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Hepatocyte-targeted gene transfection of galactosylated chitosan-graft low molecular polyethyleneimine/DNA complexes

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Abstract: Objective To investigate the hepatocyte targeted specific property of galactosylated chitosan-graft-polyethyleneimine (GC-PEI)/DNA complexes in vitro and in vivo. **Methods** With the plasmid expressing enhanced green fluorescent protein (pEGFP-C1) as the reporter gene, the formation of GC-PEI/DNA complexes was induced to self-assemble in 0.01 mol/L phosphate buffered saline(PBS), 150 mmol/L NaCl, or 5% glucose solution (GS). The complexes were characterized by the particle size, Zeta potential, DNA binding and protection capacity, and further tested for cytotoxicity and hepatocyte targeted transfection activity. **Results** With the GC-PEI/DNA mass ratio from 1∶1 to 2.5∶1, the GC-PEI/DNA complexes effectively bound and protected the DNA from degradation of DNase Ⅰ and the serum, which presented as a well-formed sphere or compacted nucleocapsid structure at a diameter of 50 – 200 nm. The GC-PEI copolymer showed no obvious toxicity in the tested cell lines. Acute toxicity assay revealed that the mice grew well in 2 weeks with GC-PEI dosage from 50 to 300 μg. The assay by flow cytometry and fluorescent microscope showed that the transfection efficiency in hepatocyte lines (L02, QSG7701/core) was higher than that in non-hepatocyte lines (SGC7901, HBE) in vitro. In vivo, the GFP was obviously expressed in the liver tissue and not expressed in other organs 48 h after the transfection. **Conclusion** GC-PEI copolymer may carry the exogenous gene specifically to hepatocytes in vitro and in vivo, which has very good liver targeted specific property.

Key words: galactosylated chitosan-graft-polyethyleneimine; liver-targeting; receptor-mediated gene transfer

半乳糖化壳聚糖-低分子聚乙烯亚胺/DNA 复合物的肝靶向性研究

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[摘要] **目的:**研究半乳糖化壳聚糖-低分子聚乙烯亚胺(galactosylated chitosan-graft-polyethyleneimine, GC-PEI)/DNA 复合物在体内外的肝靶向性。**方法:**GC-PEI 与增强型绿色荧光蛋白(enhanced green fluorescent protein, EGFP) 质粒(pEGFP-Cl)在 0.01 mol/L PBS, 150 mmol/L NaCl, 5% 葡萄糖溶液中自组装成 3 种不同

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Biography TAN Pingping, master, mainly engaged in the research of liver pathology. LI Man is an equal contributor to this work.

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溶媒介导的 GC-PEI/ DNA 复合物,检测复合物粒径大小与形态,Zeta 电位以及结合和保护 DNA 的能力;并进一步测定 GC-PEI 聚合物的毒性,研究复合物的肝靶向转染效率。**结果**:在 GC-PEI 与 DNA 质量比为 1∶1 ~ 2.5∶1时,GC-PEI 聚合物能有效地结合和保护所携带的 DNA 免受核酸酶和血清的降解。复合物粒子呈规则的球形,有明显的核壳结构。GC-PEI 聚合物在检测细胞中未显示出明显毒性;动物体内急性毒性实验显示:通过尾静脉注射 50 ~300 μg 的 GC-PEI 聚合物入小鼠后,实验小鼠 2 周内无急性毒性反应和死亡发生。荧光显微镜和流式细胞仪检测证实 GC-PEI/DNA 复合物在肝细胞系(QSG7701/core,L02)中的绿色荧光蛋白(green fluorescent protein, GFP)表达明显高于非肝细胞系(SGC-7901,HBE)细胞。体内实验表明转染 48 h 后,小鼠肝组织在荧光显微镜下可以检测到明显的绿色荧光,而其他主要脏器未见明显荧光。**结论**:GC-PEI 聚合物能够在体内外特异性将外源基因或 DNA 导入肝细胞,具有良好的肝靶向性。

[**关键词**] 半乳糖化壳聚糖-低分子聚乙烯亚胺; 肝靶向性; 受体介导的基因转移
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With the development of molecular biological techniques and the continuous understanding of the pathogenesis of Hepatitis C, gene therapy was introduced in the treatment of Hepatitis C. To choose a highly-effective, specific, and safe hepatic-targeting gene vector is the key to the success of genetic treatment of hepatic disease. Gene vector is divided into viral vectors and non-viral vectors. Viral vectors include retrovirus, adenovirus, herpesvirus, etc. Their application was limited because of low security and other flaws[1-2]. Non-viral vectors include cationic polymers, cationic peptides, cationic liposomes, etc, and get more and more important in recent years[3-5]. Recently, cationic polymer is extensively applied for its stability, being easy to modify, and low immunogenicity, especially the polyvalent cationic polymer polyethyleneimine (PEI) with the characteristic of intracellular degradation in physiological environment[6-8]. Chitosan (CS), a natural polycation gene vector, which has a low toxicity, good biocompatibility, high transfection efficiency and other characteristics[9], has attracted extensive attention[2-12]. In PEI/CS mediated hepatic-targeting gene introduction, actively recognizing and endocytosing the transfection compound containing its specific ligand through asialoglycoprotein receptor (ASGP-R) is the primary mechanism of improving the efficiency of the gene transfer. Also it is a receptor-mediated gene transfer system which is widely studied at present.

In this research, we grafted the low-molecular-weight PEI2K to galactosylated CS following the document[12], synthesized galactosylated CS-PEI2K (GC-PEI), and studied the targeting of GC-PEI/DNA complex in hepatic cell lines (L02, QSG7701, and QSG7701/core) and hepatic cells in mouse. To optimize the transfection efficiency of gene vectors, we prepared GC-PEI/DNA complexes in 3 different solvents, and selected appropriate gene vector for experiment in vitro and in vivo.

1 MATERIALS AND METHODS

1.1 Main reagents and equipments

Branched PEI (relative molecular weight 25 kD, no water, PEI25K), branched PEI (relative molecular weight 2 000 D, 50% water, PEI2K), N-hydroxysuccinimide (NHS), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), lactobionic acid (LA), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and potassium periodate were purchased from Sigma-Aldrich Corporation (USA); CS (deacetylation degree 93%) was from Shanghai Bio Science and Technology Co., Ltd. (China); DNase Ⅰ was from Roche Corporation in Switzerland; DMEM medium was from Gibco Corporation (USA); and plasmid of enhanced green fluorescent protein (pEGFP-C1) was from Clontech Corporation (USA); all other reagents were of analytical pure.

Dialysis bags of 12 000-14 000 D and 3 500 D were purchased from Pierce Corporation (USA); transmission electron microscope was from Simens (Germany); freeze drier was from Thermo Corporation (Germany), and laser scattering particle and potential analyzer was from Malvern Corporation (England).

1.2 Methods

1.2.1 Preparation of GC-PEI2K[12]

Chitosan (0.8 g), EDC (1.456 g), and NHS (0.8 g) were dissolved in 24 mL tetramethyl ethylene diamine/hydrochloric acid buffer (10 mmol/L, pH 4.7) and mixed with LA. The mixture was fully magnetic-stirred for 72 h and vacuum freeze-dried. Freeze-dried GC (0.20 g) and potassium periodate (0.287 g) was separately dissolved in 12 mL sodium

acetate buffer (pH 4.5). The 2 were mixed in nitrogen, reacted in 4℃, dark sealed condition for 48 h before terminated by adding ethylene glycol, and then put into dialysis bag (3.5 kD) to be dialyzed in 0.2 mol/L NaCl solution (pH 4.5) and double distilled water1 (pH 4.5). Then 1.8 g PEI2K was added and the solution was magnetic-stirred at 4℃ for 48 h. Following that, 0.4 g borohydride sodium was added. The solution was put into dialysis bag (12 – 14 kD) to be dialyzed in 0.2 mol/L NaCl solution (pH 4.5) and double distilled water (pH 4.5), and then vacuum freeze-dried. The dried polymer was accurately measured and dissolved in 5% glucose solution (GS), 150 mmol/L NaCl solution, and 0.01 mol/L PBS separately to be made into 200 μg/mL solvents (pH was adjusted to 7.2), which were named GC-PEI/5% GS, GC-PEI/NaCl, and GC-PEI/PBS respectively.

1.2.2 GC-PEI2K/DNA binding test

Electrophoresis mobility shift assay (EMSA) and DNA binding efficiency test were performed to test the DNA-binding ability of GC-PEI polymer. The GC-PEI polymers and pEGFP-C1 were mixed in a vortex mixer in 3 solvents separately. The mass ratios of GC-PEI/DNA were 0.25∶1, 0.75∶1, 1∶1, 1.5∶1, and 2.5∶1. After reacted for 30 min at room temperature, the products were underwent electrophoresis in 1.0% agarose gel at 80 V for 50 min and the results were observed and photographed them with gel imaging system. The GC-PEI/DNA complexes of above ratios were stilled at room temperature for 60 min, and centrifuged at 12 000 r/min for 15 min. The supernatant was collected to measure its absorbance at the reference wavelength of 260 nm with an ultraviolet spectrophotometer. The volume of the supernatant and the content of the uncombined DNA were measured. And the ratio of uncombined DNA was calculated using the formula: uncombined ratio = uncombined DNA / total DNA × 100% (total DNA refers to pEGFP-C1 DNA added; uncombined DNA refers to DNA in the supernatant).

1.2.3 Test of GC-PEI/DNA complexes resisting DNase I digestion

GC-PEI/5% GS, GC-PEI/NaCl, and GC-PEI/PBS were mixed with pEGFP-C1 at the mass ratio of 1.5∶1, respectively. And the GC-PEI/DNA complexes were allowed to react at room temperature for 1 h. DNase I was dissolved in DNase/Mg^{2+} reaction buffer (50 mmol Tris-Cl, 10 mmol $MgCl_2$, pH 7.6) and was made into 0.25 μg/μL DNase I solution. Three microlitre of the solution was taken and mixed with naked DNA or GC-PEI/DNA complexes (the mass ratio 1.5∶1, containing 0.4 μg DNA). The mixture reacted at room temperature for 1 h, and then incubated at 37 ℃ for 30 min. Four microlitre of 25 mmol/L EDTA was added to inactivate the DNase I for 10 min. NaOH solution (0.2 mol/L, pH 7.4) containing 1% SDS was added to elute plasmid DNA from the GC-PEI/DNA complexes. The eluted DNA was extracted with isoamylol and chloroform, deposited with absolute ethyl alcohol, washed with 70% ethyl alcohol and dried, dissolved in 5 μL double distilled water, and then analyzed with 1.0% agarose gel electrophoresis. Naked DNA was served as the control.

1.2.4 Serum protection test of GC-PEI/DNA complexes

GC-PEI/5% GS, GC-PEI/NaCl, and GC-PEI/PBS were mixed with pEGFP-C1 at the mass ratio of 1.5∶1, respectively. And the GC-PEI/DNA complexes were stilled at temperature for 1 h, and then added into DMEN medium containing 10% serum, and incubated at 37 ℃ for 8 h. The plasmid DNA was eluted from the GC-PEI/DNA complexes with 0.2 mol/L NaOH solution containing 1% SDS, extracted with isoamylol and chloroform, deposited with absolute ethyl alcohol, washed with 70% ethyl alcohol and dried, dissolved in 5 μL double distilled water, and then analyzed with agarose gel electrophoresis. Naked DNA was served as the control.

1.2.5 Particle size, size distribution and Zeta potential detection

The GC-PEI polymers were mixed with pEGFP-C1 in the 3 solvents at the mass ratio of 1.5∶1, 0.75∶1, 1.5∶1, and 2.5∶1 and made into solutions of 1 mL with DNA content of 20 μg/mL. They were mixed by vortex for 15 s, stilled for 30 min at room temperature, and loaded to the Particle Size Analyzer to detect the particle size, size distribution and Zeta potential.

1.2.6 Transmission electron microscope detection

The GC-PEI polymers were mixed with pEGFP-C1 in the 3 solvents at the mass ratio of 0.25∶1 and 1.5∶1 in vortex mixer for 15 min, and stilled at room temperature for 30 min. A certain amount of the mixture was dripped to on a copper net covered with carbon membrane, and air-dried at room temperature for 10 min. Then a transmission electron microscope was used to observe the shape and size of the complexes and take photographs.

1.2.7 Cytotoxicity test of GC-PEI complexes in vitro

Human embryonic hepatic cell line L02, hepatic cell line QSG7701/core which stably expresses HCV core protein, human bronchial epithelial (HBE) cell line, and human gastric cancer cell line SGC7901 preserved in our laboratory were incubated in DMEM medium containing 10% fetal calf serum (FCS) at 37℃, 5 % CO_2, and saturated humidity.

QSG7701/core and HBE cells were made into single cell suspension with DMEM medium containing 10% FCS, and transferred to a 96-well culture plate (200 μL suspension with 5×10^3 cells in each well). Then the cells were incubated at 37℃, 5 % CO_2, and saturated humidity for 24 h. After the incubation, the medium was discarded, and 200 μL PEI2K and GC-PEI2K-DMEM medium (5, 10, 15, 20, 30, 50 μg/mL) were added separately. Wells added with DMEM medium of the same volume was served as the control, and 3 parallel samples were set up in each group. After 24 h incubation, the medium was changed into fresh medium containing 10% FCS, and the incubation continued for 48 h. Then the medium was discarded and 180 μL DMEM medium containing 7% FCS and 20 μL MTT solution was added into each well, and the incubation continued for 4 h. After that, the incubation was terminated, the medium was cleared away, and 150 μL DMSO was added into each well. The culture plate was shaken for 10 min to allow the crystal fully dissolved. The absorbance of each well was measured with ELISA at reference wavelength of 490 nm, and the relative cell viability (RCV) was calculated following the formula RCV(%) = OD of the experimental group/OD of the control group ×100% to assay the cytotoxicity of the polymers.

1.2.8 Transfection in vitro of GC-PEI/DNA complexes

The L02, QSG7701, QSG7701/core, HBE and SGC7901 cells were transferred into 24-well culture plate, 1×10^5 cells per well. The cells reached 65%-70% confluence was used for the transfection. GC-PEI/5% GS, GC-PEI/PBS, and PEI2K were mixed with DNA separately (mass ratio 2∶1) in vortex mixer for 15 s, and then stilled at room temperature for 30 min. The cells were incubated in 0.5 mL serum-free DMEM medium after being rinsed with serum-free DMEM medium twice. One microgram of the polymer/DNA complexes were added into each well. After 4 – 5 h, the medium was discarded, 0.5 mL DMEM medium containing 10% FCS was added, and the cells were incubated for 48 h. Finally, the cells were observed with fluorescence microscope, and the transfection efficiency was analyzed by flow cytometry. Lipofectamine™2000 positive control group, naked plasmid DNA negative control group and 3 parallel samples in each group were set up.

1.2.9 Galactose competitive inhibition test

QSG7701 /core cells were transferred to a 24-well culture plate. GC-PEI/PBS and DNA were mixed (mass ratio 2∶1) in a vortex mixer for 15 min, and then stilled for 30 min. The medium was changed into serum-free DMEM medium (0.5 mL per well) and GC-PEI/DNA complexes was added (1 μg plasmid per well) as well as 30 mmol/L *D*-galactose or *D*-mannose. The blank control was just added GC-PEI/DNA complexes. The mixture was fully mixed by shaking. After incubated for 4 – 5 h, the transfection medium was discarded. DMEM medium (0.5 mL) containing 10% FCS was added into each well, and the incubation was continued for 48 h. Finally, the cells were observed with fluorescence microscope, and the transfection efficiency was analyzed by flow cytometry.

1.2.10 In vivo acute toxicity test of GC-PEI polymer

Twenty-four Kunming mice (half male and half female) were divided into 6 groups, 4 in each. The first group was the negative control group, the mice were injected with sterilized PBS buffer; the rest groups were the experimental groups, and the mice were injected with GC-PEI/PBS solution of various reagent-to-mouse mass ratios (50, 100, 150, 200, and 300 μg/mouse). The volume of the solution was 400 μL. Feeding was stopped 12 h before the injection. The mice were observed for 14 d to acquire their toxi city reaction and death. The influence of the polymer on organs and tissues was detected using morphological method.

1.2.11 Transfection of GC-PEI/DNA polymer in vivo

Twenty Kunming mice (half male and half female, 6 – 8 weeks old, 18 – 22 g) were randomly divided into 4 groups, 5 in each. Mice in the control group only injected naked plasmid DNA solution; mice in the cationic liposome group were injected with Lipofectamine™2000; mice in the GC-PEI/PBS /DNA group and GC-PEI /5% GS /DNA were injec-

ted with GC-PEI/PBS/DNA and GC-PEI/5% GS/DNA, respectively. The injection was performed at tail vein; the injection volume was 400 μL; the DNA content was 30 μg; and the mass ratio (GC-PEI-to-DNA) was 2∶1. Forty-eight hours after the injection, the tissues of liver, heart, lung, spleen, and kidney were cut and immediately put into the freezing microtome to make frozen sections with thickness of 15 μm. Then fluorescence microscope was used to observe the expression of GFP and take photos.

1.3 Statistical analysis

Data were presented as mean ± standard deviation ($\bar{x} \pm s$). The statistical analyses were processed by use of the statistical software SPSS13.0. Data were analyzed by variance analysis. $P < 0.05$ was considered to be statistical significance.

2 RESULTS

2.1 Analysis of the combination of GC-PEI2K polymer and DNA

It was found that GC-PEI could combine with DNA in various solvents and different mass ratios of GC-PEI-to-DNA. The DNA binding ability increased as the concentration of GC-PEI increased. When the mass ratio of GS-PEI-to-DNA was 0.25∶1, part of the DNA combined with the polymer, and only a small part were left and moved to the positive pole; when the ratio ≥1∶1, all the DNA combined with the polymers in the 3 solvents and remained in the gel loading wells, no DNA bands appeared (Fig. 1). DNA concentration in the supernatant decreased with the increase of concentration of GC-PEI polymer. When the mass ratio was 1∶1, 1.5∶1, and 2.5∶1, more than 90% of DNA was combined in all the 3 solvents, and DNA in the supernatant was very little, and the DNA in the 3 solution was almost the same (Fig. 2). So GS-PEI-to-DNA ratio of 1.5∶1 was applied in the subsequent DNA protection test.

2.2 Protective effect of GC-PEI/DNA complexes on DNA

GC-PEI polymers combined with plasmid DNA at the ratio of 1.5∶1 to produce GC-PEI/DNA complexes. After reacted with DNase Ⅰ and serum, the complexes were stable, and obvious DNA bands was seen in the sample-loading wells and the electrophoresis tracks; while the naked DNA in the control group was mostly degraded, and no obvious DNA band could be seen (Fig. 3A, 4). Fig. 3B showed the result of electrophoresis of the DNA eluded by 1% SDS, which was consistent with that of the lanes 2 – 4 of Fig. 3A. According to the images, GC-PEI/PBS/DNA and GC-PEI/5% GS/DNA was a little more stable than GC-PEI/NaCl/DNA.

2.3 Particle size, size distribution and Zeta potential

With the increase of GC-PEI/DNA mass ratio, the particle size of GC-PEI/DNA complexes in the 3 solvents decreased, and the Zeta potential increased. When the GC-PEI/DNA mass ratio was 0.25∶1, the particle size of GC-PEI/DNA complexes was larger than 220 nm, Zeta potential was negative. The change of the complexes was obvious when the ratio varied from 0.25∶1 to 1.5∶1. When the ratio varied from 1.5∶1 to 2.5∶1, the polymer size changed little, and the Zeta potential nearly kept constant. The grain size of the GC-PEI/5% GS/DNA group was the smallest, the GC-PEI/NaCl/DNA group the largest, and the GC-PEI/PBS/DNA group between them. The particle size of GC-PEI/5% GS group was no uniform, and size distribution was wide (Fig. 5 – 7).

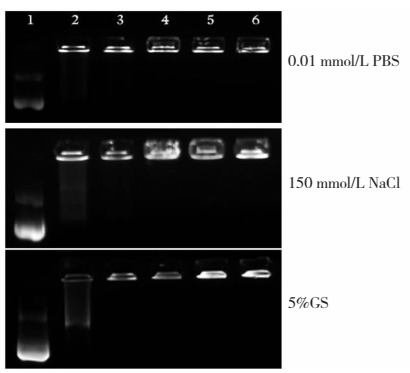

Fig. 1 **Effects of GC-PEI binding DNA in various solvents and different mass ratios of GS-PEI-to-DNA.** Lane 1: Control plasmid DNA; Lane 2 – 6: GC-PEI/DNA mass ratio was 0.25∶1, 0.75∶1, 1∶1, 1.5∶1, and 2.5∶1, respectively.

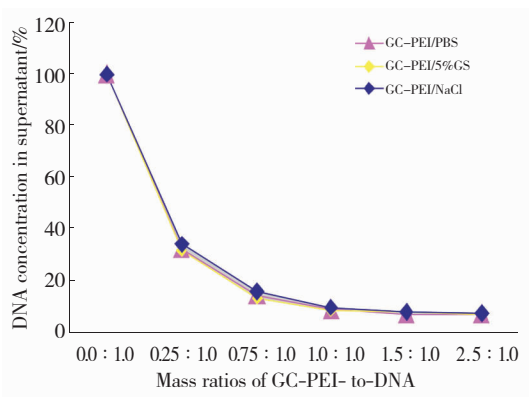

Fig. 2 **Efficiency of GC-PEI binding DNA.**

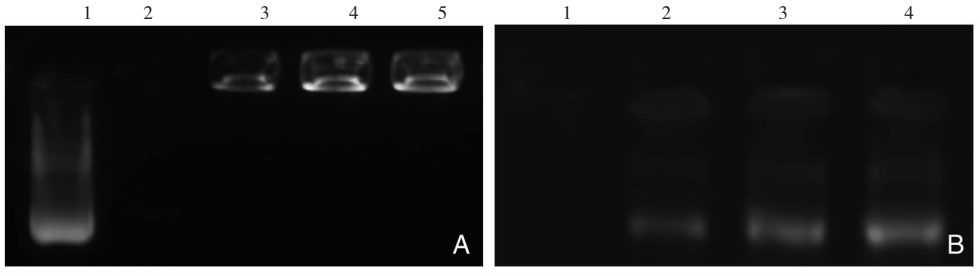

Fig. 3 Test of DNase Ⅰ digesting GC-PEI/DNA complexes. (A) Lane 1: Untreated plasmid DNA; Lane 2: Plasmid DNA + DNase Ⅰ; Lane 3 – 5: GC-PEI/NaCl/DNA, or GC-PEI/PBS/DNA, or GC-PEI/5% GS/DNA complexes + DNase Ⅰ, respectively; (B) Lane 1: Plasmid DNA + DNase Ⅰ; Lane 2 – 4: GC-PEI/NaCl/DNA, or GC-PEI/PBS/DNA, or GC-PEI/5% GS/DNA complexes + DNase Ⅰ, respectively.

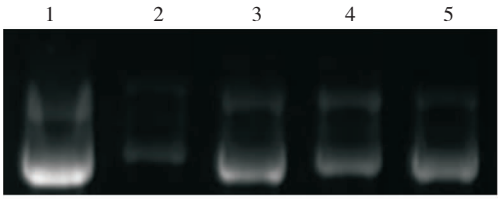

Fig. 4 GC-PEI/DNA complexes treated with serum. Lane 1: Untreated plasmid DNA; Lane 2: Naked plasmid DNA treated with serum; Lane 3 – 5: GC-PEI/5% GS/DNA, GC-PEI/NaCl/DNA, or GC-PEI/PBS/DNA complexes treated with serum, respectively.

2.4 Analysis results of transmission electron microscope

Transmission electron microscope was used to observe the form of the GC-PEI/DNA polymers. When the GC-PEI-to-DNA ratio was 1.5∶1, GC-PEI and DNA was tightly jointed; the particle was regular spheres with apparent nucleus-shell structure; the particle size and size distribution was uniform; no obvious aggregation was found (Fig. 8).

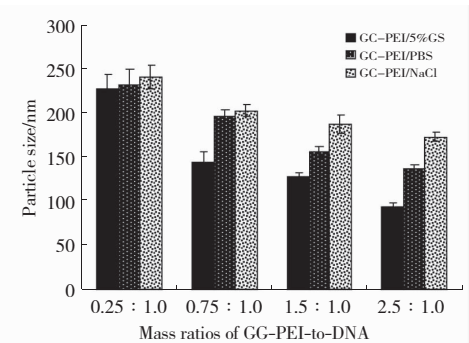

Fig. 5 Particle size distribution of GC-PEI/DNA complexes with different mass ratios of GC-PEI-to-DNA.

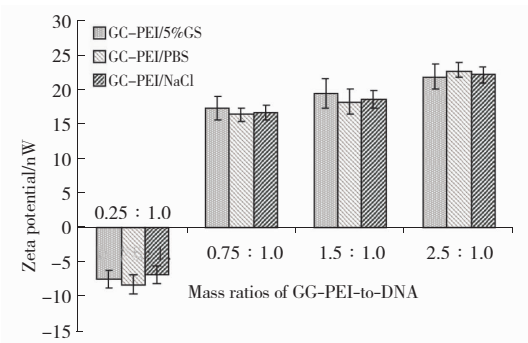

Fig. 6 Zeta potential distribution of GC-PEI/DNA complexes with different mass ratios of GC-PEI-to-DNA.

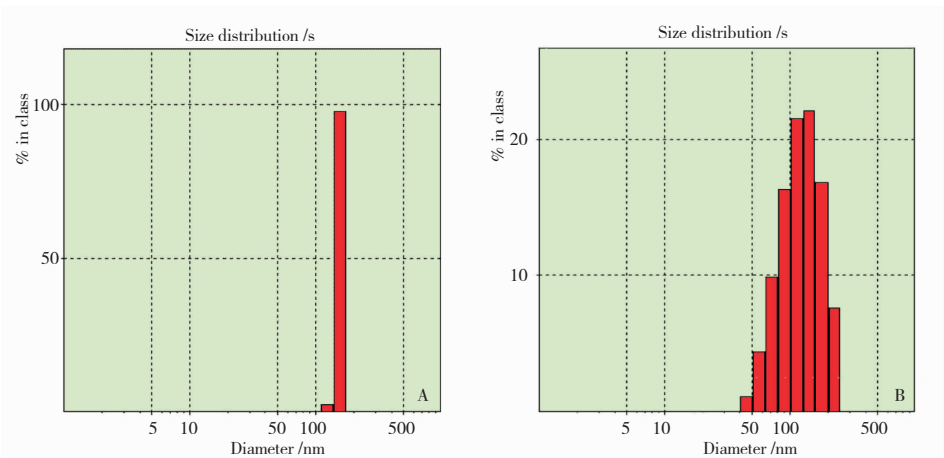

Fig. 7 Particle size distribution of GC-PEI/DNA complexes. A: GC-PEI/DNA: DNA was 1.5∶1; B: GC-PEI/5% GS: DNA was 1.5∶1.

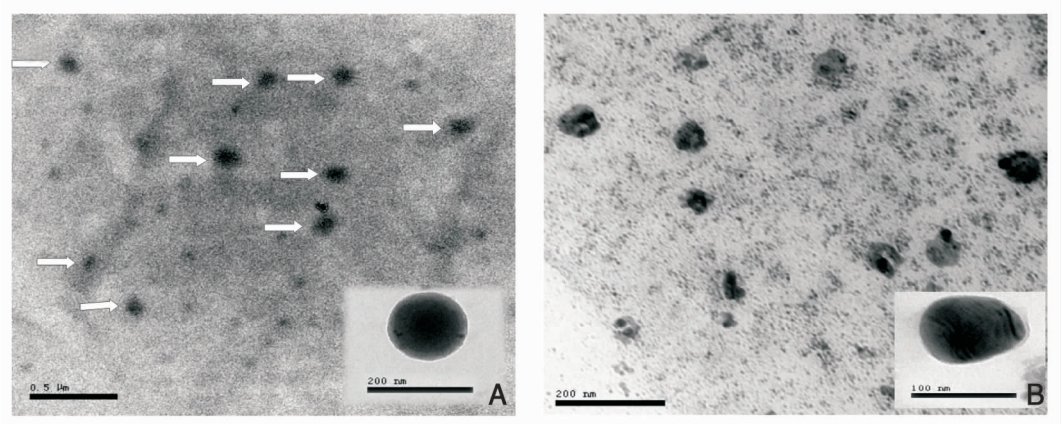

Fig. 8 GC-PEI/pEGFP-C1 complexes observed under transmission electron microscope. A: GC-PEI/PBS: DNA was 1.5:1; B: GC-PEI/5% GS: DNA was 1.5:1.

2.5 Results of MTT assay

When the concentration of the polymers was between 10 – 50 μg/mL, the value of RCV was higher in PEI2K and GC-PEI group than in PEI25K group ($P < 0.05$); when the concentration of the polymers ≤ 30 μg/mL, the influence of PEI2K and GC-PEI2K on cell viability was not significantly different ($P > 0.05$), and the values of RCV were higher than 80%; when concentration of the polymers reached 50 μg/mL, the cell viability in PEI2K group decreased significantly, the value of RCV was ≤70%; while in the GC-PEI2K group, the value of RCV > 80%; difference between the 2 groups was significant ($P < 0.05$, Fig. 9 – 10).

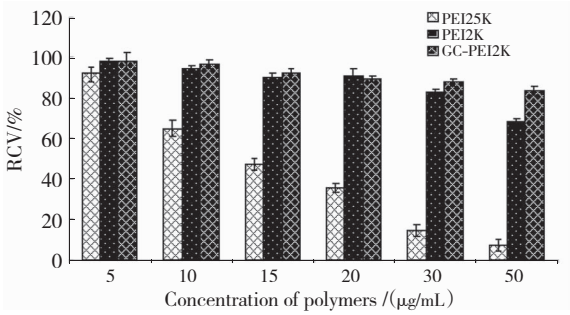

Fig. 9 Cytotoxicity of 3 polymers in HBE cells.

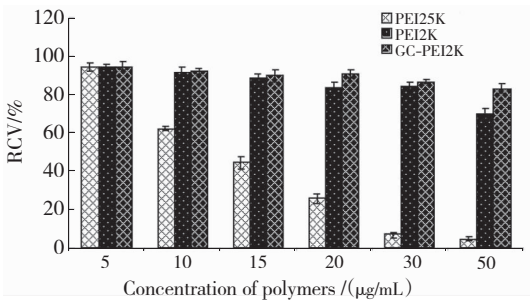

Fig. 10 Cytotoxicity of three polymers in QSG7701/core cells.

2.6 Results acute toxicity test in animals

The mice injected with GC-PEI polymers of various reagent-to-mouse mass ratios were observed for 14 d. No obvious abnormity was found, i.e., no acute toxic reaction or death occurred. There was no significant morphological difference between the tissues of the control and those of the experimental groups. And no obvious pathological change was found in tissues of the experimental groups (Fig. 11).

2.7 Influence of different cells on transfection efficiency (study of liver-targeting)

After the 48-h transfection in vitro, the flow cytometry showed that the transfection efficiency of the 3 hepatic cell line (L02, QSG7701, and QSG7701/core) in GC-PEI/PBS group was about 35%, and difference between the 3 was not significant ($P > 0.05$); the transfection efficiency in HBE and SGC7901 cells was only 3% – 7%, significantly different from that in the hepatic cells ($P < 0.05$). The GC-PEI/5% GS group also showed obvious targeting transfection. The transfection efficiency in hepatic cells was 23% approximately, lower than that of the GC-PEI/PBS group ($P < 0.05$). In the lipofectamine™2000/DNA group, clear green fluorescent spots were seen in all the 5 cell lines, and the transfection efficiency was about 50%. Transfection efficiency of the GC-PEI group was lower than that of the liposome group ($P < 0.05$), but the liposome group didn't show obvious targeting. The transfection efficiency of the naked DNA group and the PEI2K group was lower than 1% in all the 5 cell lines (Fig. 12).

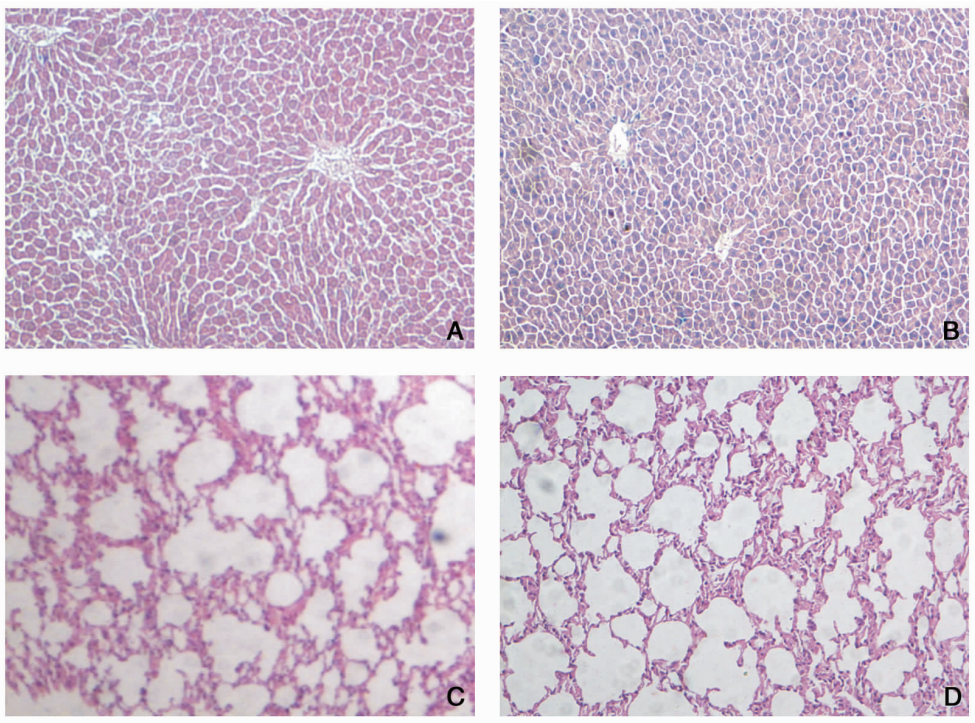

Fig. 11 Morphology of liver and lung of mice (HE, ×200). A: Liver of the control group; B: Liver of the group injected with 300 μg GC-PEI; C: Lung of the control group; D: Lung of the group injected with 300 μg GC-PEI.

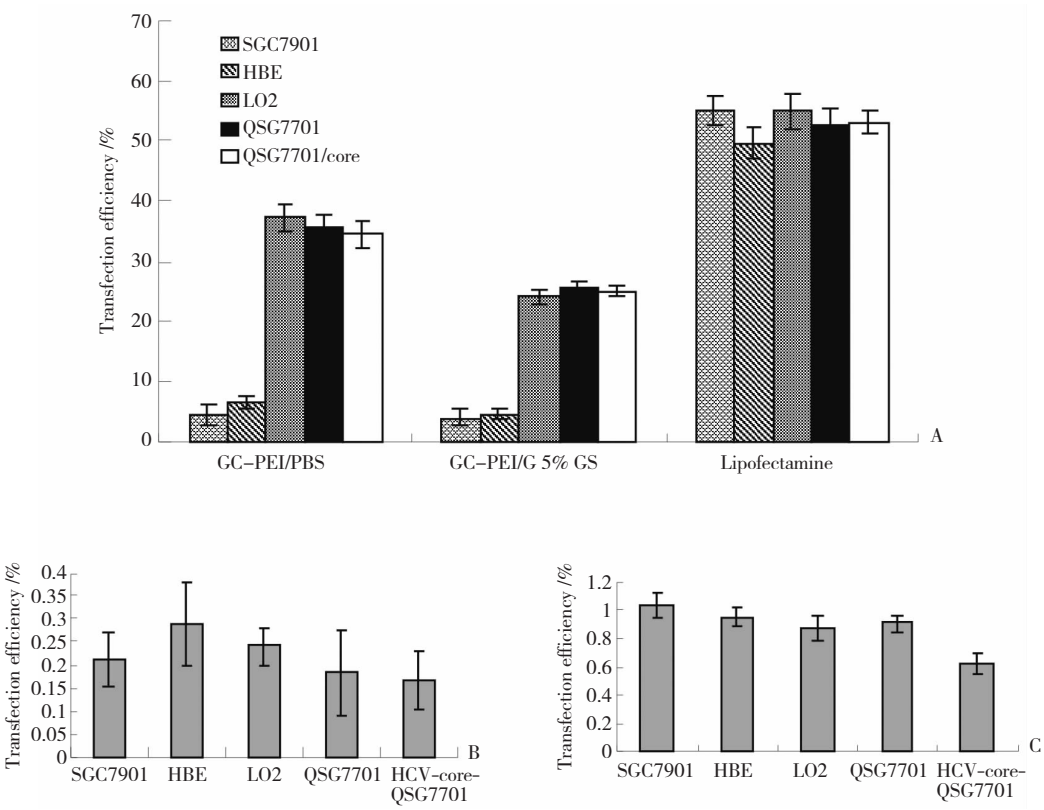

Fig. 12 Transfection efficiency of different vectors in various cells. A: Vector. The transfection efficiency of DNA mediated by GC-PEI/PBS, GC-PEI/5% GS and Lipofectamine™2000 in SGC7901, HBE, L02, QSG7701, and QSG7701/core cells, respectively; B: Naked DNA. The transfection efficiency of naked plasmid DNA in SGC7901, HBE, L02, QSG7701, and QSG7701/core cells, respectively; C: PEI2K. The transfection efficiency of DNA mediated by PEI2K in SGC7901, HBE, L02, QSG7701, and QSG7701/core cells, respectively.

2.8 Result of galactose competitive antagonism test

The transfection efficiency of GC-PEI/DNA complexes combined with 30 mmol/L galactose in QSG7701/core cells (17.1%) was evidently lower than that of the control group ($P<0.05$); whereas in those added with mannose, the transfection efficiency was not different from that of the control group ($P>0.05$, Fig. 13).

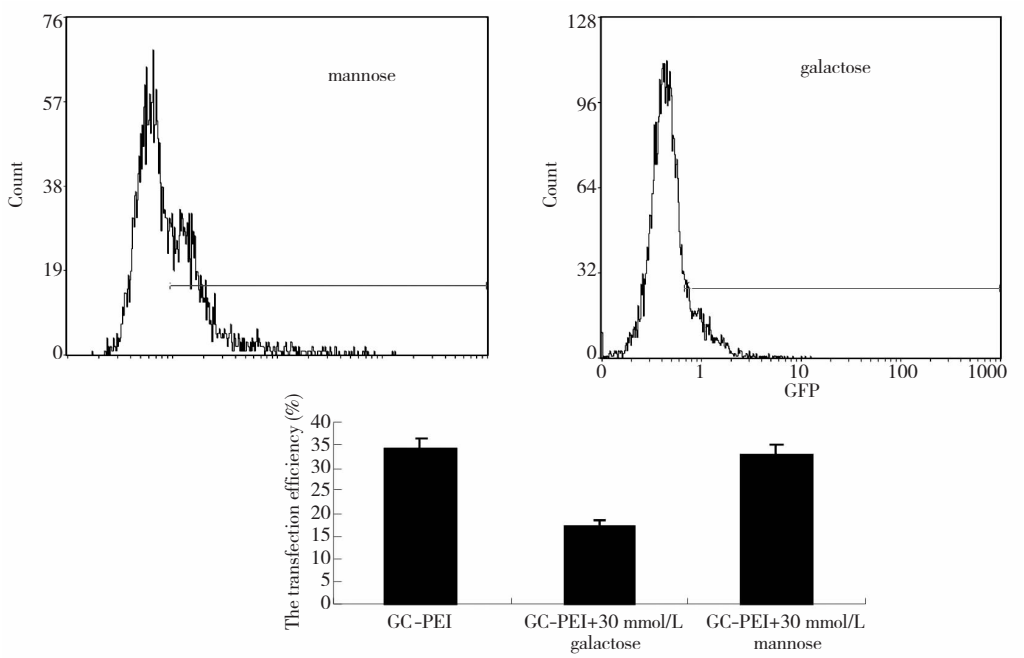

Fig. 13 Galactose competitive antagonism test.

2.9 Result of in vivo transfection

Frozen sections of the mice tissues were observed under fluorescence microscope. And the result showed that after the 48 h transfection, obvious green fluorescent spots were found in hepatic tissue sections of the 2 experimental groups, and the expression was not strong. Unclear green fluorescent spots were found in kidney tissue sections, but no spots in heart, lung and spleen tissues, only their outline could be made out (Fig. 14).

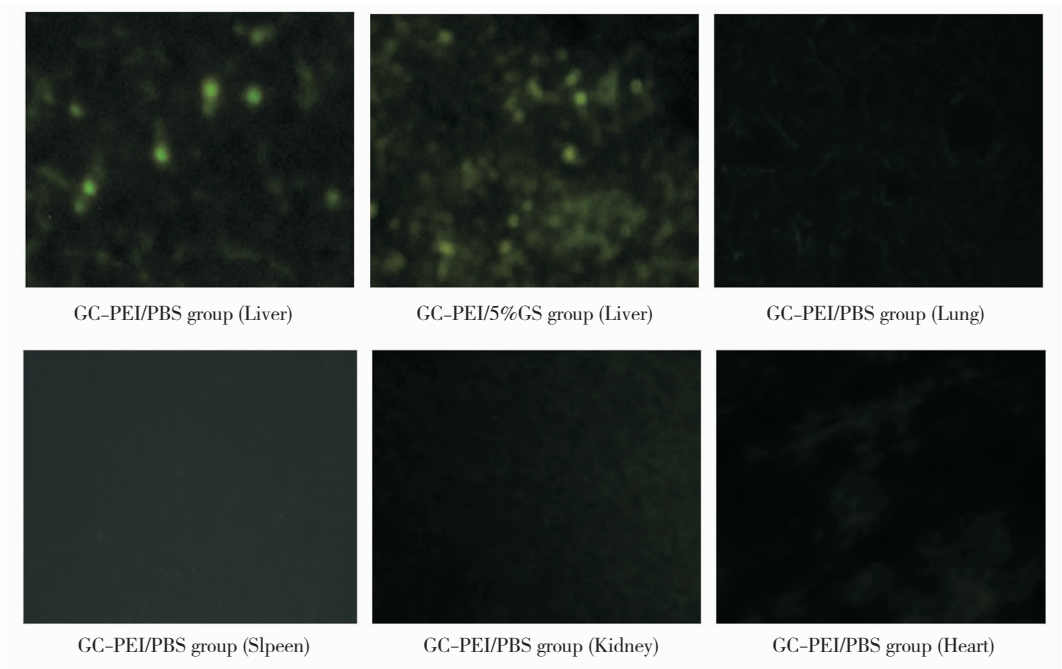

Fig. 14 Expression of GFP in the main organs of mice after 48 h transfection.

3 DISCUSSION

Cationic polymers can combine with electronegative DNA through the positive charge on their surface and produce electropositive complexes. The multivalent cations compress DNA into vectors, reducing the size of the complexes, and get positive charge their surface so that they are easy to ingestion through endocytosis[13-15]. In this experiment, galactosylated chitosan was synthesized, and low molecular weight PEI2K was grafted to it to form GC-PEI2K polymer. The polymer was mixed with pEGFP-C1 in various solvents and was made into GC-PEI/DNA complexes of various GC-PEI-to-DNA mass ratios. We then accessed their features and biological characteristics, and picked out those appropriate for transfection. The result EMSA and binding ability test showed that GC-PEI had good DNA-binding ability in the three solvents, and that the higher the GC-PEI-to-DNA ratio was, the more DNA would be left in the sample-application wells and the lower the concentration of uncombined DNA would be in the supernate after high-speed centrifugation, indicating that with the elevation of GC-PEI-to-DNA ratio within a certain range, the DNA combined by GC-PEI polymer increased, and the DNA-binding ability of the polymer enhanced. Test of GC-PEI/DNA complexes' anti-DNaseI effect also proved that the GC-PEI/DNA complexes could protect the DNA from being degraded in DNaseI and serum solution. The GC-PEI/DNA complexes synthesized in 5% GS and PBS solution were more stable than those in the 150 mM NaCl solution. Guy, et al.[16] found that particle size is an important parameter in in-vivo transfection, smaller complexes (particle size less than150 nm) were easy to engulf in mammal. It was reported that in vivo the gene transference efficiency of smaller complexes was better than that of larger ones; while in vitro, within a certain range, the gene transference of larger ones was more efficient[17-19]. Observations with particle size analyzer and transmission electron microscope showed that the mass ration of polymer to DNA has a certain influence on the particle size and Zeta potential of the complex, and that as the mass ratio increased, the particle size became smaller and the Zeta potential increased. The general complex distribution trend in the 3 solvents was basically the same, and particle size of GC-PEI/DNA/NaCl complex was a little bit larger than the others. When the ratio was 1.5:1, the GC-PEI/DNA complex particles were regular spheres with obvious nucleus-shell structure, indicating that the polymer could well combine and completely enclose the DNA. The nucleus of the complex was DNA, and shell was cationic polymer. The nucleus-shell structure improved the stability of the complex in blood, and protected the DNA from being degraded by nuclease during transference. The highly charged ions repel each other and inhibit convergence to maintain the stability of the complex, and keep the particle size small. Therefore, within a certain range, with the increasing of polymer-to-DNA ratio, the DNA-binding ability of the polymers improves, charge on the surface of the complex increased, which compress and enclose the DNA and formed spheres, and the particle size become smaller. The results were consistent with reported ones[16].

Cytotoxicity assay is an important index in the detection of gene vectors. PEI is a cationic polymer studied by many people, mainly on improving its transfection efficiency and reducing its toxicity. Isomers or different molecular weight PEI in vivo gene transfection has different efficacy and toxicity[20]. Transfection efficiency and cytotoxicity of PEI are positively correlated with its relative molecular mass. With the increase of PEI's relative molecular mass and concentration, its charge increases and cytotoxicity is enhanced. PEI with molecular mass of less than 2 000 cannot be transfected, but has mild toxicity[21-22]. Branched low molecular weight PEI has highly-branched structure, and is modifiable. In recent years, studies have been carried out on modification of PEI. For example, lots of derivatives were developed by incorporating target ligands and grafting to biomaterials, which improved the transfection efficiency and targeting ability, and reduced cytotoxicity of PEI complexes. Among PEIs of different molecular mass, branched PEI25K is the one that has been studied thoroughly so far and are used as a standard to be compared with other cationic polymers in various properties[23]. In this research, branched PEI25K of various concentrations was employed as the reference, PEI2K and GC-PEI2K of different concentrations was used in transfection of HBE, QSG7701/core cells. The MTT assay showed that cells viability of PEI25K group was significantly low-

er than that of the PEI2K and GC-PEI2K group; the RCV in PEI2K group decreased sharply when the concentration was high (50 μg/mL); RCV value of GC-PEI group was more than 80% even when the concentration was high; and no obvious cytotoxicity was found. The acute toxicity test performed in mice proved that within the dosage range of 50 – 300 μg, GC-PEI was not cytotoxic, no toxic reaction occurred in the mice, and no obvious pathological changes in the major organs were detected in HE assay. This experiment also proved that grafting low-molecular-weight PEI to galactosylated chitosan scaffold would reduce its cytotoxicity, which might related to the fact that, when galactosylated chitosan oxidized by periodic acid reacted with PEI2K, the amino on the surface of PEI was inhibited and the biocompatibility was improved.

GFP reporter gene is a common reporter that indicates gene vector's property, the product of its expression GFP gives out a green fluorescent light under fluorescence microscope. In this research, we chose pEGFP-C1 which can be easily detected as the reporter gene, and mixed it with GC-PEI/PBS to produce complex. Then fluorescence microscope and flow cytometry was employed to observe and detect the GFP positive cells and measured the transfection efficiency. The result of the in vitro experiments showed that the expression level of GFP was much higher in hepatic cell lines than in non-hepatic ones, and that no obvious fluorescent spots was found in cells of the control group and PEI2K group, proving that modifying the galactosyl in the vector could improve the compatibility of polymer and cell membrane, promote the specific combination of complex and ASGP-R on the surface of hepatic cells, and improve the translation efficiency of hepatic cells. Result of the galactose competitive inhibition test indicated that ASGP-R was an effective target for liver-target transmission. In physiological circumstances, naked plasmid DNA is a highly hydrophilic macromolecule with negative charge on the surface. It can not get through biomembrane or continuous endothelial cells without the help of external force or vectors, and its diffuse outside the cell was quite limited, so it can be easily digested by nuclease. Cationic liposome's performance was not satisfied either. In physiological circumstances, Cationic liposome is positively charged, and tends to attract electronegative serum proteins to gather up, which are quickly cleared up by macrophages, or even activate complement or cooperate with the non-methylated CpG DNA in the plasmid to induce acute toxic reaction. Ten min after the injection from tail vein, only less than 2% of the complexes remained in the blood. Most of them were taken in and preserved by pulmonary tissues. The transfection efficiency was rather poor in liver parenchymal cells[24-26]. The result of this experiment was that in hepatic tissues of the mice injected with GC-PEI/DNA complexes, obvious green fluorescent spots could be seen under fluorescence microscope, while in heart, spleen, lung and kidney no expression of GFP was detected. In the control group, on obvious fluorescence was seen in the hepatic tissues of the mice injected with naked DNA or liposome, indicating that GC-PEI/DNA complexes had good liver-targeting specificity.

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