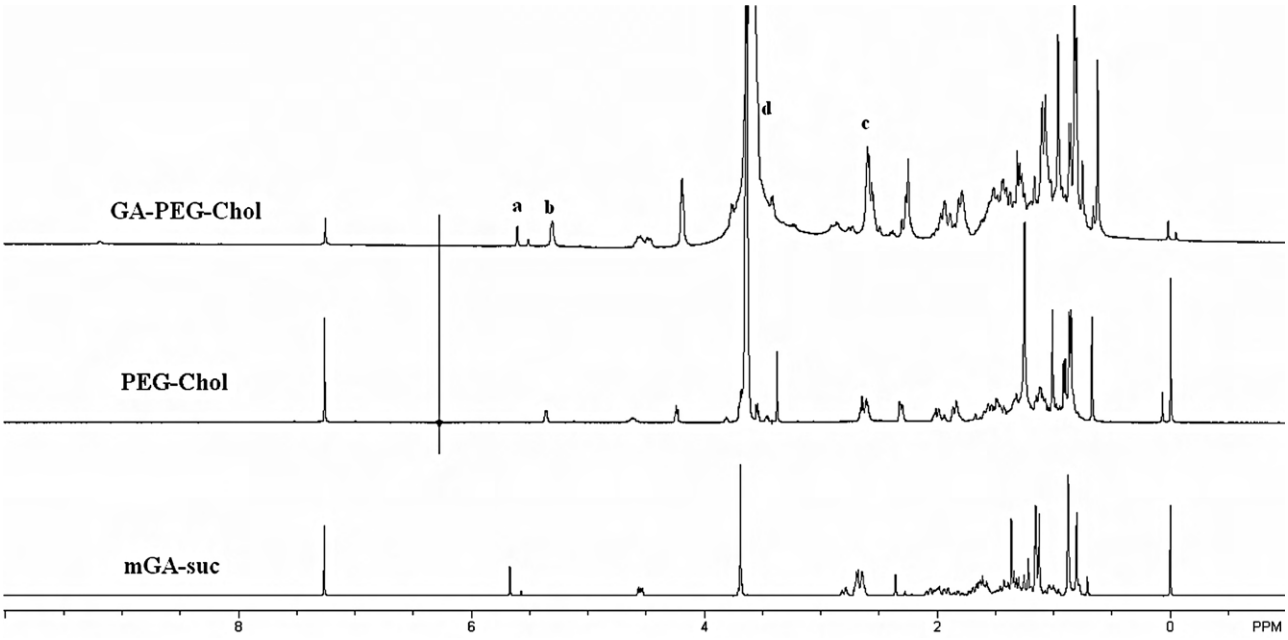


Fig. 1. Comparison of the ^{1}H NMR spectra of Chol-b-PEG, GA–PEG–Chol and mGA-suc in $CDCl_3$.

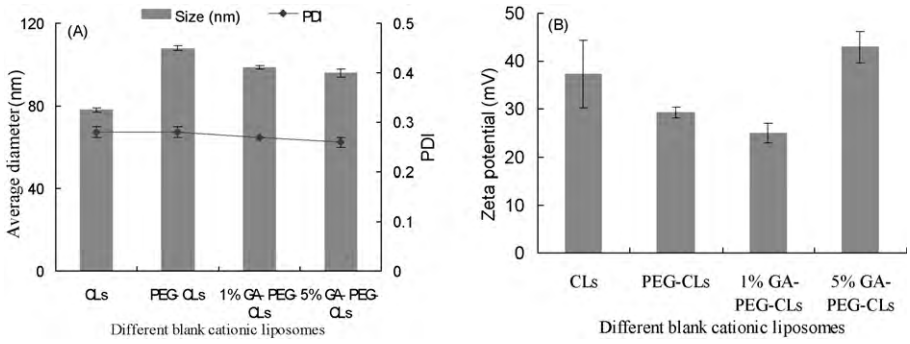


Fig. 2. The average diameters, PDI and zeta potentials of four kinds of blank cationic liposomes.

medium was changed to complete culture medium again and the cells were incubated for an additional 48 h. The expressed green fluorescent protein (GFP) was observed under an Olympus IX 70 inverted fluorescence microscope (Olympus Corp., Shinjuku, Tokyo, Japan). After that, the cell suspensions were collected after trypsinized with 0.25% trypsin-EDTA and centrifuged. And then the cell sediments were washed three times with PBS. The resuspended cell suspensions in PBS were analyzed by flow cytometry on a Coulter EPICS Elite ESP Flow Cytometer (Beckman coulter, Miami, FL, USA) to determine the transfection efficacy of the lipoplexes.

To assess the transfection ability of CLPs, PEG–CLPs and GA–PEG–CLPs in medium containing 10% serum, the transfection experiment of HepG2 cells was carried out as described above except that 800 μl DMEM was changed to 800 μl complete culture medium.

The competitive inhibition experiments mediated by GA were carried out in HepG2 cells. HepG2 cells were incubated with various

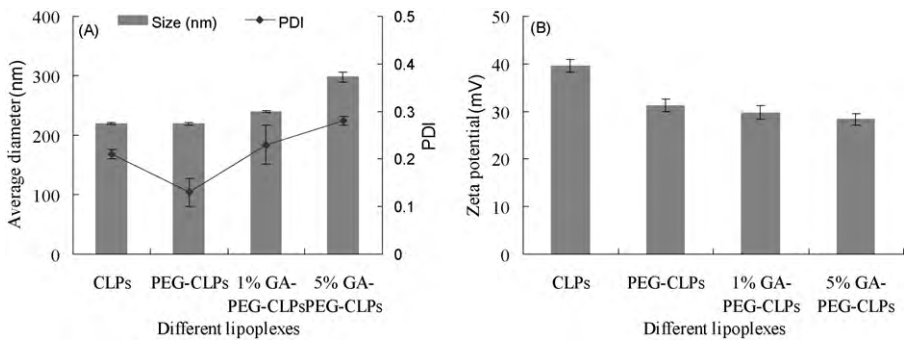


Fig. 3. The average diameters, PDI and zeta potentials of four kinds of lipoplexes.

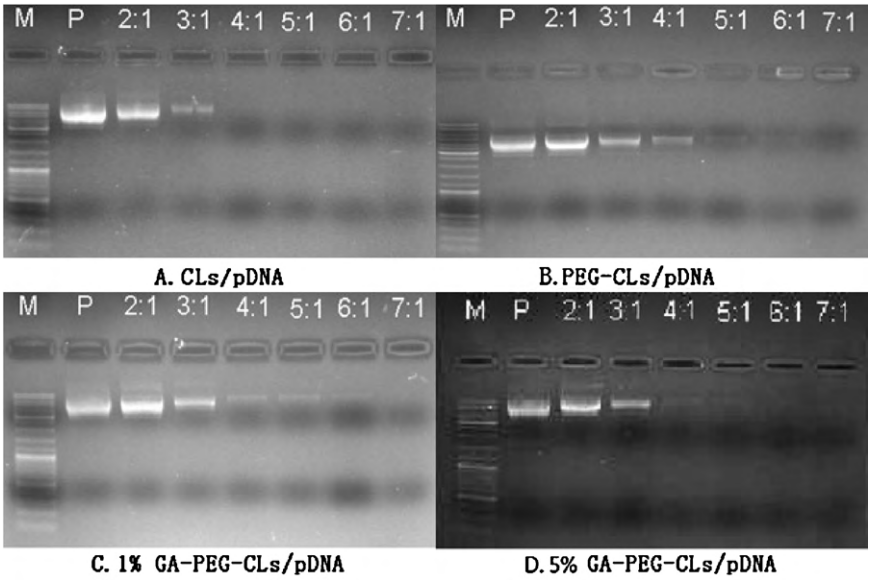


Fig. 4. Agarose gel electrophoresis of four different lipoplexes (CLs/pDNA, PEG–CLs/pDNA, 1% GA–PEG–CLs/pDNA and 5% GA–PEG–CLs/pDNA) at weight ratios ranging from 2:1 to 7:1.

amounts of GA (2.58 μM to 10.32 μM) for 30 min in serum-free medium, and then 5% GA–PEG–CLPs were added. The other process was accordant with the above-mentioned method.

2.9. Data analysis

Data analysis was performed using Student's *t*-test on Statistical Product and Service Solutions (SPSS V13.0, SPSS Inc., Chicago, USA). Differences were considered significant at $p < 0.05$.

3. Results and discussion

3.1. Synthesis of GA–PEG–Chol conjugates

Fig. 1 shows the ^{1}H NMR spectra of GA–PEG–Chol, mGA-suc and PEG–Chol in $CDCl_3$. The single peaks at δ 5.69 and 5.60 (a) were attributed to the protons of olefinic bond (–(C=O)–CH=C–) in GA. The single peak at δ 5.30 (b) was attributed to the protons of olefinic bond (–CH_2–CH=C–) in cholesterol. The peaks at δ 2.58–2.66 (c) came from the protons of succinate linkage (–(C=O)–CH_2–CH_2–(C=O)–). The peaks at δ 3.52–3.76 (d) were attributed to the protons from the glycol unit (–O-CH_2–CH_2–O–) in PEG chain. Thus GA–PEG–Chol conjugates have been successfully synthesized.

3.2. Preparation and characterization of blank cationic liposomes

Two kinds of GA–PEG–CLs containing different ligand density were prepared. One was 1% GA–PEG–CLs which were made from DOTAP/Chol/MPEG2000-Chol/GA–PEG–Chol at molar ratio of 50:45:4:1, the other was 5% GA–PEG–CLs constituted by DOTAP/Chol/GA–PEG–Chol at molar ratio of 50:45:5. As seen in Fig. 2, the size of blank cationic liposomes slightly increased with introduction of PEG, while the zeta potential decreased in contrast. This phenomenon, accordant with that reported in the literature (Morille et al., 2009), might result from the PEG steric shield around the surface of the cationic liposomes which could increase the diameter of liposomes and partly shield the positive charge.

3.3. Preparation and characterization of lipoplexes

Agarose gel electrophoresis was used to study and compare abilities of the four different cationic liposomes (CLs, PEG–CLs, 1% GA–PEG–CLs and 5% GA–PEG–CLs) to condense pDNA (Fig. 4). It turned out that CLs, PEG–CLs, 1% GA–PEG–CLs and 5% GA–PEG–CLs completely retarded DNA migration at liposomes/pDNA weight ratios of 4:1 (N/P ratio which is the ratio between the nitrogen in the cationic lipid and the phosphate in pDNA: 1.14), 7:1 (N/P ratio: 1.99), 7:1 (N/P ratio: 1.99) and 4:1 (N/P ratio: 1.14) respectively. This result might be due to the different surface potential of the four kinds of cationic liposomes. To elucidate the above phenomenon, the four kinds of lipoplexes were further characterized by average diameter, PDI and zeta potential. The dispersion medium of four cationic liposomes was 5% (w/v) isotonic glucose solution (pH 7), which simulated the physiological condition. The results were displayed in Fig. 3. The average diameter of all the four lipoplexes increased, while PDI had no significant change. Compared to corresponding blank cationic liposomes, the zeta potentials of CLPs and PEG–CLPs did not change dramatically, while that of 1% GA–PEG–CLPs increased significantly but that of 5% GA–PEG–CLPs decreased significantly. According to literatures, the interaction of cationic liposomes with pDNA was dominated by the electrostatic attachment of the negatively charged DNA and positively charged liposomes (Zuidam and Barenholz, 1997) and hydrophobic interaction. The targeting moiety GA was hydrophobic. 1% GA–PEG–CLPs and 5% GA–PEG–CLPs contained different amounts of GA, therefore the hydrophobic interaction of the two liposomes with pDNA might be different, which resulted in different zeta potential change.

3.4. In vitro cell transfection efficiency

To assess the gene transfection efficiency of GA–PEG–CLPs constructed above and optimize better density of GA, the in vitro cell transfection experiment was carried out using HCC HepG2 cell line as model. The samples investigated in the experiment were CLPs, PEG–CLPs, 1% GA–PEG–CLPs and 5% GA–PEG–CLPs at the optimal liposomes/pDNA weight ratios in which pDNA was completely entrapped. After treatment with four kinds of lipoplexes, the fluorescent photos were presented in Fig. 5 and the transfection

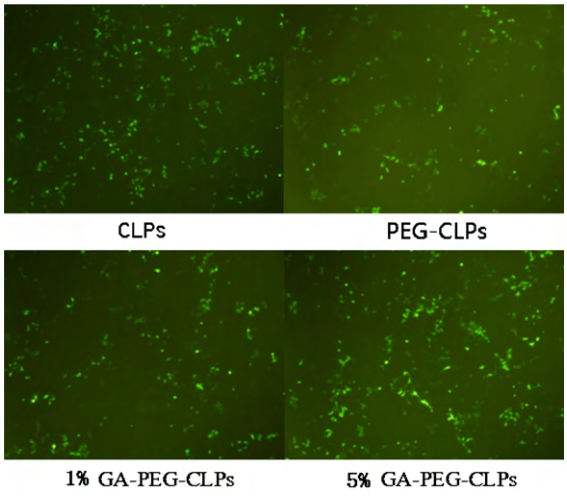


Fig. 5. The GFP expression fluorescent photos of HepG2 after treatment with four kinds of lipoplexes.

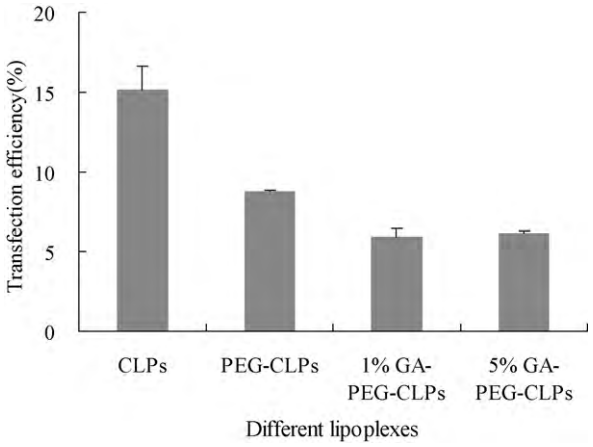


Fig. 7. GFP gene expression efficiency of HEK293 after treatment with four kinds of lipoplexes.

efficiency was displayed in Fig. 6. It was easy to see that the transfection efficiency increased when GA ligand density increased from 0% to 5%. This phenomenon indicated that GA could improve the gene transfection efficiency on HepG2 cell line with a density-dependent feature. The zeta potentials of PEG–CLPs, 1% GA–PEG–CLPs and 5% GA–PEG–CLPs had no significant difference ($p > 0.05$) (Fig. 4), while the average diameters of the above three lipoplexes increased with increase in GA–PEG–Chol content (Fig. 3). Theoretically, the endocytosis of 5% GA–PEG–CLPs might decrease due to the biggest size. However, 5% GA–PEG–CLPs achieved the highest transfection efficiency. It might be deduced that the uptake of 5% GA–PEG–CLPs was enhanced by the over-expressing GA receptors PKCα on the membrane of hepatocellular carcinoma cells.

The transfection efficiency of PEG–CLPs was one time lower than CLPs, probably resulting from the steric shielding of the vector's cationic charge from the highly anionic cell surface proteoglycans (as seen in Fig. 3, the zeta potential of CLPs was higher than that of PEG–CLPs $p < 0.05$) (Mounkes et al., 1998). After introduction of GA–PEG–Chol, the transfection efficiency increased. When the content of GA–PEG–Chol was up to 5%, the transfection efficiency of stealth lipoplexes was not dramatically different from that of CLPs. This suggested that the PEG-dependent shielding of charge interactions may have been compensated by the high affinity binding of GA to PKCα on HepG2 cells (Reddy et al., 2002).

In order to demonstrate the mechanism of GA–PEG–CLPs HCC targeting, the transfection efficiency in HEK 293 cell line and the competitive inhibition experiments mediated by GA in HepG2 cell line were performed. HEK 293 cell line does not express the GA receptor according to the HEK 293 cell database record. The GFP gene expression efficiency of HEK293 after treatment with four kinds of lipoplexes (Fig. 7) showed that two GA–PEG–CLPs had lower transfection efficiency than CLPs and PEG–CLPs. GA–PEG–CLPs could achieve higher gene transfection efficiency in HCC other than normal tissues, which indicated that GA–PEG–CLPs might be lower toxic in normal tissues than CLPs and PEG–CLPs. The competitive inhibition experimental result mediated by GA in HepG2 cell line was presented in Fig. 8. Before transfected by 5% GA–PEG–CLPs, HepG2 cells was firstly treated with four different concentrations of GA (2.58 μM, 5.16 μM, 7.74 μM and 10.32 μM) for 30 min in order to saturate the GA binding site of PKCα. The GFP gene expression efficiency in HepG2 cells pretreated by GA dramatically decreased ($p < 0.05$). As seen in Fig. 8, 2.58 μM GA which was two times GA that 5% GA–PEG–CLPs contained, was enough to inhibit the PKCα activity. Further increase in GA concentration, no further decrease in GFP gene expression efficiency was observed. Thus, it might be concluded that HepG2 cells over-expressed PKCα and GA–PEG–CLPs HCC targeting was mediated by the specific interaction of PKCα with GA targeting moiety.

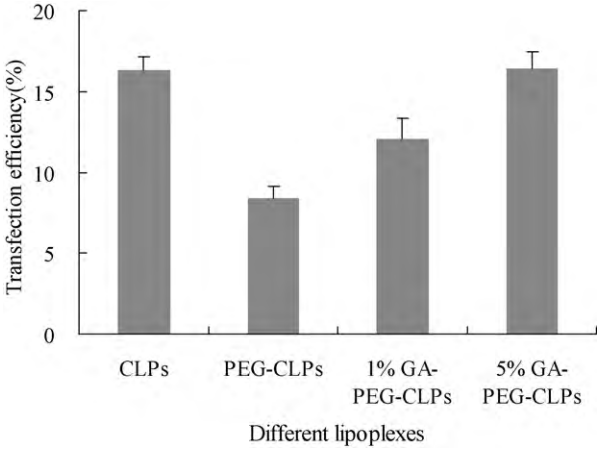


Fig. 6. GFP gene expression efficiency of HepG2 after treatment with four kinds of lipoplexes.

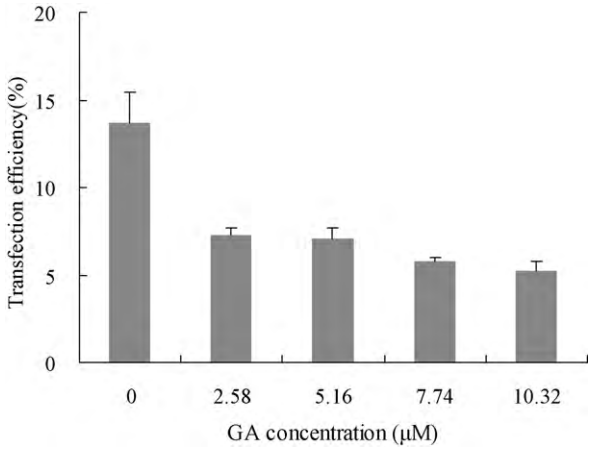


Fig. 8. GFP gene expression efficiency of HepG2 after treatment with different concentrations of GA and 5% GA–PEG–CLPs.

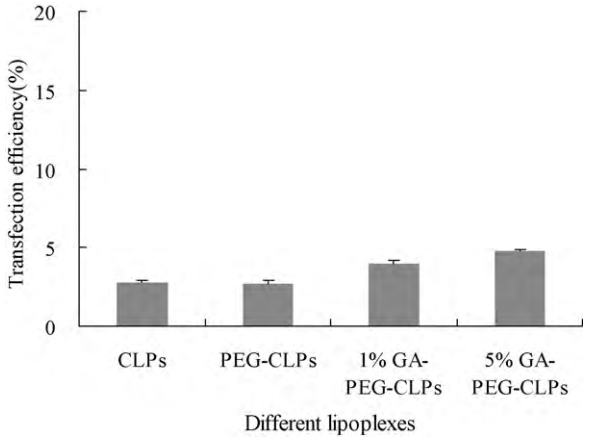


Fig. 9. GFP gene expression efficiency of HepG2 after treatment with four kinds of lipoplexes in the medium containing 10% serum.

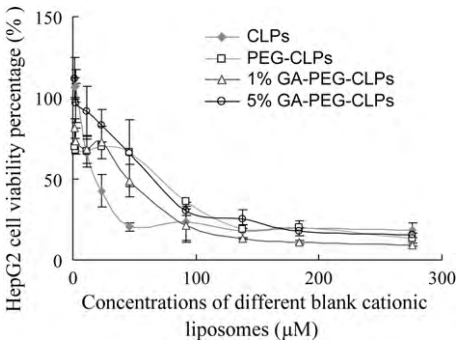


Fig. 10. The cytotoxicity of four kinds of blank cationic liposomes on HepG2.

The transfection ability of CLPs, PEG–CLPs and GA–PEG–CLPs in medium containing 10% serum was also investigated in HepG2 cells. The data in Fig. 9 displayed that all the transfection of four lipoplexes was remarkably inhibited by serum. The GFP gene expression efficiency of 5% GA–PEG–CLPs was the highest than the other three lipoplexes, which implied that the better density of GA ligand in the constructed GA–PEG–CLPs might be 5% or higher (we are currently investigating higher GA density from 5% to 20%).

3.5. Cytotoxicity assessment

5% GA–PEG–CLPs could achieve higher gene transfection efficiency, therefore, the cytotoxicity of 5% GA–PEG–CLs was further investigated on hepatoma cell line HepG2 and normal hepatocyte cell line L02 by comparing with CLs, PEG–CLs and 1% GA–PEG–CLs. The results were shown in Figs. 10 and 11. On both cell lines, the cytotoxicity of four blank cationic liposomes were dependent on the concentration. The cytotoxicity of 5% GA–PEG–CLs was similar as PEG–CLs and 1% GA–PEG–CLs on HepG2 cell line, but lower than CLs, PEG–CLs and 1% GA–PEG–CLs on L02 cell line. This indicated that 5% GA–PEG–CLs was a low-toxicity gene carrier suitable for hepatoma targeting therapy.

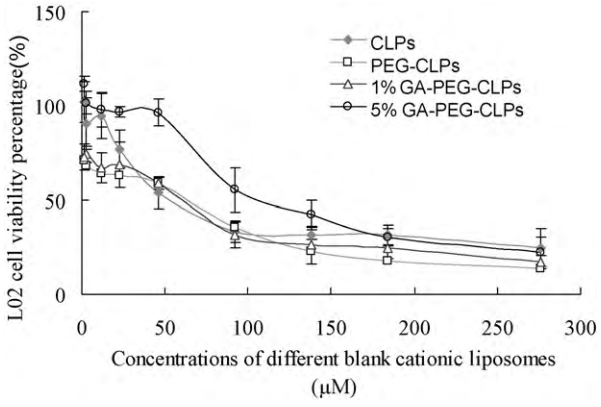


Fig. 11. The cytotoxicity of four kinds of blank cationic liposomes on L02.

4. Conclusion

As a novel hepatoma targeting gene vector, GA–PEG–CLs could entrap pDNA with high efficiency and enhance the uptake of pDNA in hepatocellular carcinoma cells and then achieve high transfection efficiency. The gene entrapment and transfection efficiencies of GA–PEG–CLs were dependent on the amount of GA ligand and had a positive relationship. 5% GA–PEG–CLs, comprising DOTAP/Chol/GA–PEG–Chol at molar ratio of 50:45:5, had higher gene transfection efficiency and lower cytotoxicity of normal hepatocyte cells, which might be one of the most potential gene vectors as hepatoma targeting therapy.

Acknowledgements

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