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Research Article

Mannan-Modified PLGA Nanoparticles for Targeted Gene Delivery

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The studies of targeted gene delivery nanocarriers have gained increasing attention during the past decades. In this study, mannan modified DNA loaded bioadhesive PLGA nanoparticles (MAN-DNA-NPs) were investigated for targeted gene delivery to the Kupffer cells (KCs). Bioadhesive PLGA nanoparticles were prepared and subsequently bound with *pEGFP*. Following the coupling of the mannan-based PE-grafted ligands (MAN-PE) with the DNA-NPs, the MAN-DNA-NPs were delivered intravenously to rats. The transfection efficiency was determined from the isolated KCs and flow cytometry was applied for the quantitation of gene expression after 48 h post transfection. The size of the MAN-DNA-NPs was found to be around 190 nm and the Zeta potential was determined to be −15.46mV. The *pEGFP* binding capacity of MAN-DNA-NPs was (88.9 ± 5.8)% and the *in vitro* release profiles of the MAN-DNA-NPs follow the Higuchi model. When compared with non-modified DNA-NPs and Lipofectamine 2000-DNA, MAN-DNA-NPs produced the highest gene expressions, especially *in vivo*. The *in vivo* data from flow cytometry analysis showed that MAN-DNA-NPs displayed a remarkably higher transfection efficiency (39%) than non-modified DNA-NPs (25%) and Lipofectamine 2000-DNA (23%) in KCs. The results illustrate that MAN-DNA-NPs have the ability to target liver KCs and could function as promising active targeting drug delivery vectors.

1. Introduction

Biodegradable nanoparticles have been used frequently as gene delivery vehicles due to their extensive bioavailability, better encapsulation, high stability, and minimal toxicity [1]. They can be tailor made to achieve both controlled drug release and tumor targeting by tuning the polymer characteristics and shaping the surface through nanoengineering [2]. A number of different polymers, both synthetic and natural, have been utilized in formulating biodegradable nanoparticles [3–5]. One of the most extensively investigated polymers for nanoparticles is the biodegradable and biocompatible poly(D,L-lactide-co-glycolide) (PLGA), which has been approved by the FDA for certain human clinical uses [6].

Active targeting is a noninvasive approach to transport drugs and genes to target sites with the help of site-specific ligands [7]. Nanocarriers with active targeting properties could potentially be delivered to specific organs, tissues, cells, or even cellular organelles. Various kinds of targeting residues such as antibodies, peptides, and saccharides have been applied towards the modification of nanocarriers to achieve active targeting [8, 9]. Nanocarriers modified with saccharides, have seen advancement due to their high specificity, low toxicity, and low immunogenicity. Recently, bioadhesive PLGA nanoparticles were established as promising drug delivery systems [6, 10], and mannan-based PE-grafted ligands (MAN-PEs) were synthesized and applied for the surface modification of nanocarriers to achieve active targeting [11]. In this study, MAN-PE-modified bioadhesive PLGA nanoparticles were investigated as active targeting gene delivery system using plasmid enhanced green fluorescent protein (*pEGFP*) as the model gene.

In the present study, MAN-PEs were applied for the surface modification of PLGA nanoparticles to achieve active targeting to the liver. *In vitro* and *in vivo* behavior

of mannan-modified DNA-loaded PLGA nanoparticles was investigated in comparison with nonmodified DNA-loaded PLGA nanoparticles.

2. Materials and Methods

2.1. Materials. Poly(d,l-lactic-co-glycolic) (PLGA, 50 : 50, Av.MW 25,000) was obtained from Shandong Institute of Medical Instrument (China). Mannan and L-α-phosphatidylethanolamine (PE) were purchased from Sigma-Aldrich Co. (USA). *pEGFP*-N1 was provided by Shandong University (China). PicoGreen reagent and Lipofectamine 2000 were obtained from Invitrogen Corporation (USA). MTT (3-[4,5-dimehyl-2-thiazolyl]-2,5-diphenyl-2H-tetrazolium bromide) was purchased from Sigma-Aldrich (China). All other chemicals were of analytical grade or higher.

2.2. Animals. Adult male Sprague-Dawley rats (10-to-15-week old) were purchased from the Medical Animal Test 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).

2.3. Preparation of Bioadhesive PLGA Nanoparticles. Bioadhesive PLGA nanoparticles (NPs) were prepared following the methods described by Zou et al. [6, 10]. Carbopol 940 (CP) was dispersed in distilled water at room temperature and left overnight to swell. Neutralization of the CP dispersion was achieved by adding the required amount of 1 M NaOH until pH 7.0 was reached. The resulting CP stock solution was diluted with distilled water to afford a 0.02% (w/v) CP solution. Bioadhesive PLGA nanoparticles were prepared under optimized conditions by a nanoprecipitation method (solvent displacement technique) [12, 13]. 50 mg of PLGA polymer was accurately weighted and dissolved in 3 mL acetone. The organic phase was added dropwise into the 0.02% CP solution being stirred at 600 rpm and room temperature. When complete evaporation of the organic solvent had occurred, the redundant stabilizers and the nanoparticles were separated by ultracentrifugation at 1000 g, 4°C for 20 min. The pellet was resuspended in Milli-Q water, washed three times, and filtered through a 0.45 μm membrane. The resulting nanoparticle suspensions were stored at 4°C until use.

2.4. Fabrication of DNA-Loaded Nanoparticles. The reporter gene *pEGFP* was mixed with the PLGA nanoparticles by vortexing the nanoparticle suspension with a 1 mg/mL solution of DNA for 20 s. Incubation of the mixture for 30 min at RT facilitated the formation of the DNA-loaded PLGA nanoparticles (DNA-NPs).

2.5. Surface Modification of DNA-Nps with Mannan-PE. Mannan-PE was synthesized in accordance with the method described by Yu et al. [11]. Surface modification of DNA-NPs with mannan-PE ligands was accomplished according to the procedure developed by Vyas et al. [14]. Mannan-PE ligands were dissolved in 5 mL of phosphate buffered saline (PBS, pH 7.4). Then, the solution was added at dropwise into 10 mL of DNA-NPs that were stirred at 800 rpm by a laboratory magnetic stirrer at RT. The suspension was continually stirred at 600 rpm until the completion of modification. The excessive nonmodified ligands were removed by spinning the resultant suspension through a Sephadex G-50 column at 2,000 rpm for 5 min. The pellet was resuspended in Milli-Q water, washed three times, and filtered through a 0.80 μm membrane to obtain the desired mannan-modified DNA-loaded PLGA nanoparticles (MAN-DNA-NPs).

As a result of the modification process, the hydrophobic PE domains coated the surface of bioadhesive DNA-NPs, masking the inherent charge on the carriers. The total ligand-to-carrier weight ratio was optimized by measuring the change in Zeta potential. The optimum ratio was found to occur when increasing the ligand-to-carrier weight ratio resulted in no significant change in Zeta potential.

2.6. Characterization of MAN-DNA-NPs and DNA-NPs. The surface morphologies of both the MAN-DNA-NPs and the nonmodified DNA-NPs were examined by transmission electronic microscopy (TEM). The mean particle size and Zeta potential of the NPs were analyzed by photon correlation spectroscopy (PCS) and laser Doppler anemometry, respectively.

PicoGreen-fluorometry assay was carried out to measure the adsorption efficiency of the DNA loaded NPs [9, 15]. The *pEGFP* was isolated from the MAN-DNA-NPs and DNA-NPs by centrifugation at 1000 g, 4°C for 20 min. Analysis of the *pEGFP* containing supernatants by a fluorescence spectrophotometer afforded the concentration of plasmid DNA.

2.7. In Vitro DNA Release Studies. The MAN-DNA-NPs and DNA-NPs were placed in phosphate buffered solution (PBS, pH 7.4) to characterize the DNA release profile [16]. Typically, aliquots of complexes (equivalent to 1 μg DNA) were suspended in Eppendorf tubes containing 1 mL of PBS and vortexed. The tubes were continuously shaken in a 37°C water bath at 100 rpm. Separate tubes were used for each data point. At predetermined time intervals, the nanoparticle suspensions were centrifuged (1000 g, 4°C for 20 min) and the amount of DNA released in the supernatant was analyzed by the PicoGreen assay mentioned above. Background readings were obtained using the supernatants from the blank PLGA-NPs.

2.8. Isolation and Culture of Kupffer Cells. Kupffer cells (KCs) were isolated from SD rats under pentobarbitone anaesthesia [17–20]. The rats' portal vein were cannulated and perfused with HBSS for 10 min at RT. During this time, the liver was excised and the perfusate discarded. The liver was then perfused with 0.2% pronase (60 mL at RT) which was discarded. Then, the liver was perfused with a recirculating solution of 0.05% pronase and 0.05% collagenase (60 mL at RT) until the liver was digested as judged by the softening of the liver parenchyma beneath the capsule. The liver was then cut into

small pieces, suspended in 100 mL solution containing 0.02% pronase, 0.05% collagenase, and 0.005% DNase, and agitated at RT for 20 min. Following digestion, the liver homogenate was filtered through sterile gauze and centrifuged (1000 g, 4°C for 10 min). The supernatant was removed and the pellet resuspended in 10 mL Percoll gradient. Aliquots (5 mL) of this cell suspension were added to 5 mL aliquots of Percoll gradient. These were carefully overlaid with 5 mL HBSS and centrifuged at 1000 g, 4°C for 20 min. The nonparenchymal cell-enriched layer observed at the interface between the two layers was carefully harvested and diluted with 10 mL of HBSS. The suspension was then centrifuged (1000 g, 4°C for 20 min) to precipitate the KCs, which were then seeded into a 96-well microtiter plate at a density of 2×10^4 cells/well in 200 μL RPMI 1640 supplemented with 10% fetal bovine serum (FBS) and antibiotics. After incubation at 37°C for 2 h under 5% CO_2 atmosphere, the culture medium was replaced by 200 μL fresh RPMI 1640 to yield the purified KCs.

2.9. In Vitro Cytotoxicity Evaluation and Transfection Analysis. The *in vitro* cytotoxicity of MAN-NPs was evaluated by MTT assay [21] in the above cultured KCs. After incubation at 37°C for 24 h under 5% CO_2 atmosphere, the culture medium was replaced with 200 μL of fresh RPMI 1640 containing various concentrations of the MAN-NPs or Lipofectamine 2000 for comparison, and the cells were incubated for another 24 h. The cell viability was then assessed by MTT assay. 5 mg/mL of MTT in PBS was then added to each well, and the plate was incubated for an additional 4 h at 37°C under the aforementioned 5% CO_2 atmosphere. Then, the MTT containing medium was removed, and the crystals formed by living cells were dissolved in 100 μL DMSO. The absorbance at 570 nm was determined by a 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 to control cells was calculated using $(\text{Abs}_{\text{sample}}/\text{Abs}_{\text{control}}) \times 100$.

For transfection efficiency analysis, the KCs were seeded into 24-well plates at a density of 1×10^5 cells/well in 1 mL of RPMI-1640 with 10% FBS, 24 h prior to transfection. When the cells were at about 80% confluence, the media was replaced with 200 μL serum-free media containing MAN-DNA-NPs and DNA-NPs. Naked DNA was used as a negative control. Lipofectamine 2000 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 h under a 5% CO_2 atmosphere. The cells were incubated and were studied every 12 h until 48 h after transfection. The fluorescent cells were observed using an inversion fluorescence microscope, at which time pictures were taken for the record.

2.10. In Vivo Delivery and Transfection. Adult male Sprague-Dawley rats (10-to-15-week old) were divided into five groups (six rats in each group) and injected intravenously with 1 mL of both MAN-DNA-NPs and DNA-NPs. Identical intravenous doses of blank NPs, naked *pEGFP*, and Lipofectamine 2000-DNA served as controls. At predetermined time intervals, the rats' KCs were isolated under pentobarbitone anaesthesia as described in Section 2.8. The KCs were then seeded into 24-well plates in 1 mL of RPMI 1640 with 10% FBS. The fluorescent cells were observed using an inversion fluorescence microscope, and the pictures were captured.

For further quantitation of the transfection efficiency, flow cytometry was used. Cells were washed with 1 mL of PBS (100 g, 4°C for 5 min) and were detached with trypsin/EDTA. The supernatant was discarded and resuspended with 300 μL of PBS, mixed well, and added into the flow cytometer to determine the amount of KCs which has been successfully transfected.

2.11. Statistical Analysis. All studies were repeated a minimum of three times and all measurements were carried out in triplicate. Results were reported as means ± SD (SD = standard deviation). Statistical significance was analyzed using the Student's t-test. Differences between experimental groups were considered significant when the P value was less than 0.05 ($P < 0.05$).

Table 1: Particle size and Zeta potential of NPs, DNA-NPs, and MAN-DNA-NPs (mean ± SD, $n = 3$).

Sample	Mean particle size (nm)	Polydispersity index (PDI)	Zeta potential (mV)
NPs	116.7 ± 3.2	0.19 ± 0.05	−29.18 ± 3.54
DNA-NPs	145.3 ± 8.7	0.23 ± 0.08	−36.58 ± 1.32
MAN-DNA-NPs	189.7 ± 5.3	0.15 ± 0.07	−15.46 ± 1.09

3. Results

3.1. Characterization of DNA Loaded NPs. The transmission electron micrograph (TEM) pictures of the MAN-DNA-NPs and DNA-NPs were shown in Figure 1. DNA-NPs had spherical shapes (Figure 1(a)) while the MAN-DNA-NPs had dark coats on the white particles (Figure 1(b)). Mean particle size, polydispersity index (PDI), Zeta potential of blank PLGA nanoparticles (NPs), DNA-NPs, and MAN-DNA-NPs were characterized and summarized in Table 1.

To optimize the mannan-PE to DNA-NPs weight ratio, the change in Zeta potential was measured and the optimum ratio was obtained when no significant change in Zeta potential was observed after increasing the mannan-PE percent. The optimum ratio was obtained at 1/2 (mannan-PE/DNA-NPs, w/w) (Figure 2).

3.2. Gene Loading Capacity and In Vitro Release Studies. PicoGreen fluorometry assay was carried out to quantitate the loading efficiencies of the gene delivery systems. DNA binding quantity (%) = (total amount of DNA − the amount of free DNA)/total amount of DNA × 100. The *pEGFP* binding capacities of MAN-DNA-NPs and DNA-NPs were (88.9 ± 5.8)% and (89.5 ± 4.5)%, respectively.

The *in vitro* release profiles of modified and nonmodified DNA-NPs are illustrated in Figure 3 and the release behavior of the MAN-DNA-NPs and DNA-NPs follow the Higuchi model best.

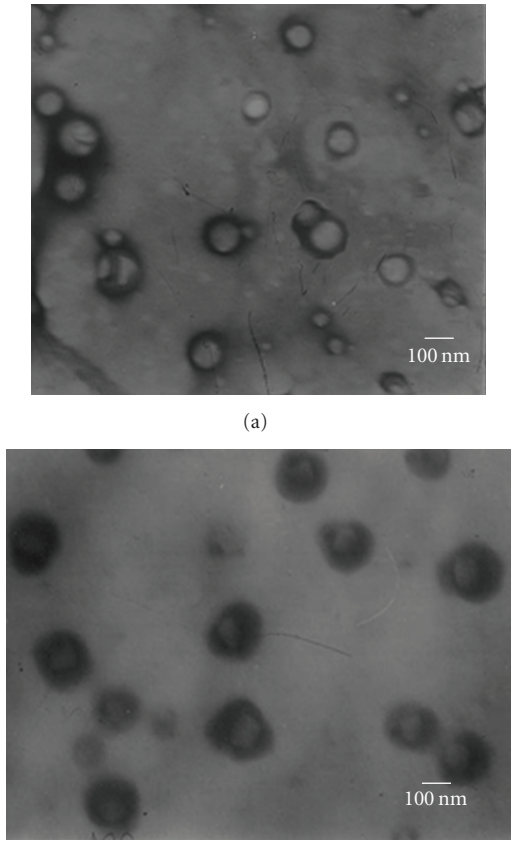


Figure 1: TEM imaging of DNA-NPs (a) and MAN-DNA-NPs (b).

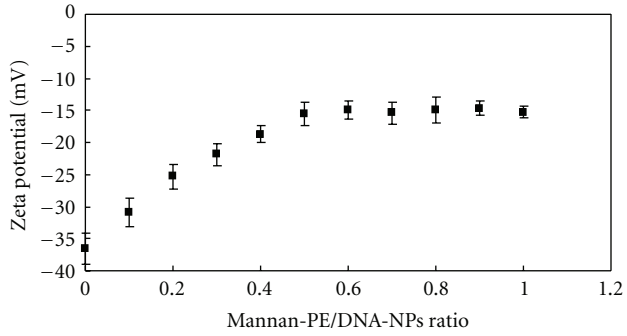


Figure 2: Optimization of mannan-modified DNA-NPs: MAN-PEs to DNA-NPs weight ratio (w/w).

3.3. In Vitro Cytotoxicity and Transfection. The isolated Kupffer cells were defined on the basis of the presence of phagocytosis of latex beads and or positive immunostaining with antibody to the epitope ED2. With the above-mentioned method, about 81. 52×10^6/rat liver Kupffer cells were obtained, purity was around 92% (to 88.69×10^6/rat liver NPC), and more than 95% cells were alive. The isolated and purified rat Kupffer cells retained their *in vivo* morphological, biological and immunological characteristics.

In vitro toxicity of modified and nonmodified NPs was evaluated by MTT assay in rat KCs. The cytotoxicity of

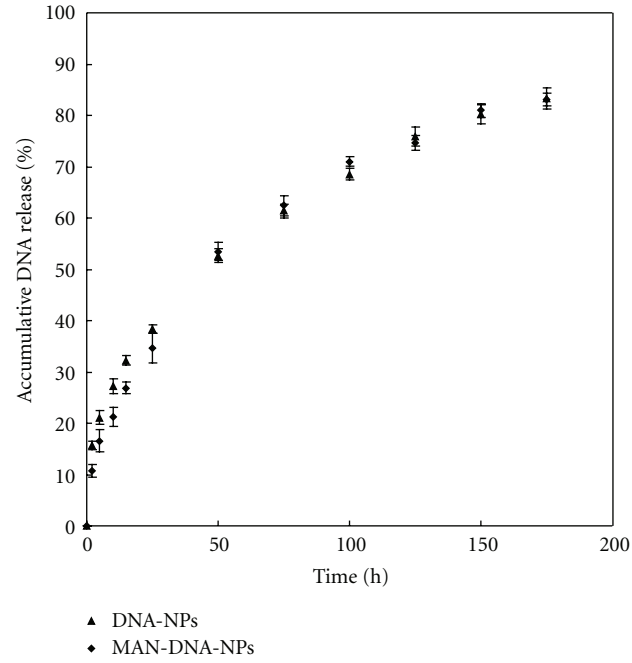


Figure 3: *In vitro* release profile of MAN-DNA-NPs and DNA-NPs (mean ± SD, $n = 3$).

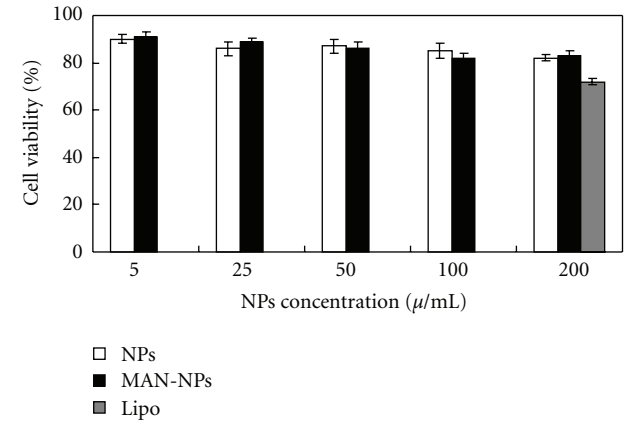
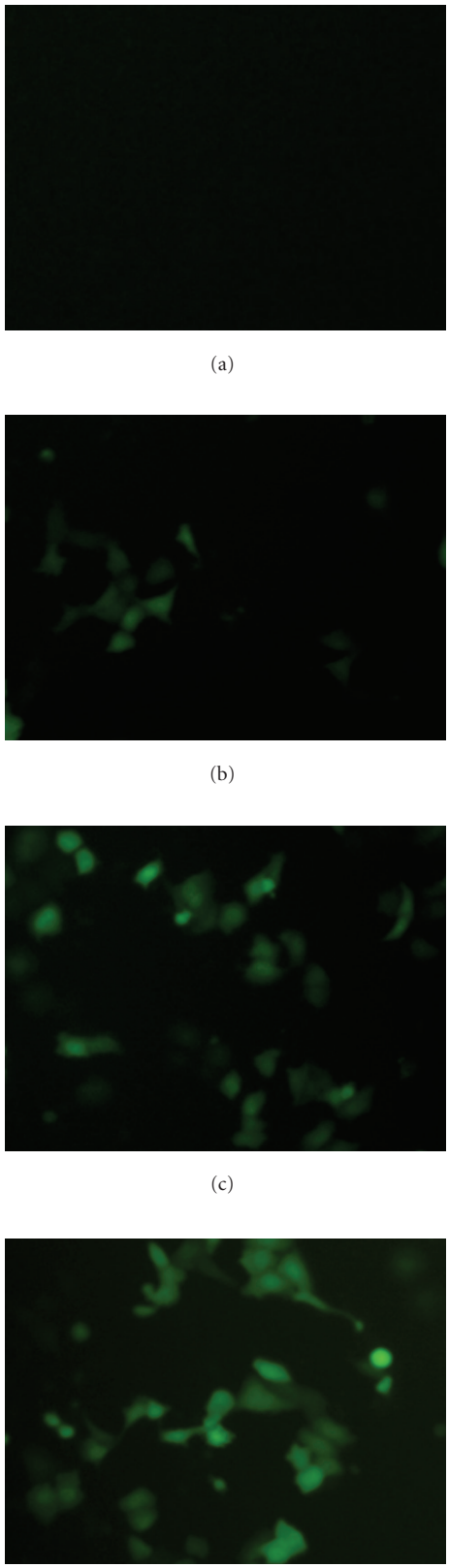


Figure 4: Cell viabilities of MAN-DNA-NPs and DNA-NPs (mean ± SD, $n = 3$).

MAN-NPs and NPs at various concentrations (5, 25, 50, 100, and 200 μg/mL) was evaluated. Lipofectamine 2000 at the concentration of transfection was used as comparison. The cell viabilities in the presence of NPs and MAN-NPs over the studied concentration range (5–200 μg/mL) were between 80 and 120% compared with controls (Figure 4). MAN-NPs and NPs exhibited a lower cytotoxicity than Lipofectamine 2000 at all concentrations ($P < 0.05$).

The *in vitro* transfection efficiencies of MAN-DNA-NPs and DNA-NPs in KCs after 48 h of transfection are shown in Figure 5. When compared with naked DNA, DNA-NPs, and Lipofectamine-DNA, MAN-DNA-NPs had higher transfection efficiency.



(a)

(b)

(c)

(d)

Figure 5: Fluorescent micrographs of KCs transfected by plasmid EGFP with DNA-NPs (c) and MAN-DNA-NPs (d). The KCs transfected by naked DNA (a), and Lipofectamine-DNA (b) was used as control.

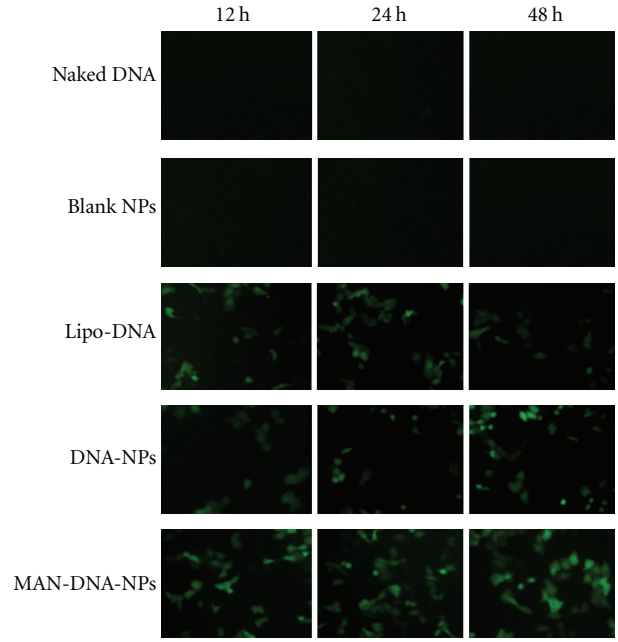


Figure 6: Fluorescent micrographs of KCs transfected by plasmid EGFP *in vivo*. Gene expression was examined after 12, 24 and 48 h post injection.

3.4. In Vivo Gene Delivery in Rats. After intravenous injection of naked DNA, blank NPs, Lipofectamine 2000-DNA, DNA-NPs, and MAN-DNA-NPs, the KCs were isolated at 12 h, 24 h, and 48 h. The fluorescent cells were observed using an inverted fluorescence microscope (Figure 6). The pictures apparently showed that MAN-DNA-NPs had better transfection efficiency in rats compared with nonmodified DNA-NPs and Lipofectamine 2000-DNA. Flow cytometry was applied for the further quantitation of gene expression after 48 h following transfection. As shown in Figure 7, the percentage of KCs transfected with *pEGFP* appeared at the UR and LR quadrants, MAN-DNA-NPs displayed a remarkably higher transfection efficiency than nonmodified DNA-NPs ($P < 0.05$) and Lipofectamine 2000-DNA ($P < 0.05$) in KCs.

4. Discussion

In the present study, novel mannan-modified bioadhesive PLGA nanocarriers were constructed as active targeting gene delivery system. As illustrated in Figure 1 and Table 1, nonmodified DNA-NPs had spherical shapes while the mannan-modified MAN-DNA-NPs had a dark coat on the white balls, which may be employed to identify the successful coating of mannan-PE. The mean particle size of NPs was around 100–200 nm, which is ideal for the nanoparticulate system.

During the modification procedure, mannan containing ligands were extensively coated onto the DNA-NPs' surface, which covered their original charge and caused the change in Zeta potential. The optimization of ligand-to-carrier ratio was carried out by measure the Zeta potential. The optimum ratio was obtained at 1/2 (mannan-PE/DNA-NPs, w/w)

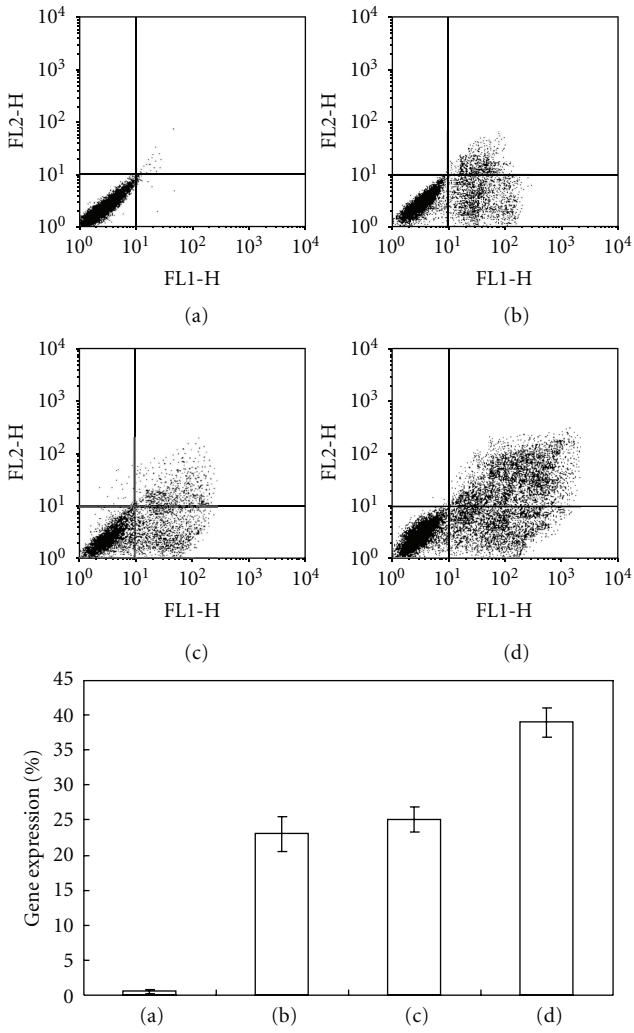


Figure 7: Flow cytometry analysis of KCs transfected by plasmid EGFP *in vivo*. Gene expression was examined after 48 h following injection. (a) Naked DNA, (b) Lipofectamine-DNA, (c) DNA-NPs, and (d) MAN-DNA-NPs (mean ± SD, $n = 3$).

(Figure 2) and the optimum MAN-DNA-NPs had a Zeta potential of −15.46 mV (Table 1).

PicoGreen fluorometry method was applied to quantitate the loading capacity and *in vitro* release behavior of MAN-DNA-NPs. The binding efficiency of MAN-DNA-NPs and DNA-NPs was 88.9% and 89.5%, which had no significant difference. The results demonstrated that binding of mannan containing ligand onto the NPs surface did not detach the DNA from the complexes. The *in vitro* release profiles (Figure 3) of MAN-DNA-NPs and DNA-NPs were nearly identical. These findings support the notion that the coating of mannan did not hinder the release of DNA. The release profiles of the MAN-DNA-NPs and DNA-NPs both follow the Higuchi model, MAN-DNA-NPs: $Q\% = 0.0654t^{1/2} + 0.0206$, $r = 0.9952$; DNA-NPs: $Q\% = 0.063t^{1/2} + 0.05848$, $r = 0.9958$.

Macrophages play a major role in the immune response to foreign antigens, targeting macrophages is one of the important therapeutic ways to treat genetic metabolic diseases such as Gaucher's disease and human immunodeficiency virus (HIV) infection [22]. KCs are liver-specific resident macrophages that play an integral part in the physiological homeostasis of the liver. Kuppfer cells 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 [23]. In this study, rat KCs were isolated and used as model cells for *in vitro* toxicity and transfection evaluation. For cell viability study, both MAN-NPs and blank NPs showed lower cytotoxicity compared with Lipofectamine 2000 ($P < 0.05$, Figure 4), most probably due to the better biodegradability and bioavailability. The *in vitro* transfection of MAN-DNA-NPs and DNA-NPs was carried on KCs (Figure 6). After transfection for 48 h, MAN-DNA-NPs gained better transfection results than DNA-NPs, which may be explained by the sugar-lectin-mediated active targeting mechanism. The modified *pEGFP* loaded NPs were bound to the KCs and delivered the DNA into the cells to express EGFP.

The *in vivo* gene delivery and expression studies were applied in rats. After intravenous injection, the KCs were isolated, cultured, and analyzed with fluorescence microscope and flow cytometer. As shown in Figure 6, MAN-DNA-NPs achieved the best transfection efficacy in every data point. For further quantitation of the fluorescent KCs, flow cytometry was applied and the KCs with green fluorescence appeared at the UR and LR quadrants (Figure 7). The modified MAN-DNA-NPs showed higher transfection efficiency in rats compared with nonmodified DNA-NPs and Lipofectamine 2000-DNA ($P < 0.05$) which demonstrates that the mannan modified PLGA NPs had the ability to target liver KCs and could function as promising active targeting drug delivery vectors.

5. Conclusions

In conclusion, MAN-PEs-modified *pEGFP*-loaded bioadhesive PLGA-NPs could be targeted delivered to the liver and successfully transfected the KCs. This may be explained by the sugar-lectin-mediated active targeting mechanism. The results indicate that this kind of modification could be generalized to other kinds of nanocarriers and construct many kinds of active targeting drug delivery systems.

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