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RESEARCH PAPER

Mannan-Modified Solid Lipid Nanoparticles for Targeted Gene Delivery to Alveolar Macrophages

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ABSTRACT

Purpose Cationic solid lipid nanoparticles (SLN) have established themselves during the past decades. They can efficiently bind DNA directly via ionic interaction and mediate gene transfection. One major problem with SLN is the lack of cell-targeting ability. In the present study, a mannan-based PE-grafted ligand was synthesized and used for the surface modification of DNA-loaded cationic SLN to prepare Man-SLN-DNA.

Methods For *in vitro* test, the cytotoxicity and transfection investigation was carried out on murine macrophage cell line RAW 264.7. For *in vivo* evaluation, Man-SLN-DNA was delivered into the lung of the rats, and the alveolar macrophages (AM) were isolated for the fluorescence determination of transfection efficiency.

Results When compared with non-modified SLN-DNA and Lipofectamine 2000-DNA, Man-SLN-DNA produced the highest gene expressions, especially *in vivo*.

Conclusion These results demonstrated the active targeting ability of this kind of mannan-modified DNA-loaded vehicles, which may have great potential for targeted gene delivery.

KEY WORDS active targeting · cationic solid lipid nanoparticles · gene delivery · mannan-based PE-grafted ligand · surface modification

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INTRODUCTION

Lung cancer is one of the most aggressive solid cancers, causing the highest morbidity and mortality among all cancers in many parts of the world (1,2). Other pulmonary conditions, including inflammation and infectious diseases, are also critical health problems (3). Gene therapy is an exciting prospect for treatment of lung diseases because genes can be transduced into specific cells to correct genetic lesions or inactivate oncogenes (4,5). Successful gene therapy depends on the development of efficient delivery systems, which calls for the development of safe and effective gene vehicles (6). Compared to viral vectors, non-viral vectors are potentially less immunogenic, relatively easy to produce in clinically relevant quantities, and associated with fewer safety concerns. Moreover, non-viral vectors provide flexibility in formulation design and can be tailored to the size and topology of the DNA cargo and the specific route of vector administration (7).

With the incredible developmental rate in chemical and biological engineering of polymeric materials and nanoparticles over the past couple decades, a wide range of non-viral vectors have been developed for use in drug and gene delivery (8–10). Our group has made some efforts in several vector systems, including cationic solid lipid nanoparticles (SLN) (11), anionic SLN (12), and polymeric nanoparticles (13), which achieved relatively high loading capacity and low toxicity. Among these carriers, we mainly focus on SLN, applying new structures (12) and novel modifiers (11) to reduce the cytotoxicity and improve the transfection efficiency. However, there remains a critical gap in the development of facile and effective targeting techniques for SLN.

Surface modification of nanocarriers with specific ligands can assist in targeting and internalization of the nanocarriers to specific cell populations, such as cancers and disease organs (14–17). It is reported that alveolar macrophages (AM) are key effector cells in the first-line host defense and lung homeostasis (18), which make macrophages important targets for gene therapy (19). Macrophages are also a key component of cancer-promoting inflammatory reactions (20). Tumor-associated macrophages (TAM) represent the major inflammatory component of the stroma of many tumors and can affect different aspects of the neoplastic tissue (21). Thus, therapeutic targeting of macrophages-derived mediators may provide innovative therapeutic strategies against tumor invasion and metastasis. It has been shown that human and murine macrophages express mannose receptor (MR) on their surface (22), and several studies have confirmed the feasibility of using mannose- or mannan-modified nanocarriers to target macrophages (23–26).

Pulmonary delivery of pharmacologically effective drugs has been employed for years, since it was discovered that the large and highly absorptive surface area of the lungs might be utilized as an effective target for the treatment of local or systemic diseases (27). Pulmonary delivery offers a number of advantages, such as providing an enormous absorptive area, thin alveolar epithelium membrane, extensive vasculature, low extracellular and intracellular enzyme activities, and little first-pass metabolism, thereby permitting rapid absorption and onset of action.

In our previous study, a novel single-tailed cationic lipid, named LHLN, was synthesized and used as a modifier to prepare cationic SLN for gene delivery (11). This kind of nanocarrier achieved relatively lower toxicity and higher transfection capability than their CTAB counterparts. Thus, we decided to apply surface modification of this nanocarrier to achieve active targeting. Since mannose residues have the ability to target AM, and polysaccharides have higher content of sugar residues compared with monosaccharides and exhibit more efficient cellular recognition and internalization, mannan was employed as a candidate (28). It is also reported that the saccharide derivatives with lipids can easily be incorporated into the nanocarrier surface (29); hence, phosphatidylethanolamine (PE) was selected as the lipophilic anchor.

In this study, mannan-based PE-grafted ligands (Mannan-PE) were synthesized and applied for the surface modification of SLN to prepare mannan-modified SLN-DNA (Man-SLN-DNA). The active targeting ability of the Man-SLN-DNA was evaluated by transfer into murine macrophage cell line (RAW) and by pulmonary delivery to rats. *In vivo* and *in vitro* studies showed enhanced uptake of Man-SLN-DNA in contrast with non-modified SLN-DNA. The results indicated that Man-SLN-DNA could have the ability to actively target AM and may have enormous potential for gene delivery to the lung.

MATERIALS AND METHODS

Materials

Mannan, L-α-phosphatidylethanolamine (PE) and concanavalin A (Con A) were purchased from Sigma-Aldrich Co. (USA). Glyceryl monostearate was provided by Shanghai Chemical Reagent Co. Ltd. (China). Injectionable soya lecithin was obtained from Shanghai Pujiang Phospholipids Co. Ltd. (China). pEGFP-N1 was provided by Zhejiang University (China). PicoGreen reagent was obtained from Invitrogen Corporation (USA). MTT (3-[4,5-dimehyl-2-thiazolyl]-2,5-diphenyl-2H-tetrazolium bromide) were purchased from Sigma-Aldrich (China). Lipofectamine 2000 was purchased from Invitrogen (USA). RAW 264.7 cells were obtained from the American type culture collection (USA). Mouse anti-rat CD11c:Alexa Fluor® 647 was purchased from AbD Serotec (UK). All other chemicals were of analytical grade or higher.

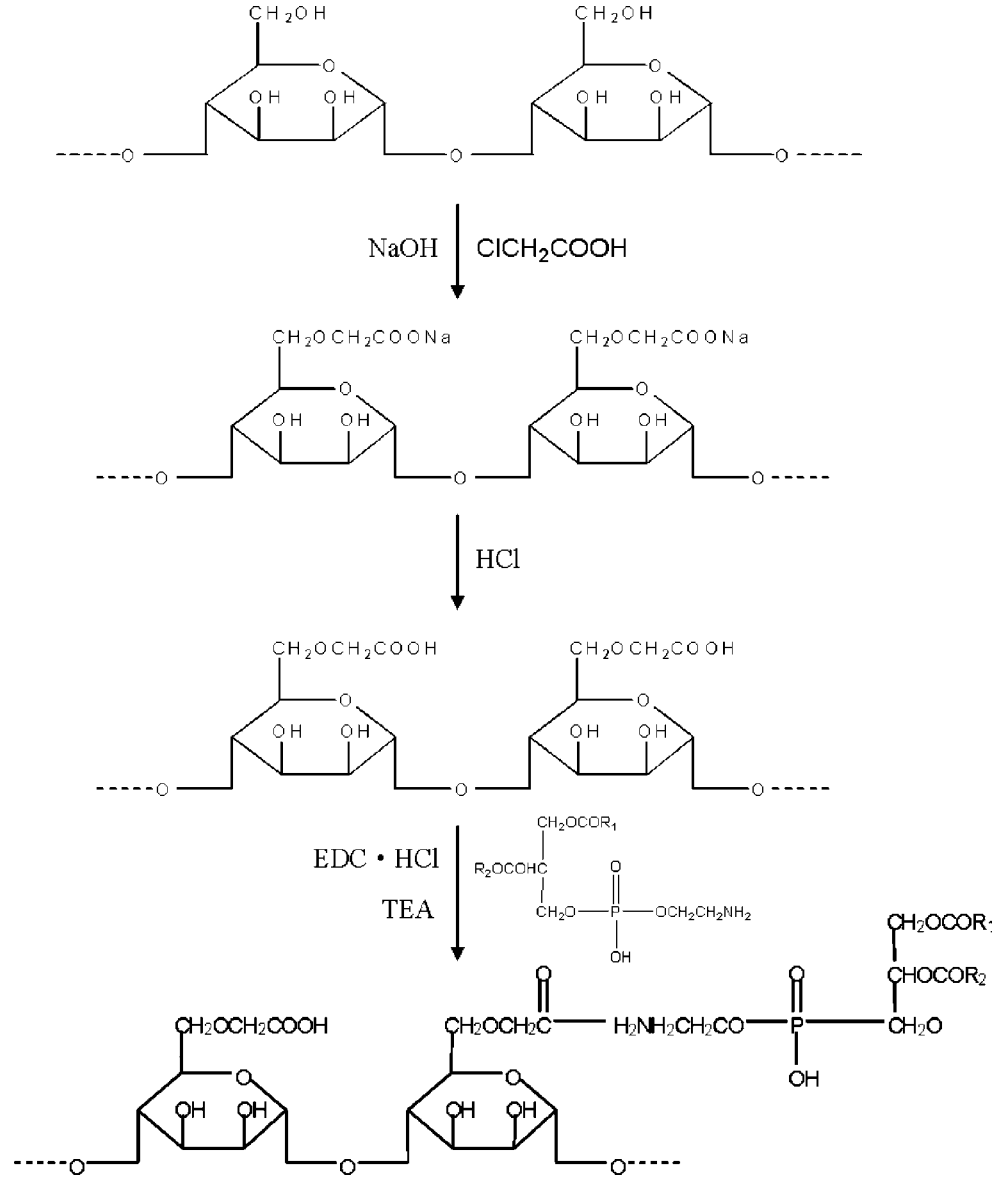
Animals

Ten-week-old male wistar rats weighing 250–300 g (purchased from the Medical Animal Test Center of the New Drugs Evaluation Center, Shandong University) were 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 Mannan-Based PE-Grafted Ligands

Mannan-based PE-grafted ligand (Mannan-PE) was synthesized (Scheme 1) in accordance with the method described by Yang *et al.* (30). Mannan (100 mg) was dissolved with sodium hydroxide (3 M, 1 mL) and stirred for 10 min for alkalinization, then chloroacetic acid (16 g/100 mL, 1.2 mL) was added into the solution and stirred in an oil bath (55°C) for 7 h. After that, hydrochloric acid (1 M) was added until pH 2–3 to produce carboxymethylated mannan. Carboxymethylated mannan was dissolved with dimethyl sulfoxide (DMSO) and stirred with PE (6 mg, in DMSO). To the mixture, 1-[3-(dimethylamino) propyl]-3-ethylcarbodiimide (EDC·HCl) (12 mg, 1 equivalent of triethylamine, in DMSO) was added dropwise in an ice bath, then the mixture was stirred at room temperature (RT) for 2 days. DMSO was moved by rotary evaporation, and the product was dissolved with Milli-Q water and then dialyzed against Milli-Q water for 3 days.

Scheme 1 Synthesis of mannan-based PE-grafted ligands.



The Mannan-PE was characterized using IR and ^{1}H NMR spectroscopy.

Preparation of SLN and SLN-DNA Complexes

SLN formation was performed under optimal conditions by solvent displacement technique (nanoprecipitation method) using a cationic lipid LHLN as surfactant as described in our previous study (11). Glyceryl monostearate (25 mg) and 15 mg of soya lecithin were dissolved in 3 mL of acetone to form the diffusing phase. This phase was added at 12 mL/h using a micro-injection pump (Kd Scientific, Fabriqe'auxetats-unis, USA) into an aqueous solution containing LHLN (0.1%, *w*/*v*). The solution was then stirred at 400 rpm with a magnetic stirrer (ETS-D4 stirrer, IKA, Germany) at RT until complete evaporation of the organic solvent. The remaining surfactant was removed from the SLN by centrifugation at 15,000 rpm and 4°C for 30 min (Cetra-MP4 centrifuge, International Equipment Company, Miami, USA). The sediment was redissolved in Milli-Q water, washed three times, syringe filtered (0.45 μm pore size), and adjusted to pH 7.2–7.4 with NaOH using a pH meter (FE20 pH meter, Mettler-Toledo Instruments Co. Ltd., Switzerland). The obtained SLN suspensions were stored at 4°C.

The reporter gene, encoding-enhanced green fluorescence protein (pEGFP) was added to the SLN suspensions at the ratio of 8:1 (SLN/DNA, *w*/*w*) under gentle vortexing for 20 s and incubated for 30 min at RT to facilitate formation of the SLN-DNA complexes.

Characterization of SLN and SLN-DNA Complexes

The surface morphology of the SLN and SLN-DNA complexes was examined by transmission electronic microscopy (TEM) (JEM-1200EX, Japan). Samples were prepared by placing a drop of SLN or SLN-DNA suspensions onto a copper grid and air-drying, followed by negative staining with one drop of a 3% aqueous solution of sodium phosphotungstate. The air-dried samples were then directly examined under the TEM.

The mean particle size and zeta potential of SLN and SLN-DNA suspensions were analyzed by photon correlation spectroscopy (PCS) and laser Doppler anemometry, respectively, using a particle sizer (Zetasizer 3000 HAS, Malvern Instruments, Malvern, UK). All measurements were carried out in triplicate. The average particle size was expressed in volume mean diameter, and the reported value was represented as mean ± SD ($n=3$).

Surface Modification of SLN-DNA Complexes with Mannan-PE

Surface modification of SLN-DNA complexes with Mannan-PE ligands was accomplished according to the procedure developed by Jain and Vyas (31). Briefly, Mannan-PE ligands were dissolved in 1 mL of phosphate-buffered saline (PBS, pH 7.4). Then the solution was added at 18 mL/h using micro-injection pump into 2 mL of SLN-DNA complexes that was stirred at 800 rpm by a laboratory magnetic stirrer (ETS-D4 stirrer, IKA, Germany) at RT. The suspension was continually stirred at 400 rpm until the completion of modification. The excessive non-modified 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 membrane with 0.80 μm pore size to obtain Man-SLN-DNA. During the modification process, the hydrophobic PE domains absorbed onto the positively charged SLN-DNA, thereby reducing the inherent charge on the carriers. Two process variables (total ligand-to-carrier weight ratio and incubation time) were optimized by measuring the change in zeta potential. For optimization of total ligand-to-carrier weight ratio, formulations with different ratios were prepared and incubated for a fixed time period of 12 h. The optimum ratio was determined at which no significant change in zeta potential was recorded on further increasing the ligand-to-carrier weight ratio. Similarly, for optimization of incubation time the formulations with optimum ligand-to-carrier weight ratio were prepared and incubated for different time intervals and their zeta potential measured. After completion of the modification, no significant change in zeta potential was recorded.

Man-SLN complexes were prepared following the same procedure using the blank SLN. They were used for the background reading of Man-SLN-DNA and utilized in the cell viability studies.

Characterization of Man-SLN-DNA Complexes

The physicochemical properties of Man-SLN-DNA were characterized following the methods described in "Characterization of SLN and SLN-DNA Complexes" section.

In Vitro Concanavalin A (Con A) Agglutination Assay

The Con A agglutination assay was performed as described by Cui *et al.* (24,32). Briefly, 200 mL of Man-SLN-DNA complexes was added into 1 mL of Con A (1 mg/mL) in PBS (pH 7.4) with 5 mM of calcium chloride ($CaCl_2$) and 5 mM of magnesium chloride ($MgCl_2$) (RT). The increase in turbidity at 360 nm (OD_{360}) was monitored (UV-2100 UV/Vis Spectrophotometer, UNICO, Spain) at RT for 300 s.

DNA Binding Capacities of Man-SLN-DNA Complexes: PicoGreen Assay

The DNA binding efficiency of the complexes was determined by the PicoGreen-fluorometry method as previously described (11). Briefly, free DNA was isolated from the Man-SLN-DNA and SLN-DNA complexes by centrifugation at 15,000 rpm and 4°C for 30 min, the supernatants were collected, and the concentration of plasmid DNA was assessed by a fluorescence spectrophotometer (ex/em 480/520 nm) (HITACHI F2500, Japan). The amount of DNA loaded in the Man-SLN-DNA was calculated according to the linear calibration curve of DNA (5 to 2,000 ng/mL, $R^2=0.9991$).

In Vitro Release of DNA from Man-SLN-DNA Complexes

The *in vitro* release studies of Man-SLN-DNA and SLN-DNA were performed in PBS (pH 7.4) (11). Typically, aliquots of complexes (equivalent to 2 μg DNA) were suspended in Eppendorf® tubes containing 1 mL of PBS and vortexed. Separate tubes were used for each data point. The tubes were then placed in a shaking water bath (37°C, 100 rpm). At predetermined time intervals, the suspensions were centrifuged (15,000 rpm, 30 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 Man-SLN.

Cell Viability Studies

The cytotoxicity of Man-SLN was evaluated by MTT assay (13) in RAW 264.7 cells. RAW 264.7 cells were seeded into a 96-well microtiter plate at a density of 1×10^4 cells/well in 200 μL of DMEM supplemented with 10% fetal bovine serum (FBS) and antibiotics. After incubation in a 5% CO_2 incubator at 37°C for 24 h, the culture medium was replaced with 200 μL of fresh DMEM containing various concentrations of the Man-SLN complexes or Lipofectamine 2000 for comparison, and the cells were incubated for another 24 h. The cell viability was then assessed by MTT assay. Briefly, 5 mg mL^{-1} of MTT in PBS was added to each well, and the plate was incubated for an additional 4 h at 37°C in a 5% CO_2 incubator. 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 (Model 680, BIO-RAD, USA). 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 $(Abs_{sample}/Abs_{control}) \times 100$.

In Vitro Transfection Efficiency Analysis

The transfection efficacies of Man-SLN-DNA and unmodified SLN-DNA complexes were evaluated in the RAW 264.7 cells. The RAW 264.7 cells were seeded into 24-well plates at a density of 1×10^5 cells/well in 1 mL of DMEM with 10% FBS, 24 h prior to transfection. When the cells were at about 80% confluence, the media were replaced with 200 μL of serum-free media containing Man-SLN-DNA and SLN-DNA complexes at 37°C. Naked DNA was used as a negative control. Lipofectamine 2000 was used as a positive control, and the formulation of Lipofectamine/DNA complexes was carried out according to the manufacturer's protocol. The original incubation medium was replaced with 1 mL of complete medium after incubation for 4 h at 37°C in a 5% CO_2 incubator, and then cells were incubated sequentially until 48 h post transfection. The fluorescent cells were observed using an inverted fluorescence microscope (OLYMPUS, ZX71, Japan), and the picture was captured.

In Vivo Uptake Study After Intratracheal Instillation

Wistar rats were divided into 4 groups, 6 rats in each group. Man-SLN-DNA and SLN-DNA complexes were intratracheally instilled into the rat lungs, respectively (300 μl per rat, containing 6 μg of DNA, 48 μg of SLN). Naked DNA was used as a negative control, and Lipofectamine 2000-DNA was used as a positive control, both of which were instilled into the rat lungs the same dosage as Man-SLN-DNA. Blank SLN was also applied as control to exclude fluorescence background coming from the SLN itself.

At predetermined time intervals, rats were euthanized and the thoraces were opened. The tracheal intubation was applied to the rats, and then 5 mL of cold normal saline (4°C) was used to lavage the lungs with gentle massage. This procedure was repeated 10 times, and the AM were preliminary isolated. The AM were obtained after gradient centrifugation (4°C 4,000 rpm 10 min, 1,000 rpm 5 min, 800 rpm 5 min) and washed three times with cold PBS (4°C). The AM were seeded into 24-well plates in 1 mL of DMEM with 10% FBS. The fluorescent cells were observed using an inversion fluorescence microscope, and the pictures were captured.

For identification of AM and quantitation of green fluorescent cells, flow cytometry was carried out. After the fluorescent pictures were captured, cells were washed once with 1 mL of PBS and were detached with trypsin/EDTA. Then, the cells were centrifuged at 1,500 rpm, 4°C for 5 min, the supernatant discarded, cells washed once with 1 mL of PBS (1,500 rpm, 4°C for 5 min), the supernatant discarded, and resuspended with 270 μl of PBS. Then 30 μl of mouse anti-rat CD11c:Alexa Fluor® 647 working dilution was added into the cells to the total volume of 300 μl, mixed well and incubated at room temperature for 30 min. Cells were washed with 1 mL of PBS (1,500 rpm, 4°C for 5 min), the supernatant discarded, and resuspended with 300 μl of PBS and directly introduced to flow cytometer. All the experiments were carried out in triplicates.

Statistical Analysis

All studies were repeated a minimum of three times and measured at least 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 ($P<0.05$).

RESULTS

Synthesis of Mannan-Based PE Grafted Ligands

The chemical structure was confirmed by IR and ^1H NMR spectroscopy. IR v/cm^{-1}: 3,441(–OH, –NH–); 2,927 (–CH_2–, –CH–); 1,651(–HN–CO–, –NH–); 1,419 (–CH_2CO–); 1,090(–C–O–C–, secondary alcohol structure in mannan). The main absorption peaks were in accordance with the IR spectroscopy of mannan and PE, which

could initially identify the product as Mannan-PE. ^{1}H NMR spectroscopy was applied in DMSO-D6 and D_2O, respectively. ^{1}H NMR:δ(3.0~6.0), the similar peaks were observed as the spectra of mannan;δ(0~3.0); the peaks were in concord with PE. When δ was about 5.2, the peak of Mannan-PE in DMSO-D6 was obviously larger than that in D_2O; this could confirm the existence of amide linkage. According to the percentage of hydrogen atom located in mannan and PE (and δ below or above 3), calculation could be made that about one of every 150 mannose monosaccharide units linked one PE molecule (slightly various from batches).

Physicochemical Characterization of SLN, SLN-DNA and Man-SLN-DNA

The results of physicochemical characterization of SLN, SLN-DNA and Man-SLN-DNA were summarized in Table I. The TEM pictures (Fig. 1) showed that they all had spheroidal shapes. Mean particle sizes of SLN, SLN-DNA and Man-SLN-DNA were 53.7, 89.3, and 125.7 nm, respectively. Zeta potential of SLN, SLN-DNA and Man-SLN-DNA decreased progressively at the same condensation, 39.48, 18.75, and 4.37 mV, respectively.

Optimization of Mannan-SLN-DNA Complexes and Con A Agglutination Analysis

During the modification procedure, mannan was coated onto the cationic SLN-DNA surface. This process causes the reduction of zeta potential. Therefore, two variables (total ligand-to-carrier weight ratio and incubation time) were optimized by measuring the change in zeta potential. For optimization of total ligand-to-carrier weight ratio, formulations with different ratios were prepared and incubated for a fixed time period of 12 h. The optimum ratio was obtained at 2/5 (Mannan-PE/SLN-DNA, w/w) (Fig. 2A). Similarly, for optimization of incubation time, the formulations with optimum ligand-to-carrier weight ratio (2/5) were prepared and incubated for different time intervals, and their zeta potential measured. The optimal time was 4 h (Fig. 2B).

Mannan containing ligand modification was verified by Con A agglutination assay. Binding of the terminal α-mannose residues of the mannan to the Con A causes agglutination of the complex in solution resulting in an increase in turbidity. As shown in Fig. 3, Man-SLN-DNA exhibited increasing turbidity, while non-modified SLN-DNA displayed nearly no increase in turbidity. Blank SLN without DNA showed slight increase in turbidity.

Table I Characterization of SLN, SLN-DNA and Man-SLN-DNA (mean ± SD, $n=3$)

Sample	Mean particle size (nm)	Zeta potential (mV)
SLN	53.7 ± 4.2	39.48 ± 1.48
SLN-DNA	89.3 ± 9.6	18.75 ± 1.32
Man-SLN-DNA	125.7 ± 7.5	4.37 ± 1.09

Binding Assays and *In Vitro* Release of DNA from Man-SLN-DNA Complexes

The DNA binding assays of the complexes were observed by PicoGreen- fluorometry method. DNA binding quantity (%) = (total amount of DNA−the amount of free DNA)/total amount of DNA×100. The absorption efficiencies of Man-SLN-DNA and SLN-DNA were (90.3±2.7)% and (91.5±1.9)%, respectively.

The *in vitro* release studies of DNA from Man-SLN-DNA and SLN-DNA were carried out in PBS (pH 7.4) at 37°C. Release profiles of modified and non-modified formulations are illustrated in Fig. 4.

In Vitro Cytotoxicity Evaluations

In vitro toxicity of Man-SLN and blank SLN was evaluated by MTT assay in RAW 264.7 cells. The cytotoxicity of Man-SLN and blank SLN at various concentrations (5, 25, 50, 100 and 200 μg/mL) was evaluated. Lipofectamine 2000 at the concentration of transfection was used as comparison. As shown in Fig. 5, the cell viabilities in the presence of SLN and Man-SLN over the studied concentration range (5–200 μg/ml) were between 80 and 120% compared with controls. Man-SLN and SLN exhibited a lower cytotoxicity than Lipofectamine 2000 at all concentrations ($P<0.05$). At the highest concentration, the cell variability of Man-SLN was higher than blank SLN ($P<0.05$).

In Vitro Transfection Investigation

The transfection efficiencies of Man-SLN-DNA and unmodified SLN-DNA in RAW 264.7 cells are shown in Fig. 6. When compared with naked DNA and SLN-DNA, Man-SLN-DNA had higher transfection efficiency. When compared with Lipofectamine-DNA complexes, after transfection for 24 h, the images indicate that the transfection efficiency of Man-SLN-DNA was similar to that of Lipofectamine-DNA complexes, while higher transfection efficiency was obtained at 48 h.

In Vivo Transfection Study in Rats

After intratracheal instillation of Naked DNA, Lipofectamine 2000-DNA, SLN-DNA and Man-SLN-DNA, rats

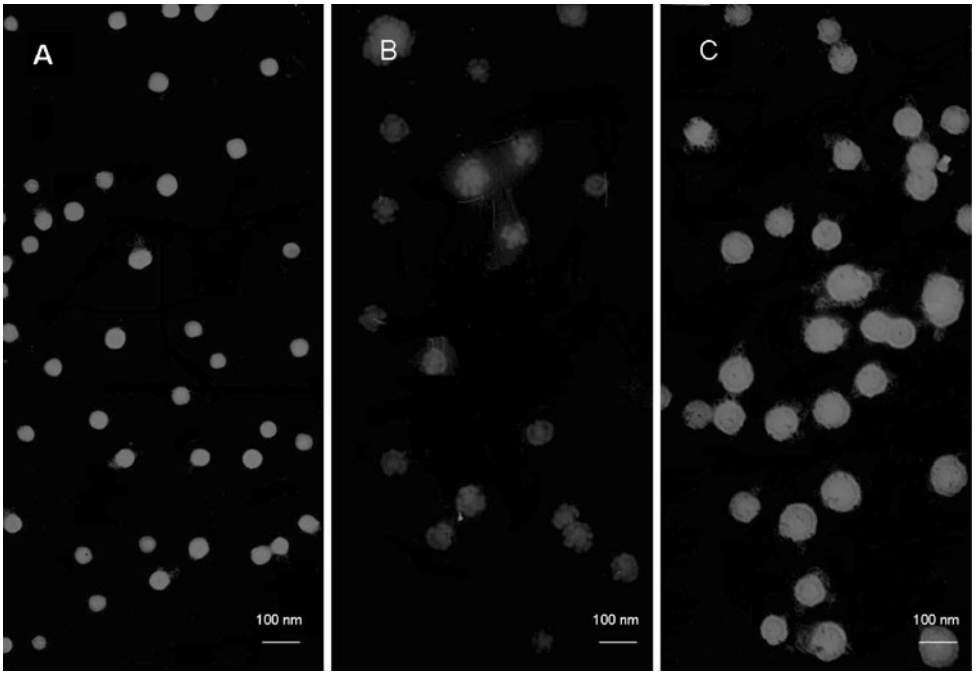


Fig. 1 The TEM pictures of SLN (**A**), SLN-DNA (**B**) and Man-SLN-DNA (**C**).

were euthanized, and the AM were isolated at different time intervals. The fluorescent cells were observed using an inverted fluorescence microscope. As illustrated from Fig. 7, control AM cells of the rats without any treatment had some slight green autofluorescence and were used as a negative control. The highest transfection efficiencies were obtained with Lipofectamine 2000-DNA, SLN-DNA and Man-SLN-DNA at 24 h, 24 h and 36 h, respectively. The *in vivo* transfection efficiency of Man-SLN-DNA in rats appeared better than un-modified ones and Lipofectamine 2000.

For identification of AM, a kind of monoclonal antibody–mouse anti-rat CD11c was used. It can recognize the rat CD11c cell surface antigen and is specific for identification of the rat alveolar macrophages. Alexa Fluor® 647 was a red dye; when conjugated with mouse anti-rat CD11c and incubated with rat AM, it could provide the percentage of AM at the UL and UR quadrants in the flow cytometry analysis. The percentage of cells transfected with pEGFP appeared at the UR and LR quadrants, so the percentage of the pEGFP-transfected AM cells appeared at the UR quadrant. As shown in Fig. 8, the percentage of AM is over 70% in the total cells after lavage. Man-SLN-DNA displayed remarkably higher transfection efficiency than Lipofectamine 2000-DNA and SLN-DNA ($P<0.05$), and

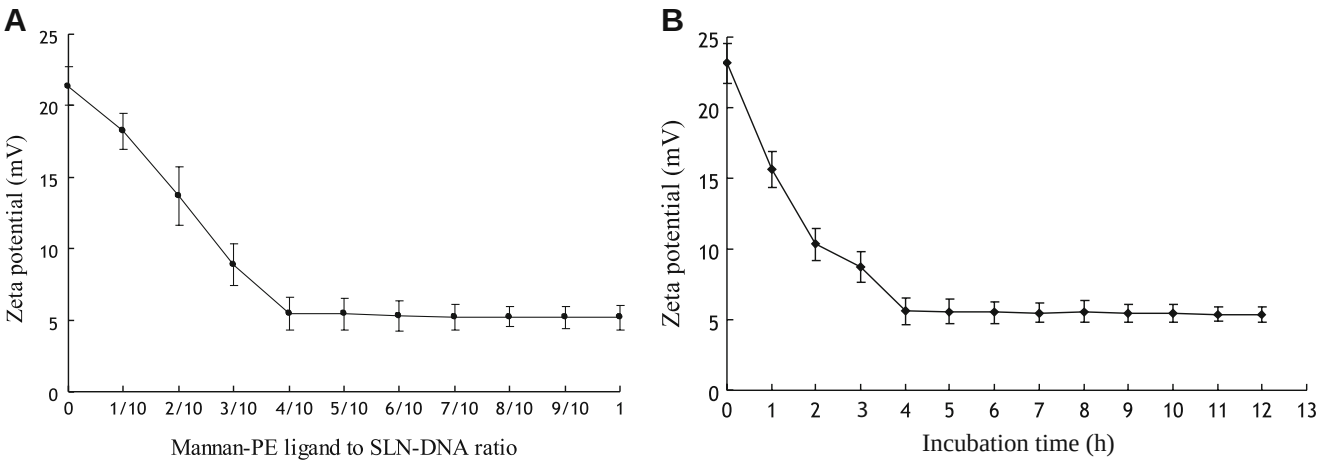


Fig. 2 Optimization of Mannan-modified SLN-DNA complexes: (**A**) ligand-to-carrier weight ratio (Mannan-PE/SLN-DNA, *w/w*); (**B**) incubation time (mean ± SD, $n = 3$).

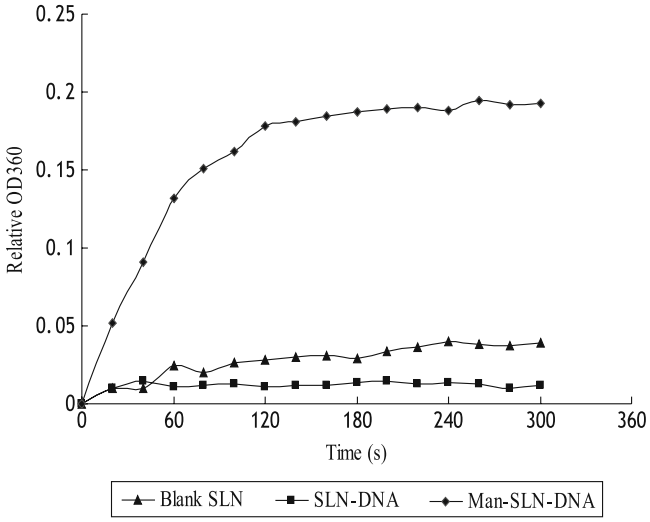


Fig. 3 Con A agglutination assay: when mixed with Con A, relative turbidity (OD_{360}) of blank SLN, SLN-DNA, Man-SLN-DNA were measured at predetermined time intervals.

transfection result at 48 h was apparently better than 24 h post transfection ($P<0.05$).

DISCUSSION

The aim of this study was to achieve active targeting to alveolar macrophages (AM) using mannan-modified DNA-loaded SLN (Man-SLN-DNA). In the present study, mannan-based PE-grafted ligand was synthesized and used for the surface modification of pre-preformed SLN-DNA. After a series of characterization and optimization steps, Man-SLN-DNA complexes were finally delivered into the lungs of the rats via intratracheal administration and successfully transfected the AM *in vivo*.

In recent years, much attention has been paid to the use of cationic solid lipid nanoparticles (SLN) as DNA carriers which may offer a number of technological advantages, including a better storage stability in comparison to liposomes, the possibility of steam sterilization and lyophilization, large-scale production with qualified production lines and the use of substances that are generally regarded as safe (GRAS) (33). Our previous study focused on synthesizing a cationic lipid named LHLN and its application as a surfactant to produce novel SLN formulation (11). It gained lower cytotoxicity and better transfection efficiency probably due to its passive targeting ability. Thus, further modification of this formulation was investigated to enhance it with an active targeting capacity.

It has been illustrated that the sugar density on the vector surface is an important factor affecting the targeting efficiency (34,35). Commonly speaking, nanocarriers modified with higher contents of sugar residues typically exhibit more efficient cellular recognition and internalization compared with lower sugar densities. Therefore, polysaccharides or multiple oligosaccharides (36) are recognized with a much higher affinity than the single sugars because of the moiety density. For this reason, which mannan, which contains a large group of mannose residues that has the ability to target AM, was employed as a targeting candidate. It is reported that saccharide derivatives with lipids could easily be incorporated onto the nanocarrier surface (29). Due to its excellent biocompatibility, PE was selected as the lipophilic anchor. During the synthesis, the positive-charged terminal amino group of PE was conjugated with carboxymethylated mannan via an amide linkage; thus, the negative-charged phosphate group was exposed and could readily absorb onto the cationic SLN-DNA surface by charge attraction. In these cases, mannan-based PE-grafted ligands (Man-PE) could be readily modified on the surfaces of SLN-DNA complexes.

During the modification procedure, Man-PE were continuously coated onto the SLN-DNA surfaces, which neutralized their original positive charge and caused a decrease in zeta potential. Two main process variables (total ligand-to-carrier weight ratio and incubation time) were optimized by measuring the change in zeta potential, and the optimum ratio was determined at which no significant change in zeta potential was recorded on further increase of the ligand-to-carrier weight ratio or incubation time. The optimum formulation had a zeta potential of

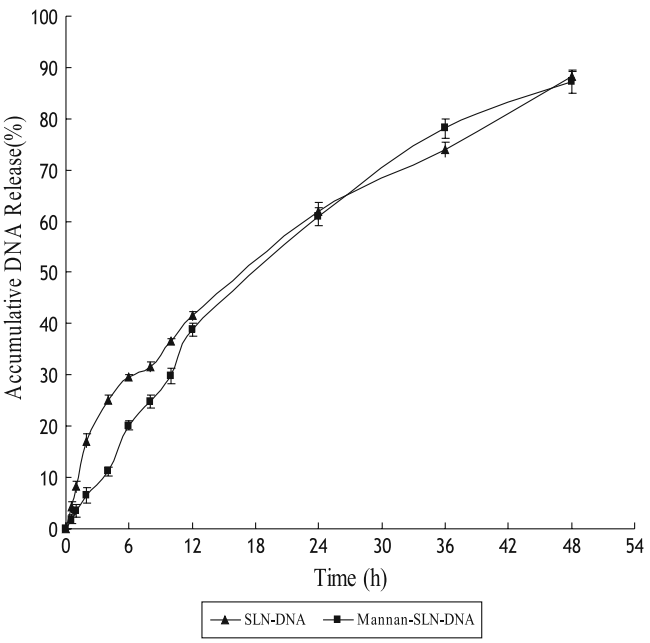


Fig. 4 The *in vitro* release of Man-SLN-DNA and SLN-DNA complexes (mean ± SD, $n=3$).

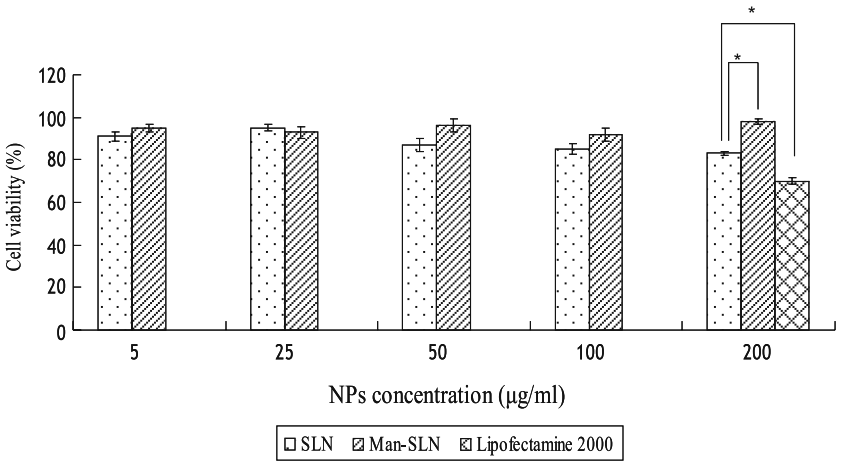


Fig. 5 Cell viability of Man-SLN and blank SLN with different concentrations and Lipofectamine 2000 against RAW 264.7 cells by MTT assay, 48 h post-treatment (mean ± SD, $n=3$). *$P<0.05$.

about 4.37 mV (Table I and Fig. 2), which was nearly neutral. This may help the vectors, to some extent, avoid binding to plasma proteins.

Concanavalin A (Con A) from jack bean (*Canavalia ensiformis*) is the first legume lectin recognized as a Glc/Man-specific protein and is a tetrameric protein with four binding sites specific for terminal α-mannose residues (32,37). Binding of the terminal α-mannose residues of the mannan to the Con A causes agglutination of the complex in solution, resulting in an increase in turbidity. As shown in Fig. 3, Man-SLN-DNA exhibited an increase in turbidity because of the specific binding between sugar and lectin. SLN-DNA showed no obviously agglutination. The slight augmentation in the OD_{360} from the blank SLN might be due to some nonspecific binding between Con A and the highly positively charged SLN (39.48 mV) (24).

After the completion of modification, PicoGreen-fluorometry method was applied to determine the binding ability and *in vitro* release of Man-SLN-DNA. The absorption efficiency of Man-SLN-DNA was (90.3± 2.7)%, which marked no significant difference from SLN-DNA (91.5±1.9)%. The results demonstrated that binding of mannan onto the SLN-DNA surface did not desorb the DNA from the complexes. The *in vitro* release profile (Fig. 4) of Man-SLN-DNA showed slower release than SLN-DNA during the first 12 h. This could be explained if the coating of mannan hindered the release of DNA initially. After that, mannan might detach from the vectors, allowing the release of DNA more freely. At the end of the release study, the total amount of DNA delivered from the two kinds of vehicles was nearly the same (around 87%). The release data were evaluated by model-dependent methods in order to determine the release mechanism of DNA from the SLN. The release profiles of the Man-SLN-DNA and SLN-DNA follow the Ritger–Peppas equation ($\ln Q\% = 1.1312 \ln t + 3.7625$, $R^2=0.9912$) and the Higuchi model ($Q\% = 0.1294\, t^{1/2} = 0.0279$, $R^2=0.9957$) best, respectively.

Alveolar macrophages (AM) were targeted in the present study. Recently, the murine macrophage cell line RAW 264.7 became available and has been used as an *in vitro*

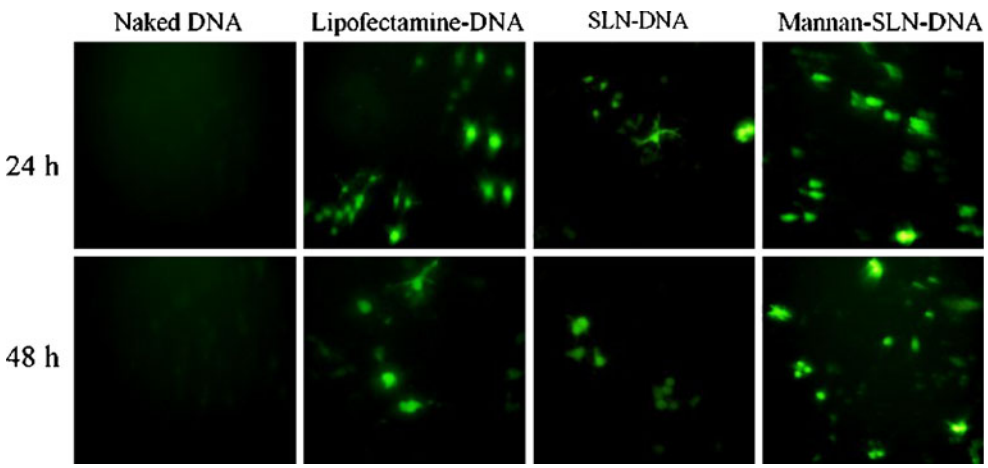


Fig. 6 Fluorescent micrographs of RAW 264.7 cells transfected by plasmid EGFP with Man-SLN-DNA and SLN-DNA complexes. Gene expression was examined after 24 h and 48 h post transfection, respectively.

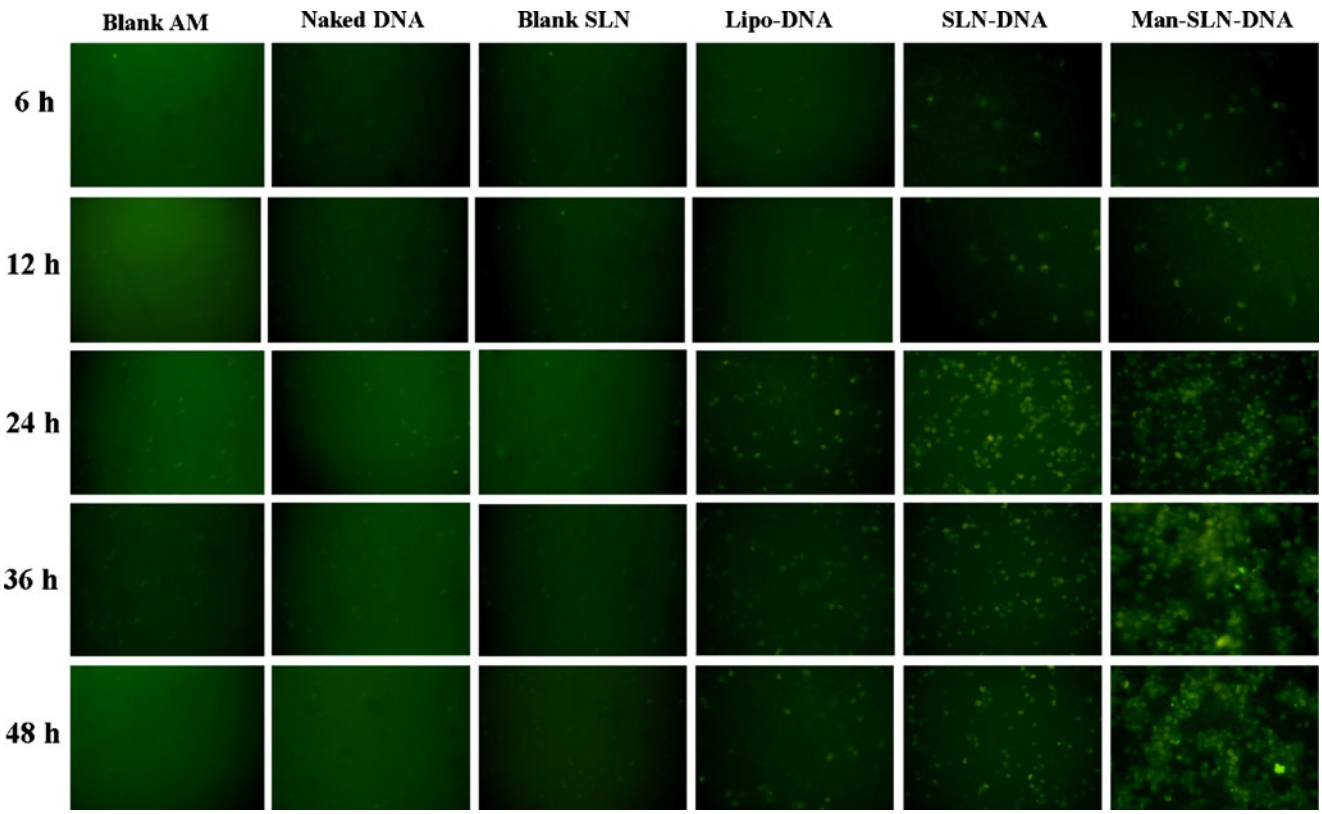


Fig. 7 Fluorescent images of the AM transfected with Man-SLN-DNA, SLN-DNA and Lipofectamine 2000-DNA complexes. Gene expression was examined after 6, 12, 24, 36, and 48 h post-transfection, respectively.

model of AM (38). Therefore, RAW 264.7 cells were applied to estimate the *in vitro* cytotoxicity and transfection efficiency of Man-SLN-DNA. There is an increasing body of evidence that the surface properties of nanoparticles can lead to considerable toxicity (39). Cationic formulations have been described to affect cell proliferation, differentiation, and pro-apoptotic genes in human epithelial cells. The polycationic nature of synthetic polycationic non-viral gene transfer systems induces cytotoxicity by necrosis and apoptosis. Therefore, generally speaking, lower positive charge density could reduce the cytotoxicity (40). This explanation is in accordance with the results illustrated in Fig. 5. The positive charge of blank SLN was covered by Man-PE resulting in a lower zeta potential, so at the highest concentration in the test, the cell viability of Man-SLN is higher than blank SLN ($P<0.05$). Both Man-SLN and SLN formulations showed lower cytotoxicity compared with Lipofectamine 2000 ($P<0.05$), most probably due to the cationic lipid (LHLN) used in the preparation which has low toxicity and good biodegradability [11]. The *in vitro* transfection efficiencies of Man-SLN-DNA and non-modified SLN-DNA on the RAW 264.7 cells are shown in Fig. 6. Man-SLN-DNA gained obviously higher transfection efficiency than SLN-DNA, which may be explained by its ligand-receptor (sugar-lectin)-mediated active targeting mechanism. After transfection for 24 h, the transfection efficiency of Man-SLN-DNA was similar to that of Lipofectamine-DNA complexes. This might be because during the first period of time, DNA had a controlled-release phase from Man-SLN-DNA due to the surface coating of mannan, which is also consistent with the *in vitro* release behavior of it. After 48 h, DNA was almost completely released from the vectors; thus, higher transfection efficiency was obtained at 48 h when compared with Lipofectamine-DNA complexes.

Pulmonary delivery offers a number of advantages, such as providing an enormous absorptive area, affluent blood volume, a thin alveolar epithelium membrane, extensive vasculature, low extracellular and intracellular enzymatic activities, and little first-pass metabolism, thereby permitting rapid absorption and onset of action (27). Therefore, pulmonary delivery through inhalation or intratracheal instillation is widely used by researchers for animal experiments. In this study, Wistar rats were established as *in vivo* model for the pulmonary gene delivery to AM. After intratracheal instillation of Naked DNA, Lipofectamine 2000-DNA, SLN-DNA and Man-SLN-DNA, rats were euthanized, and the AM were isolated at predetermined

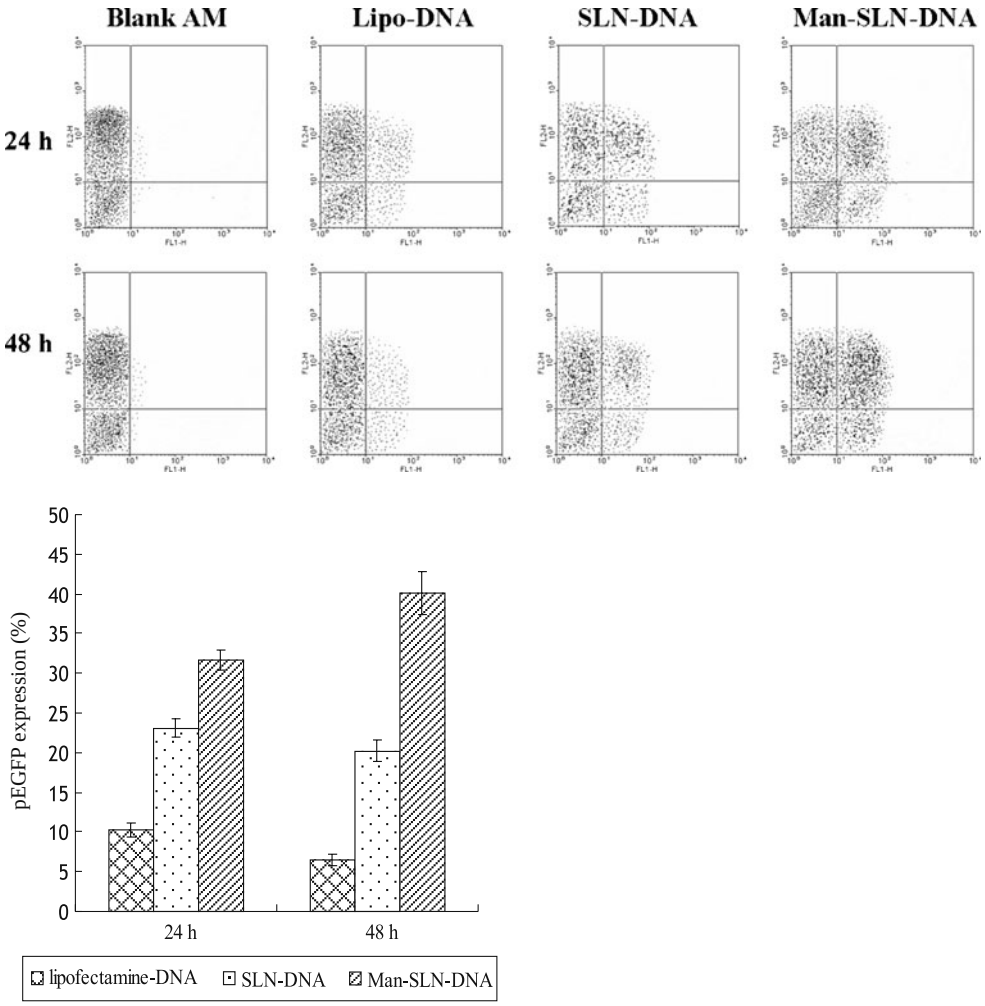


Fig. 8 Flow cytometry of rat AM transfected by plasmid EGFP with Man-SLN-DNA, SLN-DNA and Lipofectamine 2000-DNA complexes. Gene expression was examined after 24 and 48 h post transfection.

time intervals. Mouse anti-rat CD11c:Alexa Fluor® 647 is a kind of monoclonal antibody specific for identification of the rat alveolar macrophages. When incubated with rat AM, it could provide the percentage of AM at the UL and UR quadrants in the flow cytometry analysis. The flow cytometry (Fig. 8) showed the highest transfection efficiencies were reached by Lipofectamine 2000-DNA, SLN-DNA and Man-SLN-DNA at 24 h, 24 h and 48 h, respectively. The SLN-DNA got the highest transfection efficiency at 24 h, better than Lipofectamine 2000-DNA ($P<0.05$), and then performed not so well after 24 h. One explanation may be that as an *in vitro* transfection vector, Lipofectamine 2000 was not so stable and might have some toxicity *in vivo*. Man-SLN-DNA exhibited better green fluorescence protein (GFP) expression at 24 h when compared with non-modified SLN-DNA, and the plateau of the transfection efficiency was reached at 48 h. The green fluorescence in the cells of Man-SLN-DNA group of rats lasted for 24 h until 48 h. These evidences may strongly support the active targeting ability of mannan-modified DNA-loaded SLN, and the resulting particles might be very useful for *in vivo* gene delivery.

CONCLUSIONS

In this work, mannan-based PE-grafted ligands (Mannan-PE) were synthesized and applied for the surface modification of SLN-DNA complexes to prepare mannan-modified SLN-DNA (Man-SLN-DNA). The targeting ability of the Man-SLN-DNA was evaluated by transfer into RAW 264.7 cells (*in vitro*) and by pulmonary delivery to rats (*in vivo*). Man-SLN-DNA showed lower cytotoxicity in contrast with non-modified SLN-DNA. Higher gene expressions were illustrated by Man-SLN-DNA compared with Lipofectamine 2000-DNA, especially *in vivo*. The results indicated that mannan modification could enhance the active targeting ability of the carriers, and Man-SLN-DNA may be a promising non-viral vector for targeted gene delivery.

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REFERENCES

1. Jemal A, Thun MJ, Ries LA, Howe HL, Weir HK, Center MM *et al.* Annual report to the nation on the status of cancer, 1975–2005, featuring trends in lung cancer, tobacco use, and tobacco control. J Natl Cancer Inst. 2008;100:1672–94.
2. Tseng CL, Wu SY, Wang WH, Peng CL, Lin FH, Lin CC *et al.* Targeting efficiency and biodistribution of biotinylated-EGF-conjugated gelatin nanoparticles administered via aerosol delivery in nude mice with lung cancer. Biomaterials. 2008;29:3014–22.
3. Wijagkanalan W, Kawakami S, Takenaga M, Igarashi R, Yamashita F, Hashida M. Efficient targeting to alveolar macrophages by intratracheal administration of mannosylated liposomes in rats. J Control Release. 2008;125:121–30.
4. Murer B. Targeted therapy in non-small cell lung cancer a commentary. Arch Pathol Lab Med. 2008;132:1573–5.
5. Morille M, Passirani C, Vonarbourg A, Clavreul A, Benoit JP. Progress in developing cationic vectors for non-viral systemic gene therapy against cancer. Biomaterials. 2008;29:3477–96.
6. Rolland A. Gene medicines: the end of the beginning? Adv Drug Deliv Rev. 2005;57:669–73.
7. Zhao XB, Lee RJ. Tumor-selective targeted delivery of genes and antisense oligodeoxyribonucleotides via the folate receptor. Adv Drug Deliv Rev. 2004;56:1193–204.
8. Lee MK, Lim SJ, Kim CK. Preparation, characterization and *in vitro* cytotoxicity of paclitaxel-loaded sterically stabilized solid lipid nanoparticles. Biomaterials. 2007;28:2137–46.
9. Zhang HW, Bei JZ, Wang SG. Multi-morphological biodegradable PLGE nanoparticles and their drug release behavior. Biomaterials. 2009;30:100–7.
10. Karathanasis E, Chan L, Balusu SR, D'Orsi CJ, Annapragada AV, Sechopoulos I *et al.* Multifunctional nanocarriers for mammographic quantification of tumor dosing and prognosis of breast cancer therapy. Biomaterials. 2008;29:4815–22.
11. Yu W, Liu C, Ye J, Zou W, Zhang N, Xu W. Novel cationic SLN containing a synthesized single-tailed lipid as a modifier for gene delivery. Nanotechnology. 2009;20:215102.
12. Ye J, Wang A, Liu C, Chen Z, Zhang N. Anionic solid lipid nanoparticles supported on protamine/DNA complexes. Nanotechnology. 2008;19:285708.
13. Zou W, Liu C, Chen Z, Zhang N. Studies on bioadhesive PLGA nanoparticles: a promising gene delivery system for efficient gene therapy to lung cancer. Int J Pharm. 2009;370:187–95.
14. Yu W, Zhang N. Surface modification of nanocarriers for cancer therapy. Curr Nanosci. 2009;5:123–34.
15. Torchilin VP. Targeted pharmaceutical nanocarriers for cancer therapy and imaging. AAPS J. 2007;9:E128–47.
16. van Vlerken LE, Vyas TK, Amiji MM. Poly(ethylene glycol)-modified nanocarriers for tumor-targeted and intracellular delivery. Pharm Res. 2007;24:1405–14.
17. Beduneau A, Saulnier P, Benoit JP. Active targeting of brain tumors using nanocarriers. Biomaterials. 2007;28:4947–67.
18. Wijagkanalan W, Kawakami S, Takenaga M, Igarashi R, Yamashita F, Hashida M. Efficient targeting to alveolar macrophages by intratracheal administration of mannosylated liposomes in rats. J Control Release. 2008;125:121–30.
19. Kawakami S, Higuchi Y, Hashida M. Nonviral approaches for targeted delivery of plasmid DNA and oligonucleotide. J Pharm Sci. 2008;97:726–45.
20. Mantovani A, Schioppa T, Porta C, Allavena P, Sica A. Role of tumor-associated macrophages in tumor progression and invasion. Cancer Metastasis Rev. 2006;25:315–22.
21. Sica A, Schioppa T, Mantovani A, Allavena P. Tumour-associated macrophages are a distinct M2 polarised population promoting tumour progression: potential targets of anti-cancer therapy. Eur J Cancer. 2006;42:717–27.
22. Ezekowitz RA, Williams DJ, Koziel H, Armstrong MY, Warner A, Richards FF *et al.* Uptake of Pneumocystis carinii mediated by the macrophage mannose receptor. Nature. 1991;351:155–8.
23. Cui ZR, Hsu CH, Mumper RJ. Physical characterization and macrophage cell uptake of mannan-coated nanoparticles. Drug Dev Ind Pharm. 2003;29:689–700.
24. Cui ZG, Han SJ, Huang L. Coating of mannan on LPD particles containing HPV E7 peptide significantly enhances immunity against HPV-positive tumor. Pharm Res. 2004;21:1018–25.
25. Hattori Y, Kawakami S, Suzuki S, Yamashita F, Hashida M. Enhancement of immune responses by DNA vaccination through targeted gene delivery using mannosylated cationic liposome formulations following intravenous administration in mice. Biochem Biophys Res Commun. 2004;317:992–9.
26. Kaur A, Jain S, Tiwary AK. Mannan-coated gelatin nanoparticles for sustained and targeted delivery of didanosine: *in vitro* and *in vivo* evaluation. Acta Pharm. 2008;58:61–74.
27. Azarmi S, Roa WH, Loebenberg R. Targeted delivery of nanoparticles for the treatment of lung diseases. Adv Drug Deliv Rev. 2008;60:863–75.
28. Yu W, Zhang N, Li C. Saccharide modified pharmaceutical nanocarriers for targeted drug and gene delivery. Curr Pharm Des. 2009;15:3826–36.
29. Kawakami S, Wong J, Sato A, Hattori Y, Yamashita F, Hashida M. Biodistribution characteristics of mannosylated, fucosylated, and galactosylated liposomes in mice. Biochim Biophys Acta Gen Subj. 2000;1524:258–65.
30. Yang L, Cheng L, Wei Y, Tian L. Synthesis of N-[2-(cholesteryloxycarbonylamino) ethyl] carbamoylmethylated mannan. Sichuan Da Xue Xue Bao Yi Xue Ban. 2003;34:730–2.
31. Vyas SP, Sihorkar V, Jain S. Mannosylated liposomes for bio-film targeting. Int J Pharm. 2007;330:6–13.
32. Cui Z, Mumper RJ. Topical immunization using nanoengineered genetic vaccines. J Control Release. 2002;81:173–84.
33. Tabatt K, Sameti M, Olbrich C, Muller RH, Lehr CM. Effect of cationic lipid and matrix lipid composition on solid lipid nanoparticle-mediated gene transfer. Eur J Pharm Biopharm. 2004;57:155–62.
34. Managit C, Kawakami S, Yamashita F, Hashida M. Effect of galactose density on asialoglycoprotein receptor-mediated uptake of galactosylated liposomes. J Pharm Sci. 2005;94:2266–75.
35. Yeeprae W, Kawakami S, Yamashita F, Hashida M. Effect of mannose density on mannose receptor-mediated cellular uptake of mannosylated O/W emulsions by macrophages. J Control Release. 2006;114:193–201.
36. Monsigny M, Quetard C, Bourgerie S, Delay D, Pichon C, Midoux P *et al.* Glycotargeting: the preparation of glyco-amino

acids and derivatives from unprotected reducing sugars. Biochimie. 1998;80:99–108.
37. Barre A, Bourne Y, Van Damme EJ, Peumans WJ, Rouge P. Mannose-binding plant lectins: different structural scaffolds for a common sugar-recognition process. Biochimie. 2001;83:645–51.
38. Shibata K, Kudo Y, Tsunoda M, Hosokawa M, Sakai Y, Kotani M *et al.* Magnetometric evaluation of the effects of man-made mineral fibers on the function of macrophages using the macrophage cell line RAW 264.7. Ind Health. 2007;45:426–36.
39. Kabanov AV. Polymer genomics: an insight into pharmacology and toxicology of nanomedicines. Adv Drug Deliv Rev. 2006;58:1597–621.
40. Vega-Villa KR, Takemoto JK, Yanez JA, Remsberg CM, Forrest ML, Davies NM. Clinical toxicities of nanocarrier systems. Adv Drug Deliv Rev. 2008;60:929–38.