A Novel Nonviral Gene Delivery Vector: Low-Molecular-Weight Polyethylenimine-*graft*-ovalbumin

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ABSTRACT: A novel vector for gene delivery was synthesized. Here the ovalbumin (OVA) acts as a core and low-molecular-weight PEI600 was grafted to its surface. The finally product was characterized (^{1}H-NMR, UV, and TGA) and its biophysical properties such as DNA condensing, particle size, and zeta potential were determined. The agarose gel assay indicated that OVA-PEI600 could efficiently condense plasmid DNA. Its particle size was about 150 nm and zeta potential was around +20 mV. The MTT assay showed that the cytotoxicity of OVA-PEI600 was less than PEI25 kDa. Its transfection efficiency in SKOV-3 and HepG2 cell lines was higher than that of PEI600 and comparable to PEI25 kDa. *In vivo*, luciferase activity could be tested in liver, spleen, kidney, lung, and blood serum, respectively, in mice. The core-shell structure of OVA-PEI600 provided a novel strategy for nonviral gene delivery. © 2009 Wiley Periodicals, Inc. J Appl Polym Sci 114: 3744–3750, 2009

Key words: ovalbumin; poly(ethylenimine); nonviral vector; gene delivery; *in vitro*

INTRODUCTION

Gene transfer to humans required carriers that could safely and efficiently deliver DNA to the desired cells.[1,2] The ideal delivery vehicle should be stable and small enough to extravasate and could protect DNA against degradation by nucleases. In addition, the desired cells targeting ability of vectors was also favorable for practice in gene therapy.[3] Most of the used nonviral vectors had advantages over viral systems. Among the nonviral vectors, polyethylenimine (PEI) showed high transfer activity *in vitro* and it is highly recognized for its intrinsic "sponge effect."[4] However, the high-molecular-weight PEI showed high toxicity and could not degrade *in vivo*. In recent years, great attention had been paid to the low-molecular-weight PEI (M_w less than 2000).[5,6] It was imperative to seek a new strategy to modify low-molecular-weight PEI, to improve its gene delivery profile. An interesting strategy was by using conjugated ligand to activate the gene delivery procedure.[7] Ovalbumin (OVA) showed sequence identity of 30% with antitrypsin and a putative reactive center at Ala 358-Ser 359. It showed no measurable inhibitory activity and it does not undergo the typical conformational change on cleavage at this P1-P1′ site.[8,9] In this study, a novel vector was developed. OVA was selected as a core and low-molecular-weight PEI (PEI 600 Da) was conjugated to the surface of OVA to form OVA-PEI. The biophysical characterization, biocompatibility, and biological characterization of OVA-PEI/DNA complexes were studied. OVA-PEI600/DNA complexes might avoid the undesired interactions with serum and retained the advantages of low toxicity. These findings indicated that low-molecular-weight PEI600 conjugate to peptide or antibody might be a promising strategy for gene delivery.

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EXPERIMENTAL

Materials and methods

OVA (albumin from chicken egg white, Grade V), branched PEI (25 kDa, 600 Da), triethylamine, methylthiazolyldiphenyl-tetrazolium bromide (MTT), deuterium oxide (D_2O), dimethyl sulfoxide (DMSO),

and 2-iminothiolate hydrochloride were purchased from Sigma-Aldrich (St. Louis, MO). *N*-Succinimideyl-3-(2-pyridyldithio)-propionate (SPDP) was purchased from Pierce (Rockfod, IL). All reagents were used without further purification.

Plasmid preparation

The plasmid pGL3-Luc encoding luciferase was purchased from Promega (Madison, WI). The plasmid pcDNA3.1-EGFP was a gift from the Institute of Immunology, Second Military Medical University, Shanghai, China. All plasmids were amplified in *E. coli* and purified according to the supplier's protocol (Mega Endofree Plasmid Purification Kit; Qiagen, Hilden, Germany).

Cells

SKOV-3, HepG2, and A549 cell lines were purchased from American Type Culture Collection (Rockville, MD). Cell lines were cultured in DMEM supplemented with 10% FBS (Gibco) and 1% penicillin–streptomycin (Gibco) in a 37°C incubator with 5% CO_2.

Laboratory animal

ICR mice (18–25 g) were used for animal experiment. Mice were housed in standard conditions and were allowed free access to food and water. They were fasted for 12 h before the experiment.

Synthesis of OVA-PEI600

OVA (60.0 mg, 1.3 μmol) was dissolved in PBS buffer (15 mL, pH 7.2). 2-Iminothiolate hydrochloride (9.5 mg, 0.07 mmol, in 1.9 mL H_2O) was added into OVA solution at room temperature under nitrogen to form OVA-SH.

SPDP (18.0 mg, 0.06 mmol, in DMSO 1.0 mL) was added dropwise into PEI600 (0.6 g, 1 mmol, 5 mL DMSO) under nitrogen for 30 min.

The active PEI600 was added into the OVA-SH solution. The mixture was incubated for 5 h at room temperature. The ultimate product was dialyzed in running water for 2 days and then lyophilized for 3 days.

Characterization of OVA-PEI600

^{1}H-NMR assay

The ^{1}H-NMR spectra of the OVA-PEI600 in D_2O was recorded on a Bruker Avanc4 400 Spectrometer (400 MHz) with 32 scans at room temperature.

UV analysis

The samples of OVA-PEI600, OVA, and PEI600 were dissolved in water for UV detection using Hitachi U-3400 spectrophotometer to determine the linkage of OVA-PEI600.

TGA test

The samples of OVA-PEI600, OVA, and PEI600 were analyzed by TGA on a TA Instrument SDT 2960 and were heated at 10°C/min from room temperature to 600°C in a dynamic nitrogen atmosphere environment (flow rate: 70 mL/min).

Gel retardation assay

The electrophoretic mobility of the plasmid DNA (pGL3-luc) condensed with OVA-PEI600 was measured using a gel electrophoresis system. Appropriate amount of OVA-PEI600 was added to an equal volume of DNA solution. The OVA-PEI600 and DNA weight ratio were 0, 10.0, 20.0, 30.0, 50.0, 60.0, and 70.0, respectively. Gel electrophoresis was carried out at room temperature in TAE buffer in 1% (w/w) agarose gel at 80 V for 60 min. DNA bands were visualized using an UV illuminator.

TEM assay

The electroscopic evaluation of OVA-PEI600/DNA complexes was performed at a weight ratio of 200. The particles were formed in saline solution, vortexed for 1 min, placed on a copper grid coated with carbon film, and air dried at room temperature. The observation was carried out by electron microscope (JEM-2010 TEM).

Particle size and zeta potential

The particle size and zeta potential of OVA-PEI600/DNA complexes were measured by 90 Plus Particle Size Analyzer (Brookhaven Instruments Corporation, USA) at room temperature. The concentration of DNA was 1.0 mg/mL. The weight ratios of OVA-PEI600 and DNA were 1.0, 10.0, 50.0, 100.0, 200.0, 300.0, and 400.0, respectively. A volume of 3 mL was used for the measurement. The particle size of complex was measured with scattering light at 90° angles. Each measurement was repeated five times, and an average value was obtained and reported. The zeta potential measurement was reported for five runs per sample and an average value was reported.

Cell viability assay

HepG2, A549, and SKOV-3 cell lines were placed in 96-well plates at a density of 1×10^4/well with DMEM supplemented with 10% FBS. After 16 h, the culture media were replaced with 200 μL serum-free

DMEM containing different concentration of OVA-PEI600 (0, 100.0, 200.0, 300.0, 400.0, 500.0, and 600.0 μg/mL, respectively) and then incubated for 4 h. The medium was replaced with DMEM containing 10% FBS (90 μL) and 5 mg/mL MTT (10 μL) for another 4 h. The crystals were dissolved in 100 μL/well DMSO and measured spectrophotometrically in an ELISA plate reader (model 550; Bio-Rad) at a wavelength of 570 nm. The relative cell growth (%) related to control cells cultured in media without OVA-PEI600 was calculated by the following equation:

$$\text{relative cell viability } (\%) = \left(\frac{\text{OD}_{\text{treated}}}{\text{OD}_{\text{control}}}\right) \times 100\%$$

where $\text{OD}_{\text{control}}$ was obtained in the absence of copolymers, and $\text{OD}_{\text{treated}}$ was obtained in the presence of copolymers.

In vitro gene expression

Luciferase assay

HepG2 and SKOV-3 cell lines were employed to verify the gene delivery efficiency of OVA-PEI600/DNA complexes. The cells was placed in 24-well plates at a density of 5×10^4/well with 0.5 mL DMEM containing 10% FBS. After 24 h incubation, the culture media was replaced with 0.5 mL serum-free DMEM containing the complexes of OVA-PEI600/DNA at different weight ratios. The weight ratio of OVA-PEI600 and DNA were 100, 200, 250, 300, 350, and 400, respectively. The complexes were incubated with the cells for 4 h, followed by supplementation with DMEM containing 10% FBS for 36 h. After removing the incubation medium, cells were rinsed with 1× PBS and freeze-thawed in 100 μL of 1× PBS at −80°C. The luciferase activity in cell extracts was measured using a luciferase assay kit (Promega). The quantity of total protein was determined using protein assay kit (BCA; Pierce).

GFP transfection

OVA-PEI600/DNA complexes (weight ratio: 400) were incubated with the HepG2 cells in serum-free DMEM for 4 h. After that the medium was replaced with 0.75 mL of DMEM with 10% FBS and the cells were further incubated for 36 h. Cells were observed using a fluorescence microscope.

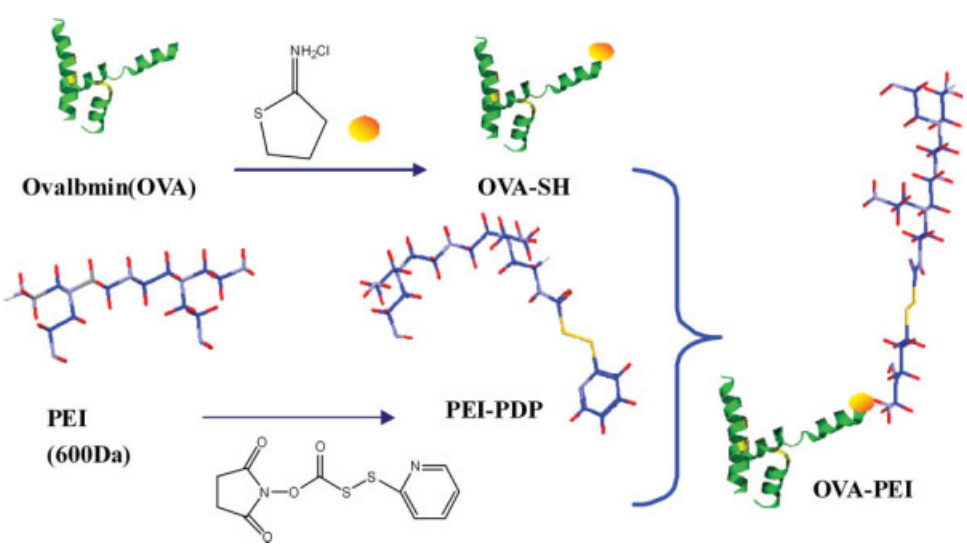
Distribution of OVA-PEI600 in organs

For examination of luciferase reporter gene expression, each animal was injected with OVA-PEI600/DNA via tail vein. Animals were killed at 24 and 48 hr by cervical dislocation. Organs were homogenized in 1× PBS. Luciferase activity in the tissues were measured using FB 12 Luminometer (Power Requirements 12V 400Ma; Berthold Detection System GmbH). The transfer activity was expressed as relative light units (RLU) per mg tissue.

RESULTS AND DISCUSSION

Synthesis and characterization of OVA-PEI600

A critical area in achieving successful gene therapy is the requirement of a vector with high transfection efficiency. Our strategy was to synthesize a vector



Scheme 1 Synthesis route of OVA-grafted PEI. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

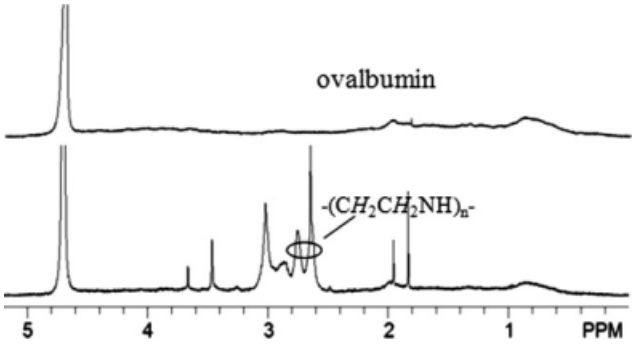


Figure 1 ^{1}H-NMR spectra of (a) OVA, (b) OVA-*g*-PEI in D_2O.

that has a core-shell structure. The synthesis step for OVA-PEI is illustrated IN Scheme 1. In the synthesis step, SPDP was used as a linker between OVA and PEI600. The advantage of SPDP was the functionalized reaction that was conducted effectively. It reacted quickly with the amino group of PEI600 to form PEI-SPDP. OVA contains 383 amino acid residues and has a molecular weight of 45 kDa. OVA was reacted with 2-iminothiolate hydrochloride, a Traut's reagent, to form OVA-SH. An important point is that precipitation may occur because the structure of OVA and PEI are both multivalent polymers. To avoid crossing, an excess of 2-iminothiolate hydrochloride must be added. Finally, OVA-SH and PEI-SPDP were reacted to form OVA-PEI600. The product was further purified by dialysis using a dialysis tube (MWCO: 8000–12,000) to remove the residues of solvent and excess of PEI600.

The successful conversion of this polymer was monitored by spectroscopic methods.[10,11] Figure 1 showed the ^{1}H-NMR spectra of OVA-PEI600, PEI600, and OVA. After grafting PEI600 to OVA, new peaks at δ 2.5–3.2 ppm appeared, which are attributed to the protons of PEI600. Figure 2(a) shows the UV spectra of OVA and OVA-PEI600. For OVA the λ_{max} was at 279.0 nm. But when PEI600 was grafted to OVA, the absorption λ_{max} was shifted from 279.0 to 277.3 nm. Figure 2(b) shows the TGA curve of OVA, PEI600, and OVA-PEI600. PEI600 started to decompose at 330°C. And three steps could be observed for OVA. The first one was due to solvent residues in OVA. The second and third steps were at 225 and 300°C, respectively. For OVA-PEI600, the decomposition temperature was 375°C, which means this compound was a new one.

The formation of OVA-PEI600/DNA complexes was examined by gel retardation assay. Figure 3(a) shows a typical result of gel retardation. For OVA-PEI600, DNA was retarded at a weight ratio of 50.0, which indicated that OVA-PEI600 can efficiently condense DNA.

The particle size depended on many parameters, including ionic strength of solvent, concentration of DNA, and sequence of addition of polymer or DNA.[12,13] Figure 3(b) shows the morphology of the OVA-PEI600/DNA by TEM. It showed that the particle size of the samples were approximately 150 nm at a weight ratio of 400, which was a suitable diameter for gene delivery.[14] The results could be confirmed by DLS measurements [Fig. 3(c)].

The surface charge of complexes played an important role in gene delivery. Figure 3(d) shows the zeta potentials of PEI600/DNA and OVA-PEI600/DNA complexes at various weight ratios. At a weight ratio of 1.0, PEI600/DNA and OVA-PEI600/DNA complexes were negatively charged, which suggested that the amount of PEI600 or OVA-PEI600 were not enough to condense the DNA completely. When the weight ratio was increased to 100, the charge of OVA-PEI600/DNA turned positive. The zeta charges kept +20 mV after the weight ratio reached 250. It was believed that high surface charge could enhance the interaction with cell membrane which was negatively charged. PEI600/DNA complexes had low zeta potentials and poor transfer activity. After conjugating PEI600 to OVA, the density of surface charge increased, and the ability of condensed DNA

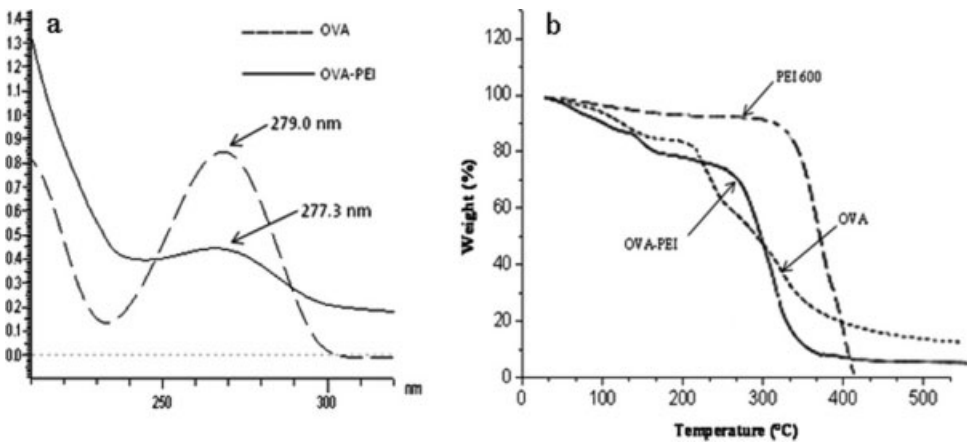


Figure 2 (a) UV detection of OVA-PEI600, OVA. (b) TGA analysis of OVA-PEI600, OVA, and PEI600.

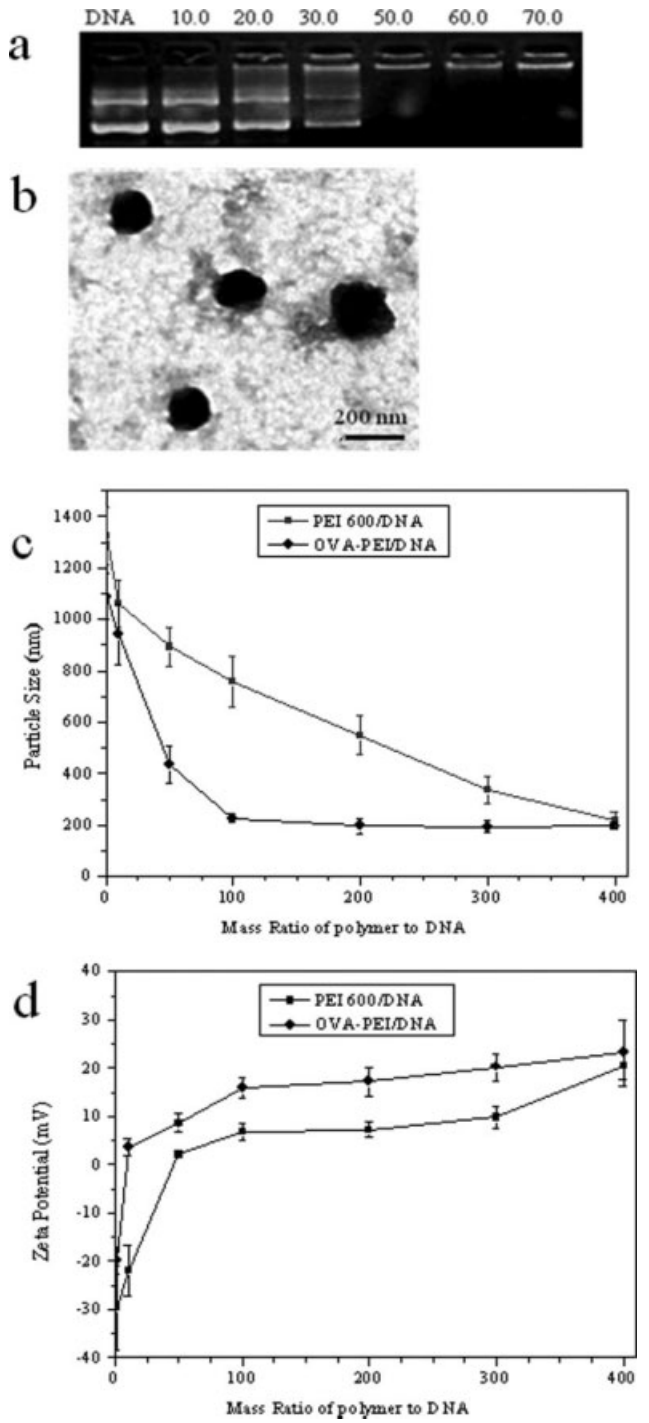


Figure 3 (a) Agarose gel electrophoresis retardation assay of OVA-PEI600/DNA complexes. OVA-PEI600 with different weights was added to an equal volume of DNA solution containing 5% glucose to achieve the desired OVA-PEI600 to DNA weight ratio (0, 10.0, 20.0, 30.0, 50.0, 60.0, and 70.0). (b) TEM micrographs of OVA-PEI600/DNA complexes. The measuring mark is 0.2 μm. (c) Particle size detection of OVA-PEI600/DNA complexes. (d) Zeta potential assay of OVA-PEI600/DNA complexes.

was increased. We thought that the surface charge of OVA-PEI600 could stabilize the complexes against aggregation *in vitro*.

Cytotoxicity of OVA-PEI600

The cytotoxicity of OVA-PEI600 was evaluated in HepG2, A549, and SKOV-3 cell lines by MTT assay and the PEI (25 kDa) and PEI600 were used as the control. In the study, OVA-PEI600 showed low toxicity in HepG2, A549, and SKOV-3 cell lines, even lower

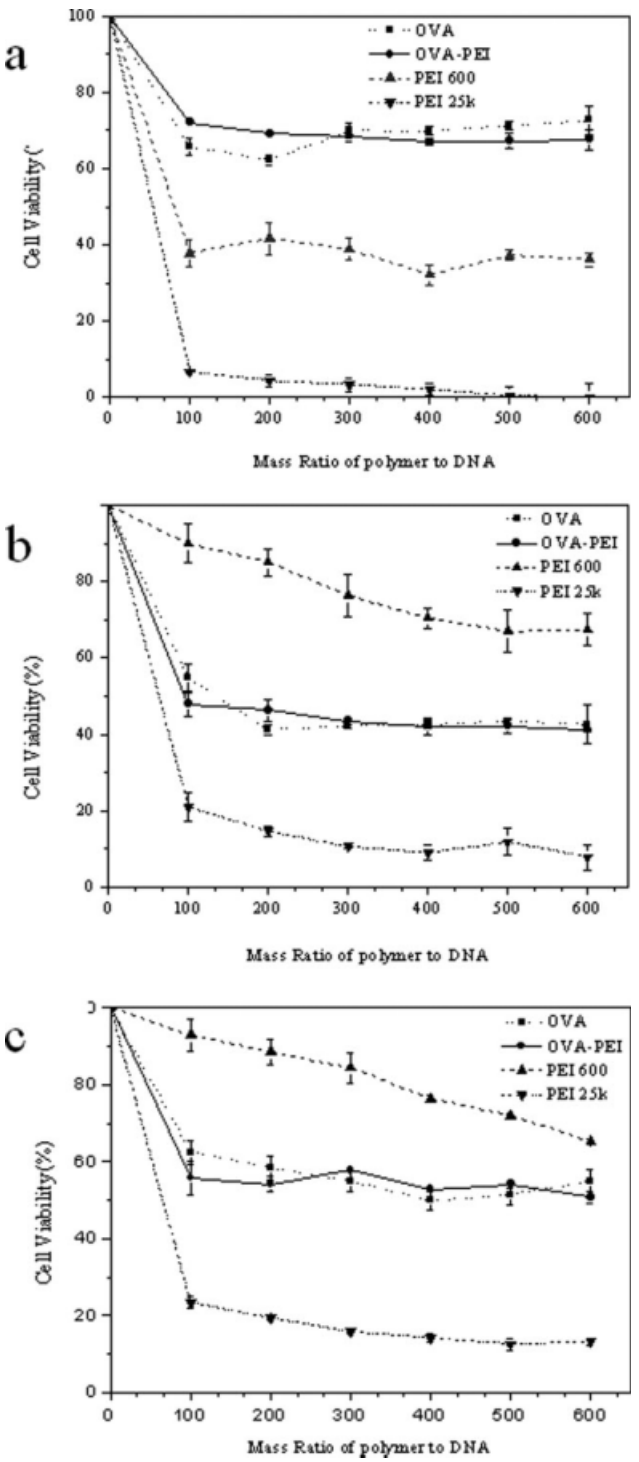


Figure 4 Cell viability: (a) HepG2, (b) A549, and (c) SKOV-3 cell lines after 4 h of incubation with OVA, OVA-PEI600, PEI25 kDa, and PEI600.

than PEI600 in HepG2 cells (Fig. 4). Even at a weight ratio of 600, the cell viability was close to 60%. But for PEI25 kDa, when the weight ratio reached 100 μg/mL, the cell viability was only 20%. It was found that after conjugating PEI600 to OVA, the property of low cytotoxicity of PEI600 was maintained.

OVA-PEI600-mediated *in vitro* gene delivery

The transfer activity *in vitro* of the OVA-PEI600 was evaluated on SKOV-3 [Fig. 5(a)] and HepG2 [Fig. 5(b)] cell lines. When the weight ratio was 400, the efficiency of OVA-PEI600/DNA reached 5.1 E +08 RLU/mg protein on HepG2 cells. The efficiency was 3.3-fold higher than PEI600/DNA complexes. The same results were obtained in SKOV-3 cell lines. These results showed that when PEI600 was conjugated to the surface of OVA, the density of positive charge on the OVA was increased, and the transfer activity was increased. And the capability of transferring across the nuclear membrane was increased because the OVA acted as a core.

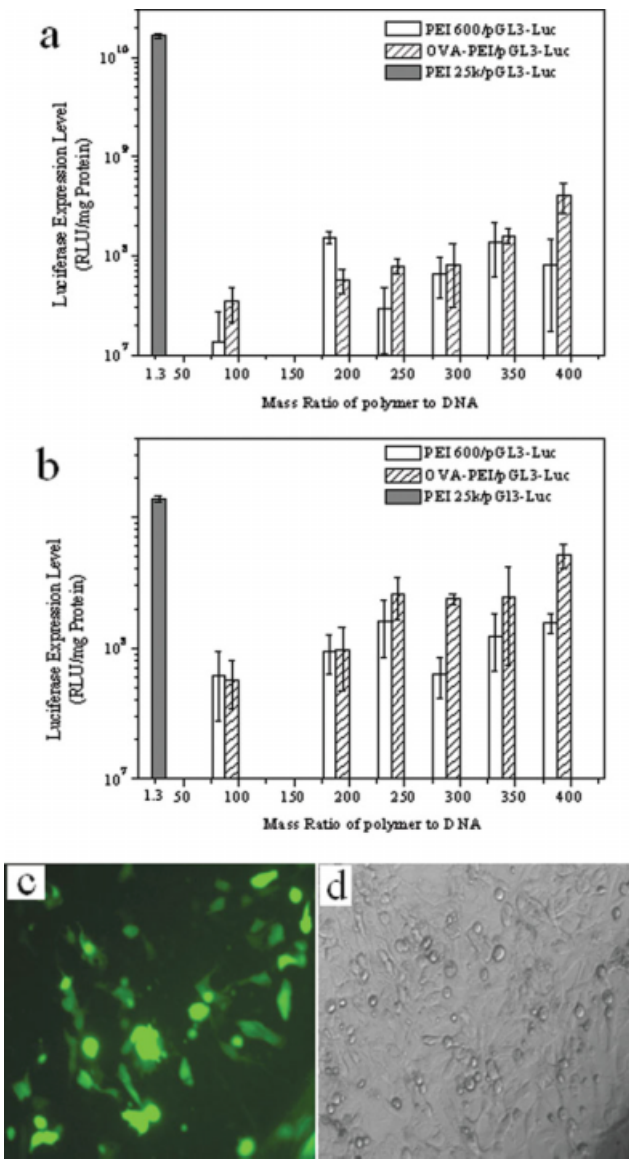


Figure 5 (a, b) Luciferase expression in HepG2 and SKOV-3 cell lines transfected by OVA-PEI600/DNA complexes at different weight ratios. Data are shown as mean ± SD ($n = 3$). For control group of PEI25 kDa, the mass ratio was 1.3. (c, d) Fluorescence microscopic images in HePG2 cell lines after administration of OVA-PEI600/DNA complexes at a weight ratio of 400. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

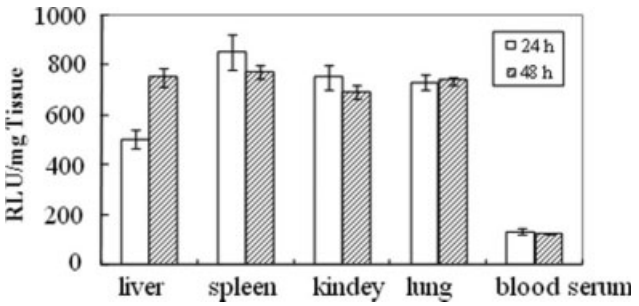


Figure 6 Luciferase gene expression at 24 and 48 h after tail vein administration of OVA-PEI600/DNA complexes at weight ratio of 400.

pEGFP transfections were performed in a similar manner as the luciferase experiments, but were scaled up to 24-well plate format. HepG2 cell lines were transfected using OVA-PEI600/DNA complexes at a weight ratio of 400, and PEI600 and PEI25KDa were used as controls in the experiment. Figure 5(c,d) shows the results of the transfer activity, which was over 30%. The experimental results suggested that the protein based on polycation might have great prospect in the gene therapy for cancer. In this experiment, we found that the presence of the protein OVA could reduce the interactions with additional serum components.

Distribution of OVA-PEI600 in organs

OVA-PEI600/DNA compleses was tested for systemic application in normal mice (Fig. 6). Transfection complexes were injected into mice via tail vein. For assessing the distribution of OVA-PEI600/DNA compleses for gene delivery, lucferase activity was measured in various organs at 24 and 48 h after injection. The results showed that no significant toxicity was found for OVA-PEI600 under experimental conditions. Luciferase activity could be tested in liver, spleen, kidney, lung, and blood serum at 24 and 48 h. Significant gene expression was observed in spleen and kindey at 24 h and in spleen and liver at 48 h. This showed that OVA-PEI600/DNA complexes could express gene in tissues. Other

section work about tumor bearing experiment were progressing.

CONCLUSIONS

More and more functional vectors with degradable, biocompatible, and chemically defined properties were synthesized for nonviral gene delivery. Except polymer, modification of protein, peptide, and antibody was attended in this study. A novel gene vector, OVA-PEI600, was prepared by grafting reaction between low-molecular-weight PEI and OVA. Though OVA was an immunostimulating protein, we used it as a model to construct a way to conjugate PEI to protein, peptide, and antibody. The gel retardation assay, particle size and zeta-potential measurements, and morphology observation of OVA-PEI600/DNA complexes showed that it was able to condense DNA efficiently. The *in vitro* cell viability assay as well as transfection indicated that OVA-PEI is a promising vector for gene delivery.

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