Mannosylated liposomes for targeted gene delivery

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Background: Liposomes can be modified with different ligands to control their biological properties, such as longevity, targeting ability, and intracellular penetration, in a desired fashion. The aim of this study was to modify liposomes with a novel mannosylated polyethylene glycol-phosphatidylethanolamine (M-PEG-PE) ligand to achieve active targeted gene delivery.

Methods: Rat Kupffer cells were isolated and used as model cells for in vitro evaluation of cytotoxicity and transfection efficiency. The modified liposomes were intravenously injected into the rats, and Kupffer cells were isolated and analyzed by flow cytometry for in vivo gene delivery and expression.

Results: The M-PEG-PE-modified liposome-enhanced green fluorescence protein plasmid (M-PEG-PE-Lipo-pEGFP) complexes had a particle size of 237 nm and a loading efficiency of 90%. The M-PEG-PE-Lipo-pEGFP complexes displayed remarkably higher transfection efficiency than unmodified Lipo-pEGFP, both in vitro (51%–30%) and in vivo (43%–27%).

Conclusion: M-PEG-PE could function as an excellent active targeting ligand, and M-PEG-PE-modified liposomes could be promising active targeted drug delivery vectors.

Keywords: gene delivery, active targeting, mannosylated, polyethylene glycol, phosphatidylethanolamine, liposomes

Introduction

Gene delivery using nonviral pharmaceutical vectors has long been considered, because these vectors are less toxic, less immunogenic, and easily modified.¹–³ Nonviral carriers, such as polymeric nanoparticles,⁴ liposomes,⁵ micelles,⁶ nanocapsules,⁷ solid lipid nanoparticles,⁸ niosomes,⁹ and other vectors have been widely used as gene delivery systems. It has been shown that liposomes can act as biocompatible, biodegradable, and nonimmunogenic drug/gene carriers, and the advantages of liposomal-encapsulated drugs/genes are a prolonged duration of exposure, selective delivery to the intended site of action, and improved therapeutic index.¹⁰,¹¹ Among the liposomes, the Lipofectamine™ 2000 transfection reagent is a proprietary formulation for transfection of nucleic acids (DNA and RNA) into cells, and has been widely used by many researchers.¹²–¹⁴

Liposomes can be modified with different kinds of ligands to control their biological properties such as longevity, targeting ability, and intracellular penetration, in a desired fashion.¹⁵ Modification of liposomes using polyethylene glycol (PEG) has emerged as a common strategy to ensure stealth shielding and long circulation of liposomal drug/gene delivery systems. PEG modification could also provide liposomes with active...
targeting properties by enabling covalent attachment of a wide assortment of targeting ligands by amide bonding or disulfide bridge formation.\(^{16}\)

A series of PEG-containing ligands, commonly known as PEG-phosphatidylethanolamine (PEG-PE) conjugates, was described by Torchilin et al who demonstrated that PEG-PE conjugates with various PEG lengths and terminal-targeted moieties can provide extremely stable, long-circulating, and actively targeted nanocarriers which spontaneously accumulate at specific sites.\(^{4,6,17}\) PEG-PE was subsequently used widely in liposomal formulations,\(^{18,19}\) and more recently, several such ligands (eg, PEG-PE and TAT-PEG-PE) have also been used for targeted delivery of drugs/gen\(e\).\(^{20–22}\)

Saccharide ligands, such as galactose, mannose, lactose, and maltose, have been extensively investigated for modification of liposomes to achieve site-specific targeting.\(^{23,24}\) In this study, a novel conjugated ligand, ie, mannose-PEG-PE (M-PEG-PE), was synthesized, and M-PEG-PE-modified Lipofectamine™ 2000 (Lipo) liposomes were investigated as an active targeting gene delivery system. Enhanced green fluorescence protein plasmid (pEGFP) was used as the model gene. The novel modified vectors were delivered intravenously into rats and the in vivo targeting behavior of M-PEG-PE-modified liposome-pEGFP (M-PEG-PE-Lipo-pEGFP) complexes was investigated in liver macrophages and compared with that of unmodified Lipo-pEGFP complexes.

**Materials and methods**

**Materials**

Butyl carbonyl (Boc)-NH-PEG\(_{2000}\)-COOH was purchased from Shanghai Yarebio Co Ltd (Shanghai, China). Mannose, L-\(\alpha\)-phosphatidylethanolamine (PE), MTT (3-[4,5-dimethyl-2-thiazolyl]-2,5-diphenyl-2H-tetrazolium bromide), and concanavalin A were purchased from Sigma-Aldrich Co, Ltd, (St, Louis, MO). pEGFP-N1 was provided by Shandong University (Shandong, China). Quant-iT™ PicoGreen\textsuperscript{a} dsDNA quantitation reagent and Lipofectamine 2000 were obtained from Invitrogen Life Technologies (Carlsbad, CA). All other chemicals were of analytical grade or higher.

**Animals**

Adult male Sprague-Dawley rats (10–11 weeks old) were purchased from the Medical Animal Testing Center of Shandong Province and housed under standard laboratory conditions. All animal experiments complied with the requirements of the National Act on the Use of Experimental Animals (People’s Republic of China).

**Synthesis of M-PEG-PE ligands**

M-PEG-PE ligands were synthesized as described in Figure 1. Boc-NH-PEG-COOH 100 mg was dissolved in dimethyl sulfoxide and stirred with 36 mg of PE to form a mixture. 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide (EDC⋅HCl) 72 mg) and triethylamine (1 equivalent of EDC⋅HCl) were dissolved in dimethyl sulfoxide and added dropwise into the mixture in an ice bath, stirred for 36 hours, and concentrated hydrochloric acid was then used to detach the Boc group and produce NH\(_2\)-PEG-CO-NH-PE. Man-

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**Figure 1** General reaction scheme for synthesis of the mannosylated polyethylene glycol-phosphatidylethanolamine ligand.

**Abbreviations:** Boc, butyl carbonyl; PE, phosphatidylethanolamine; PEG, polyethylene glycol; DMSO, dimethyl sulfoxide; TEA, triethylamine.
dimethyl sulfoxide) was added dropwise into the solution in an ice bath and stirred for 24 hours. Dimethyl sulfoxide was moved by rotary evaporation, and the product was dialyzed against Milli-Q (Billerica, MA) water for 24 hours to form M-PEG-PE.

Fabrication of Lipo-pEGFP complexes
Lipo-pEGFP complexes were prepared according to the manufacturer’s instructions for Lipofectamine 2000. Briefly, 100 µL of pEGFP 1 µg/µL was mixed with 200 µL of Lipofectamine 2000 by vortexing for 30 seconds. Incubation of the mixture for 30 minutes at room temperature-facilitated formation of the DNA-loaded liposomes (Figure 2).

Modification of Lipo-pEGFP with M-PEG-PE
M-PEG-PE-Lipo-pEGFP complexes were produced by the solvent diffusion method (Figure 2). Briefly, M-PEG-PE ligands were dissolved in 2 mL of phosphate-buffered saline (pH 7.4). The solution was then added dropwise into 10 mL of Lipo-pEGFP complexes and stirred at 600 rpm and room temperature, with immediate surface modification. Subsequently, free M-PEG-PE was removed from the modified Lipo-pEGFP by gel chromatography using a Sephadex® G-50 column (GE Healthcare, Uppsala, Sweden). The complexes obtained were resuspended in Milli-Q water, washed three times, and filtered through a membrane with a 0.80 µm pore size to obtain M-PEG-PE-Lipo-pEGFP. To optimize the modification ratio, M-PEG-PE ligands dissolved in phosphate-buffered saline were designed at different weight ratios for the Lipo-pEGFP (w/w). The particle size and zeta potential of the complexes were determined using a Zetasizer Nano ZS (Malvern Instruments, Worcestershire, UK). The shape of the M-PEG-PE-Lipo-pEGFP was examined by transmission electron microscopy. Samples were prepared by placing one drop of suspension onto a copper grid and dried, followed by negative staining with one drop of a 3% aqueous solution of sodium phosphotungstate for contrast enhancement. The air-dried samples were then examined directly under the transmission electron microscope.

M-PEG-PE modification and pEGFP-loading capacity of M-PEG-PE-Lipo-pEGFP
To confirm the success of modification and determine the optimum ratio of M-PEG-PE to Lipo-pEGFP, several experiments were carried out as follows.

PicoGreen fluorometry assay
During the modification procedure, M-PEG-PE ligands were coated onto Lipo-pEGFP carriers due to the electric charge and lipophilic interaction. A PicoGreen fluorometry assay was used to determine the appropriate modification rate of M-PEG-PE ligands and also to quantitate the amount of pEGFP carried by the optimally modified liposomes. Different ratios of M-PEG-PE to Lipo-pEGFP (from 2% to 30%, w/w) were used to prepare the M-PEG-PE-Lipo-pEGFP complexes, and the pEGFP was isolated from M-PEG-PE-Lipo-pEGFP by centrifugation at 1000 g and 4°C for 30 minutes. The concentration of pEGFP was determined by fluorescence and comparing the sample with the blank liposome supernatant. The amount of pEGFP loaded into the liposomes was calculated according to the linear calibration curve of pEGFP:

\[
\text{Gene loading quantity (\%) = \frac{\text{Total of pEGFP} - \text{free pEGFP}}{\text{total DNA}} \times 100.}
\]

Concanavalin A agglutination study
A concanavalin A agglutination assay was performed to confirm that the M-PEG-PE ligands were successfully modified onto the Lipo-pEGFP surface. Binding of the terminal

Figure 2 Preparation and modification of liposome-pEGFP complexes.
Abbreviations: M-PEG-PE, mannosylated polyethylene glycol-phosphatidylethanolamine; Lipo, Lipofectamine 2000; pEGFP, green fluorescence protein plasmid.
α-mannose residues to concanavalin A causes agglutination of the complexes in solution, resulting in an increase in turbidity. Therefore, 100 µL of M-PEG-PE-Lipo-pEGFP complexes was added into 500 µL of concanavalin A 1 mg/mL in phosphate-buffered saline (pH 7.4) with 5 mM of calcium chloride (CaCl₂) and 5 mM of magnesium chloride (MgCl₂) at room temperature. The increase in turbidity at 360 nm (OD₃60) was monitored at room temperature for 3 minutes.

Isolation and culture of Kupffer cells
Kupffer cells were isolated from Sprague-Dawley rats under pentobarbitone anesthesia using a method described elsewhere. In brief, the rat portal vein was cannulated and perfused with Hank’s buffered salt solution for 10 minutes, the liver was excised, and the perfusate was discarded. The liver was then perfused with 0.2% pronase, then with a recirculating solution of 0.05% pronase and 0.05% collagenase until the liver was digested. The liver was thereafter cut into small pieces, suspended in a solution containing 0.02% pronase, 0.05% collagenase, and 0.005% DNase, and then agitated. Following digestion, the liver homogenate was filtered through sterile gauze and centrifuged at 1000 g and 4°C for 10 minutes. The supernatant was removed and the pellet then resuspended in Percoll™ gradient. Aliquots of this cell suspension were added to aliquots of Percoll gradient. These were carefully overlaid with Hank’s balanced salt solution and centrifuged. The nonparenchymal cell-enriched layer observed at the interface between the two layers was carefully harvested and diluted with Hank’s balanced salt solution. The suspension was then centrifuged to precipitate the Kupffer cells, which were then seeded into a 96-well microtiter plate at a density of 2 × 10⁴ cells/well in RPMI 1640 supplemented with 10% fetal bovine serum and antibiotics. After incubation at 37°C for 2 hours under 5% CO₂, the culture medium was replaced with 1 mL of complete medium after incubation at 37°C for 4 hours under a 5% CO₂ atmosphere. The cells were incubated and studied at 72 hours following transfection. The fluorescent cells were observed using an inverted fluorescence microscope, at which time photographs were taken. To quantitate transfection efficiency further, the cells were washed with 1 mL of phosphate-buffered saline (100 g, 4°C for 5 minutes) and detached with trypsin-EDTA. The supernatant was discarded and resuspended in 300 µL of phosphate-buffered saline, and the fluorescence intensity of the cells was determined using a flow cytometer equipped with a 488 nm argon laser for excitation. For each sample, about 10,000 events were collected and fluorescence was detected. Signals were amplified in logarithmic mode for fluorescence to determine the positive events by a standard gating technique. The percentage of positive events was calculated as the events within the gate divided by the total number of events, excluding cell debris.

In vitro cytotoxicity evaluation and transfection analysis
The in vitro cytotoxicity of M-PEG-PE-Lipo-pEGFP in Kupffer cells was evaluated by MTT assay. The Kupffer cells were incubated with M-PEG-PE-Lipo-pEGFP and unmodified Lipo-pEGFP at various concentrations (10, 20, 50, 100, and 200 µg/mL) for 48 hours at 37°C and 5% CO₂. The cell viability was then assessed by MTT assay; 5 mg/mL of MTT in phosphate-buffered saline was then added to each well and the plate was incubated for an additional 4 hours at 37°C and a 5% CO₂ atmosphere. The medium containing MTT was then removed, and the crystals formed by living cells were dissolved in 100 µL of dimethyl sulfoxide. The absorbance at 570 nm was determined by microplate reader. Untreated cells were taken as a control with 100% viability, and cells without the addition of MTT were used as a blank to calibrate the spectrophotometer to zero absorbance. The relative cell viability (%) compared with control cells was calculated as (Abssample/Abscontrol) × 100.

For analysis of transfection efficiency, the Kupffer cells were seeded into 24-well plates at a density of 1 × 10⁶ cells/well in 1 mL of RPMI 1640 with 10% fetal bovine serum. After about 24 hours, when the cells were at about 80%–90% confluence, the media were replaced with 400 µL of transfection media containing M-PEG-PE-Lipo-pEGFP. Naked DNA was used as a negative control and unmodified Lipo-pEGFP was used as a positive control. The original incubation medium was replaced with 1 mL of complete medium after incubation at 37°C for 4 hours under a 5% CO₂ atmosphere. The cells were incubated and studied at 72 hours following transfection. The fluorescent cells were observed using an inverted fluorescence microscope, at which time photographs were taken. To quantitate transfection efficiency further, the cells were washed with 1 mL of phosphate-buffered saline (100 g, 4°C for 5 minutes) and detached with trypsin-EDTA. The supernatant was discarded and resuspended in 300 µL of phosphate-buffered saline, and the fluorescence intensity of the cells was determined using a flow cytometer equipped with a 488 nm argon laser for excitation. For each sample, about 10,000 events were collected and fluorescence was detected. Signals were amplified in logarithmic mode for fluorescence to determine the positive events by a standard gating technique. The percentage of positive events was calculated as the events within the gate divided by the total number of events, excluding cell debris.

In vivo gene delivery
Adult male Sprague-Dawley rats were divided into three groups (six rats in each group) and injected intravenously with 1 mL of M-PEG-PE-Lipo-pEGFP, Lipo-pEGFP, and naked pEGFP (300 µL per rat, containing 30 µg of pEGFP). At predetermined time intervals, rat Kupffer cells were
isolated as described earlier. The Kupffer cells were then seeded into 24-well plates, and incubated until 48 hours after transfection. The cells were then washed with 1 mL of phosphate-buffered saline (100 g and 4°C for 5 minutes) and detached with trypsin-EDTA. The supernatant was discarded and resuspended in 300 µL of phosphate-buffered saline, mixed well, and flow cytometry was performed to determine the amount of Kupffer cells that had been successfully transfected.

Statistical analysis
All studies were repeated three times and measurements were carried out in triplicate. Results are reported as the mean ± standard deviation. Statistical significance was determined using the Student’s t-test. Differences between experimental groups were considered significant when the P value was <0.05.

Results
Structural confirmation of M-PEG-PE ligands
The structure of M-PEG-PE was confirmed by infrared and 1H nuclear magnetic resonance (NMR) spectroscopy. Infrared ν/cm−1: 3521.5 (–NH–, –OH); 1818.3 (–C=O); 1665.4 (–HN–CO–); 1631.9 (–HN–CO–). 1H NMR (dimethyl sulfoxide-d6, 300 mHz) δs 2.45 (CH2CO), 3.28 (CH2 N), and 6.08 (NH). The production rate was around 65%.

Characterization of DNA-loaded liposomes
Transmission electron microscopic images of the vectors are shown in Figure 3. The mean particle size, polydispersity index, and zeta potential of Lipofectamine 2000, Lipo-pEGFP, and M-PEG-PE-Lipo-pEGFP are characterized and summarized in Table 1.

Optimization of M-PEG-PE modification rate
The quantity of pEGFP loaded into the variously modified liposomes was calculated and is displayed in Figure 4. Unmodified liposomes (0%) reached a gene loading quantity of 90%. With the increasing modification rate (from 2% to 10%), the pEGFP loading amount showed no obvious change (all around 90%). When the ratio of the ligands was above 10%, the loading capacity decreased, and only 15% gene carrying was observed at the ratio of 30%. The optimum rate was 10%, and this ratio was used for the investigations described below.

Determination of M-PEG-PE modification
A concanavalin A agglutination study was used to identify further that the M-PEG-PE ligands were successfully modified onto the Lipo-pEGFP surface. As shown in Figure 5, the turbidity of M-PEG-PE-Lipo-pEGFP appeared to increase, while the Lipofectamine 2000 and Lipo-pEGFP formulations appeared not to show any significant increase in turbidity.

In vitro cytotoxicity evaluation
The in vitro cytotoxicity of M-PEG-PE-Lipo-pEGFP and unmodified Lipo-pEGFP at various concentrations was evaluated by MTT assay. The cell viability of the liposomes

<table>
<thead>
<tr>
<th>Sample properties</th>
<th>Lipofectamine™</th>
<th>Lipo-pEGFP</th>
<th>M-PEG-PE-Lipo/pEGFP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean particle size (nm)</td>
<td>128.5 ± 2.9</td>
<td>205.9 ± 9.6</td>
<td>237.2 ± 10.1</td>
</tr>
<tr>
<td>Polydispersity index</td>
<td>0.09 ± 0.01</td>
<td>0.16 ± 0.03</td>
<td>0.18 ± 0.07</td>
</tr>
<tr>
<td>Zeta potential (mV)</td>
<td>39.56 ± 2.85</td>
<td>26.21 ± 1.69</td>
<td>18.92 ± 1.18</td>
</tr>
</tbody>
</table>

Abbreviations: Lipo, Lipofectamine™; pEGFP, green fluorescence protein plasmid; M-PEG-PE, mannosylated polyethylene glycol-phosphatidylethanolamine.
over the studied concentration range (10–200 µg/mL) was between 80% and 100% compared with the controls (Figure 6). M-PEG-PE-Lipo-pEGFP showed no greater cytotoxicity than Lipo-pEGFP at any concentration \((P > 0.05)\).

**In vitro transfection analysis**

The in vitro transfection efficiency of M-PEG-PE-Lipo-pEGFP and Lipo-pEGFP in Kupffer cells was analyzed after 72 hours of transfection. Naked DNA was used as a negative control. As shown in the fluorescent images in Figure 7, when compared with naked DNA and Lipo-pEGFP, M-PEG-PE-Lipo-pEGFP showed higher transfection efficiency at the different time intervals. The cells exhibited highest efficiency at 48 hours. Flow cytometry results showed that the highest in vitro efficiency of M-PEG-PE-Lipo-pEGFP at 48 hours was over 50%. Compared with around 30% efficiency for Lipo-pEGFP, the advantage of the modified vectors was significant.

**In vivo gene delivery**

After intravenous injection of 1 mL of M-PEG-PE-Lipo-pEGFP, Lipo-pEGFP, and naked pEGFP, the rats were euthanized and the Kupffer cells were isolated at 48 hours. Flow cytometry was applied to quantitate the amount of

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**Figure 4** Optimization of the M-PEG-PE modification rate. 
**Abbreviations:** M-PEG-PE, mannosylated polyethylene glycol-phosphatidylethanolamine; Lipo, Lipofectamine™; pEGFP, green fluorescence protein plasmid.

**Figure 5** Turbidity of Lipofectamine™ 2000, Lipo-pEGFP, and M-PEG-PE-Lipo-pEGFP at different time intervals. 
**Abbreviations:** M-PEG-PE, mannosylated polyethylene glycol-phosphatidylethanolamine; Lipo, Lipofectamine™; pEGFP, green fluorescence protein plasmid.
molecules that had been successfully transfected. As shown in Figure 8, M-PEG-PE-Lipo-pEGFP showed a remarkably higher transfection efficiency than unmodified Lipo-pEGFP ($P < 0.05$) and naked pEGFP ($P < 0.05$).

**Discussion**

Liposome-based delivery systems have long been identified as safe and effective drug carriers that are amenable to modification with cell-specific targeting ligands. In regards to gene delivery, one of the most popular liposomes is Lipofectamine 2000 which is a proprietary formulation for DNA from the complexes.

The investigation began with the synthesis of M-PEG-PE, in which mannose was the target moiety which could bind to the mannose receptor in the macrophage, and PEG-PE was the spacer linked into the surface of the liposomes. It is known that the method of connection between the carbohydrate and carrier surface through a flexible spacer influences the targeting ability and cellular uptake of the vectors. For example, the length and flexibility of the spacer between the carbohydrate head group and liposome surface mainly influence the target specificity and uptake of liposome by macrophages. PEG-PE conjugates have been reported and TATp-PEG$_{2000}$-PE conjugates have been used for the modification of liposomes to form active targeting vectors. Therefore, PEG$_{2000}$-PE was used as the anchor.

It is also known that the saccharide group density is an important factor for targeting efficiency. Generally, vectors modified with a higher content of sugar residues exhibit more efficient cellular recognition and internalization compared with those having a lower sugar density. Therefore, theoretically, the more M-PEG-PE ligands modified onto the carriers, the better the targeted delivery achieved. However, excessive modification could trigger the disintegration of liposomes, pEGFP could leak out of the vector and cause a notable decrease of the gene loading capacity. A PicoGreen fluorometry assay was used to determine the appropriate modification rate of M-PEG-PE ligands and also to quantify the amount of pEGFP carried by the optimally modified liposomes.

Nonmodified liposomes reached a gene loading quantity of 90.34% (Figure 4). With an increase in the modification rate, the loading amount of pEGFP showed no obvious change until the ratio of the ligands was above 10%. The loading capacity was decreased to 10%–30%, indicating that the optimal modification rate would be 10% (ligands/carrier, w/w). Therefore, 10% was finally determined as the ligand to carrier ratio and the gene loading amount of the modified liposomes was 90%. There was no significant difference in loading capacity between the modified and unmodified liposomes, indicating that binding of a mannose-containing ligand onto the liposome surface did not release DNA from the complexes.

Concanavalin A was the first legume lectin recognized as being mannose-specific, and is believed to play a role in recognition of mannose-containing vectors. It has been used extensively for the evaluation of glycoconjugates. In our research, a concanavalin A agglutination study was used to identify further that the M-PEG-PE ligands were successfully modified onto the Lipo-pEGFP surface. M-PEG-PE-Lipo-pEGFP showed an apparent increase in turbidity (Figure 5), whereas the Lipofectamine 2000 and Lipo-pEGFP formulations appeared to show no significant increase in turbidity. This could be taken as evidence of the success of the mannose-containing ligands modified onto the liposome surface.

Macrophages play a major role in the immune response to foreign antigens, and targeting macrophages is one of the important therapeutic methods used to treat genetic metabolic diseases such as Gaucher’s disease and human immunodeficiency virus infection. Kupffer cells are liver-specific resident macrophages that play an integral part in physiological homeostasis of the liver. They have significant roles in acute and chronic responses of the liver to bacterial and viral infections, toxic or carcinogenic attack, as well as mediating hepatotoxicity. In this study, rat Kupffer cells were isolated and used as model cells for in vitro cytotoxicity evaluation and transfection analysis. The cell
viabilities of the liposomes over the studied concentration range were between 80% and 100% compared with controls (Figure 6). M-PEG-PE-Lipo-pEGFP showed no greater cytotoxicity than Lipo-pEGFP at all concentrations. When compared with naked DNA and Lipo-pEGFP, M-PEG-PE-Lipo-pEGFP had greater transfection efficiency at all time points (Figure 7). This may be explained by the sugar-lectin-mediated active targeting mechanism. The M-PEG-PE-modified pEGFP-loaded liposomes were more likely to bind to Kupffer cells via the mannose receptor on the cells and deliver DNA more easily into the cells to express EGFP.

The gene delivery ability of the modified gene vector could be tested further in animal models. In vivo gene delivery and expression studies were used in rats. After intravenous injection, the rats were euthanized and the Kupffer cells were isolated at 48 hours and analyzed by flow cytometry. M-PEG-PE-Lipo-pEGFP displayed remarkably higher transfection efficiency (43%) than unmodified Lipo-pEGFP (26%) and naked pEGFP (1.5%), as shown in Figure 8. This could

**Figure 7** Fluorescent images and flow cytometry analysis of Kupffer cells transfected with naked pEGFP, (A), Lipo/pEGFP (B), and M-PEG-PE-Lipo/pEGFP (C) at 24, 48, and 72 hours following transfection, respectively.

*Abbreviations:* M-PEG-PE, mannosylated polyethylene glycol-phosphatidylethanolamine; Lipo, Lipofectamine™; pEGFP, green fluorescence protein plasmid.
only be explained by the mannosylated PEG-PE-modified liposomes having the ability to target liver Kupffer cells and to deliver more genes into cells, displaying higher transfection efficiency. These observations strongly support the active targeting ability of M-PEG-PE-modified liposomes, and the resulting vectors would be very useful for in vivo gene delivery.

Conclusion
This study showed that mannose-mediated targeting can successfully transfer genes into cells expressing the mannose receptor. It could also demonstrate that having a targeting ligand, such as M-PEG-PE, in liposome formulations can significantly improve the transfection efficiency of a liposomal formulation. M-PEG-PE-Lipo-pEGFP had remarkably higher transfection efficiency both in vitro and in vivo than unmodified Lipo-pEGFP. It could be concluded that mannosylated PEG-PE could function as an excellent active targeting ligand, and M-PEG-PE-modified Lipofectamine 2000 could be a promising active targeting drug delivery vector.

Disclosure
The authors report no conflicts of interest in this work.

References


Supplementary in vivo gene delivery data

Details of the experiments and data analysis are as follows. Cells were washed in cold phosphate-buffered saline three times and the fluorescent intensity of the cells was determined using a flow cytometer equipped with a 488 nm argon laser for excitation. For each sample, about 10,000 events were collected and fluorescence was detected. Signals were amplified in the logarithmic mode for fluorescence to determine the positive events by a standard gating technique. The percentage of positive events was calculated as the events within the gate divided by the total number of events, excluding cell debris.

The pictures of flow cytometry analysis are shown in two ways, ie, as in Figure 8 and as a quadrantal rule diagram. The percentage of cells transfected with pEGFP appear at the upper right and lower right quadrants of the diagram.

The data in the quadrantal rule diagram for the in vivo study are as follows:

<table>
<thead>
<tr>
<th>Quad</th>
<th>X, mean</th>
<th>Y, mean</th>
<th>Events</th>
<th>% Total</th>
<th>% Gated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 8A: UR + LR = 1.75</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
1 UL | 8.8 | 10.1 | 3 | 0.02 | 69.09 |
2 UR | 146.4 | 29.5 | 26 | 0.21 | 1.10 |
3 LL | 2.8 | 2.3 | 121 | 96.34 | 29.25 |
4 LR | 73.4 | 4.7 | 44 | 0.35 | 0.65 |
| Figure 8B: UR + LR = 27.91 |
1 UL | 8.8 | 10.1 | 3 | 0.02 | 49.83 |
2 UR | 146.4 | 29.5 | 26 | 0.21 | 20.17 |
3 LL | 2.8 | 2.3 | 121 | 96.34 | 22.28 |
4 LR | 73.4 | 4.7 | 44 | 0.35 | 0.65 |
| Figure 8C: UR + LR = 44.20 |
1 UL | 8.8 | 10.1 | 3 | 0.02 | 38.35 |
2 UR | 146.4 | 29.5 | 26 | 0.21 | 31.64 |
3 LL | 2.8 | 2.3 | 121 | 96.34 | 17.43 |
4 LR | 73.4 | 4.7 | 44 | 0.35 | 12.56 |

Data on dose optimization are as follows (gene expression was analyzed at 48 hours after intravenous injection):

<table>
<thead>
<tr>
<th>Quad</th>
<th>X, mean</th>
<th>Y, mean</th>
<th>Events</th>
<th>% Total</th>
<th>% Gated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dose: 5 µg of pEGFP per rat gene expression: 2.59</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
1 UL | 8.8 | 10.1 | 3 | 0.02 | 66.01 |
2 UR | 146.4 | 29.5 | 26 | 0.21 | 2.24 |
3 LL | 2.8 | 2.3 | 121 | 96.34 | 31.40 |
4 LR | 73.4 | 4.7 | 44 | 0.35 | 0.35 |
| Dose: 10 µg of pEGFP per rat gene expression: 4.92 |
1 UL | 8.8 | 10.1 | 3 | 0.02 | 67.06 |
2 UR | 146.4 | 29.5 | 26 | 0.21 | 3.10 |
3 LL | 2.8 | 2.3 | 121 | 96.34 | 28.37 |
4 LR | 73.4 | 4.7 | 44 | 0.35 | 1.82 |
| Dose: 15 µg of pEGFP per rat gene expression: 16.96 |
1 UL | 8.8 | 10.1 | 3 | 0.02 | 59.12 |
2 UR | 146.4 | 29.5 | 26 | 0.21 | 10.31 |
3 LL | 2.8 | 2.3 | 121 | 96.34 | 23.08 |
4 LR | 73.4 | 4.7 | 44 | 0.35 | 0.65 |
| Dose: 20 µg of pEGFP per rat gene expression: 26.38 |
1 UL | 8.8 | 10.1 | 3 | 0.02 | 49.83 |
2 UR | 146.4 | 29.5 | 26 | 0.21 | 20.17 |
3 LL | 2.8 | 2.3 | 121 | 96.34 | 22.28 |
4 LR | 73.4 | 4.7 | 44 | 0.35 | 0.65 |
| Dose: 25 µg of pEGFP per rat gene expression: 32.96 |
1 UL | 8.8 | 10.1 | 3 | 0.02 | 46.92 |
2 UR | 146.4 | 29.5 | 26 | 0.21 | 23.07 |
3 LL | 2.8 | 2.3 | 121 | 96.34 | 21.10 |
4 LR | 73.4 | 4.7 | 44 | 0.35 | 9.98 |
| Dose: 30 µg of pEGFP per rat gene expression: 44.20 |
1 UL | 8.8 | 10.1 | 3 | 0.02 | 38.35 |
2 UR | 146.4 | 29.5 | 26 | 0.21 | 31.64 |
3 LL | 2.8 | 2.3 | 121 | 96.34 | 17.43 |
4 LR | 73.4 | 4.7 | 44 | 0.35 | 12.56 |
| Dose: 40 µg of pEGFP per rat gene expression: 39.91 |
1 UL | 8.8 | 10.1 | 3 | 0.02 | 39.83 |
2 UR | 146.4 | 29.5 | 26 | 0.21 | 30.17 |
3 LL | 2.8 | 2.3 | 121 | 96.34 | 20.28 |
4 LR | 73.4 | 4.7 | 44 | 0.35 | 9.74 |
| Dose: 50 µg of pEGFP per rat gene expression: 44.38 |
1 UL | 8.8 | 10.1 | 3 | 0.02 | 39.88 |
2 UR | 146.4 | 29.5 | 26 | 0.21 | 30.12 |
3 LL | 2.8 | 2.3 | 121 | 96.34 | 16.72 |
4 LR | 73.4 | 4.7 | 44 | 0.35 | 14.26 |
| Dose: 60 µg of pEGFP per rat gene expression: 40.91 |
1 UL | 8.8 | 10.1 | 3 | 0.02 | 40.15 |
2 UR | 146.4 | 29.5 | 26 | 0.21 | 32.33 |
3 LL | 2.8 | 2.3 | 121 | 96.34 | 19.14 |
4 LR | 73.4 | 4.7 | 44 | 0.35 | 8.58 |

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