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# Preparation and Characterization of Cationic PLGA-PEG-Lf/DOPE Nanoparticles for HO-1 Gene Delivery

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**Abstract:** Cationic nanoparticles (NPs) for gene delivery were successfully prepared by assembling carboxylation poly(lactic-co-glycolic acid) (PLGA), polyethylene glycol (PEG), L-α-Phosphatidylethanolamine (DOPE) and octadecyl quaternized carboxymethyl chitosans (OQCMC). Lactoferrin (Lf) was selected as a targeting ligand conjugated to PLGA via bifunctional PEG, yielding PLGA-PEG-Lf/DOPE NPs to be used for gene vectors. Fourier transform infrared spectroscopy (FTIR), UV and nuclear magnetic resonance (NMR) spectroscopy were performed to evaluate the synthesis of the vectors. The characteristics of the vectors loaded heme oxygenase (HO-1) gene were evaluated by transmission electron microscope (TEM), particle size analyser and fluorescent microscopy. The experimental results showed that the obtained vectors were spherical in shape with average particle size of 142.2 nm and zeta potentials of +16.4 mV. The vectors could protect the loaded gene from the degradation by nuclease. For 293T cells, there is high transfection efficiency of the vectors similar to liposome-2000. It can be concluded that the established cationic PLGA-PEG-Lf/DOPE NPs have potential gene delivery ability for gene therapy.

**Key words:** PLGA nanoparticles; PEGylation; gene delivery; lactoferrin

## 1 Introduction

Gene therapy has rapidly become an advancing field with enormous potential to treat different vital human diseases[1]. It is very important for successful gene therapy to develop potent gene transfer systems that can deliver foreign gene efficiently and safely into target cells and region. The non-viral vector, with significantly low safety risk and convenient preparation, has been suggested as an alternative to viral vector[2]. Therefore, to develop a safe and effective nonviral vector system become urgent. Among non-viral vectors, cationic gene delivery polymers include poly(ethylenimine) (pEI), chitosan, poly(2-dimethylaminoethyl methacrylate), and poly-L-lysine (pLL) have been studied a lot[3,4]. However, there are also some drawbacks for these polymers, such as bad degradable property, low biocompatibility and high cytotoxicity[5]. Consequently, there is a need for biodegradable gene delivery systems. Moreover, the degradation of the polymer can be used as a tool to release the plasmid DNA into the cytosol. Recently, the use of nanoparticles prepared with biocompatible and biodegradable poly(lactic-co-glycolic acid) (PLGA) polymer has attracted much attention due to their excellent physicochemical characteristics in terms of safety, stability, the relative ease of large-scale production, and low immunogenicity[6, 7].

Although PLGA nanoparticles might be a promising and suitable candidate for gene delivery application[8, 9], there also exist several drawbacks[10]. The key problem is that low DNA encapsulation or adsorption ratio due to its negative electricity surface. The other is that PLGA nanoparticles tend to bind serum protein in systemic application due to the hydrophobic surface, which can lead to opsonization and clearance by the reticuloendothelial system (RES). Therefore, it is necessary to treat the surface property of PLGA NPs systems by modification to PLGA or by



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adding some specific materials.

Polyethylene glycol (PEG) is commonly a useful modifier due to its high hydrophilicity, low cytotoxicity and high cell permeability[11,12]. Herein, PEG was selected to modify carboxylation PLGA. Furthermore, lactoferrin ligand was used to increase the ability of uptake for cells with related protein receptor[13]. Other strategy is to add cationic surfactants and lipid, such as cetyltrimethyl ammonium bromide (CTAB)[14,15], octadecyl quaternized carboxymethyl chitosans (OQCMC)[16,17] and L-α-Phosphatidylethanolamine (DOPE). In this study, the physicochemical properties and biological properties of these nanoparticles were investigated and characterized.

# 2 Experimental

## 2.1 Materials

HO-PLGA-COOH (LA/GA=50/50, MW 5000) was purchased from Jinan Daigang Biomaterial Co., Ltd (Jinan, China). The plasmid pEGFP (Clontech, Palo Alto, CA, USA) vector was purified using QIAGEN Plasmid Mega Kit (Qiagen GmbH, Hilden, Germany). PVA, L-α-Phosphatidylethanolamine (DOPE), 5,5-Dithiobis (2-nitrobenzoic acid) (Ellmann's reagent) and 2-iminothiolane hydrochloride (Traut's reagent) were purchased from Sigma-Aldrich. α-Malemidyl-ω-N-hydroxysuccinimidyl polyethyleneglycol ($NH_2$-PEG-Mal, MW 3500) was obtained from Jenkem technology Co., Ltd (Beijing, China). DNase I was purchased from Takara (Dalian, China).

## 2.2 Synthesis of PLGA derivatives

HO-PLGA-COOH was reacted with $NH_2$-PEG3500-Mal 1:2 (mol/mol) in DMSO for 24 hours at room temperature by adding 1,1'-Carbonyldiimidazole (CDI). The primary carboxyl groups on the surface of HO-PLGA-COOH were specifically reacted with the amino groups of the bifunctional PEG derivative. The resulting conjugate, PLGA-PEG-Mal, was purified by dialysis method (cut off = 5 kDa) against PBS (pH 7.4) buffer. At the same time, Lf was thiolated using Traut's reagent as described[18], and the extent of thiolation was determined by Ellmann's reagent[19] (3 thiol groups per Lf). Then PLGA-PEG-Mal was reacted with thiolated Lf, 1:1 (mol/mol) in PBS (pH 7.0) for 24 h at room temperature. The Mal groups of PLGA-PEG-Mal were specifically reacted with the thiol groups of thiolated Lf.

## 2.3 Characterization of PLGA-PEG-Lf

The characteristics of PLGA-PEG-Lf were analyzed by Fourier transform infrared spectroscopy (FTIR), UV-Vis and nuclear magnetic resonance (NMR) spectroscopy. FTIR spectra were recorded with KBr pellets on a Bio-Rad FTS 6000 spectrometer (Bio-Rad Company, Hercules, California, USA) at room temperature. For the UV-Vis study, five samples (Lf, PLGA, $NH_2$-PEG-Mal, PLGA-PEG and PLGA-PEG-Lf) were scanned in the range of 200–600 nm in a UV 2401PC spectrophotometer (Shimadzu, Japan). For NMR analysis, PLGA-PEG-Lf was solubilized in $CDCl_3$ and analyzed in a 400 MHz spectrometer (Varian, Palo Alto, CA, USA).

## 2.4 Formulation of PLGA NPs

The PLGA NPs were prepared by a modified solvent extraction/evaporation double emulsion method. In brief, 2 mg pluronic F-68 were dissolved in 1.0 mL deionized water as the internal aqueous phase. Organic phase was prepared by dissolving 20 mg PLGA in 2.5 mL dichloromethane/acetone (4:1 *v/v*) solution. Then, the internal aqueous solution was slowly dropwise added into organic phase to form stabilized original emulsion under sonication for 120 s at 100 W output. The formed emulsion was poured into 10ml of an aqueous phase containing 5 mg PVA, which was sonicated for 180 s at 200 W output. The double emulsion was evaporated to allow the organic solvent to completely evaporate. The particles were collected for further use. For PLGA-PEG/DOPE NPs and PLGA-PEG-Lf/DOPE NPs, 1 mg DOPE was dissolved in organic phase and PVA was replaced by equal weight of OQCMC.

## 2.5 Particle size and size distribution

Average particle size and size distribution of the NPs were measured by laser light scattering (LLS, 90 Plus Particle Size, Brookhaven Instruments Co. USA). NPs was diluted with deionized water and sonicated before measurement.

## 2.6 Surface morphology

The shape and surface morphology of PLGA-PEG-Lf/DOPE NPs were investigated by transmission electron microscopy (TEM). TEM observation of the NPs was carried out at an operating voltage of 150 kV with a JEOL-100CXII (Japan) in bright-field mode and by electron diffraction. Dilute suspensions of NPs in water were dropped onto a carbon-coated copper grid by negatively staining with 2% phosphotungstic acid and then air dried.

## 2.7 HO-1 gene expression in vitro

293T cells were seeded in a 6-well plate at $2.0\times10^5$ cells per well. Cells were incubated in DMEM medium supplemented with 10% serum at 37 °C. When the cells reached about 70%–80% confluence, different wells of cells were transfected with naked HO-1 plasmid and various NPs loaded HO-1 plasmid at differentmolar ratios of total Materials/DNA of 5/1, 10/1, 15/1 and 20/1 at a dose of 1 μg of DNA per well. After a 48 h-incubation at 37 °C, a fluorescent microscope (Leica) was used to qualitatively visualize expression of the enhanced green fluorescent protein (EGFP).

# 3 Results and discussion

## 3.1 Structural characterization of PLGA-PEG-Lf

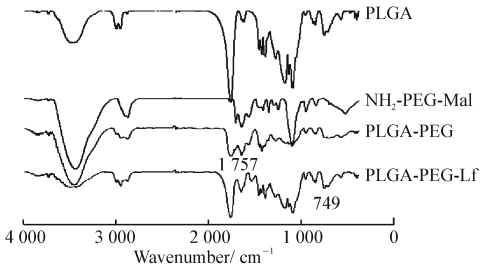


Fig.1 FTIR spectra of PLGA, PEG-Mal, PLGA-PEG and PLGA-PEG-Lf

The FTIR spectra of of PLGA, $NH_2$-PEG-Mal, PLGA-PEG and PLGA-PEG-Lf are shown in Fig.1. PLGA-PEG shows the weaken peak of the COOH-associated band at 1757 $cm^{-1}$, which can be ascribed to the part reaction of $NH_2$ on $NH_2$-PEG-Mal and COOH on HO-PLGA-COOH. Compared with spectra of PLGA-PEG, characteristic peaks of $NH_2$ for surface external bending between 600 $cm^{-1}$ and 900 $cm^{-1}$ in PLGA-PEG-Lf spectra have been strengthened for appearance of Lf.

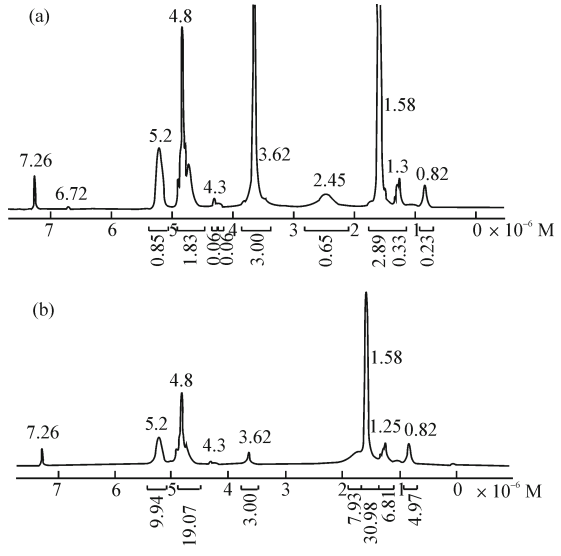


Fig.2 $^1$H-NMR spectra of PLGA-PEG (a) and PLGA-PEG-Lf (b)

The $^1$H-NMR spectrum of PLGA-PEG and PLGA-PEG-Lf are shown in Fig.2. In spectra of PLGA-PEG, the peak at δ=7.26 was attributed to the solvent of $CDCl_3$. The most intensive signals at $\delta$=0.82-1.58, and 4.3-5.2 were attributed to the framework peaks of PLGA. Two peaks at $\delta$=3.62 and 6.7 were attributed to the characteristic peaks of methylene group on PEG and hydrogen on Mal, respectively, which showed the successfully graft modification of $NH_2$-PEG-Mal to PLGA. In spectrum of PLGA-PEG-Lf, the framework peak of PLGA still existed. The intensity of the characteristic peak at δ=3.62 (methylene group on PEG) is weakened by the modification of Lf. The disappearance of peak at δ= 6.7 can be ascribed to the specific reaction of Mal to sulfhydryl group of Lf.

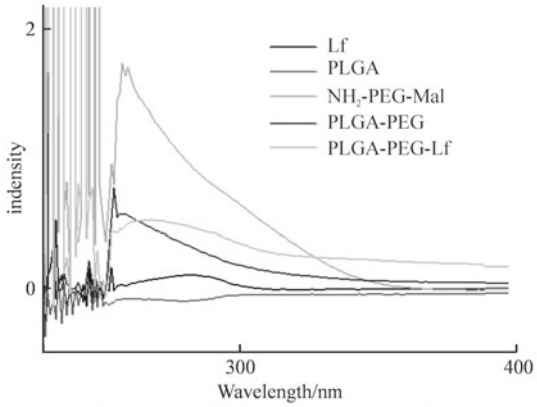


Fig.3 UV-vis spectra of Lf, PLGA, PEG-Mal, PLGA-PEG and PLGA-PEG-Lf in DMSO

UV-vis spectra of Lf, PLGA, PEG-Mal, PLGA-PEG and PLGA-PEG-Lf in DMSO are shown in Fig.3. It can be seen that the characteristic broaden peak at 280 nm of Lf changed little in DMSO solvent, although with weakened intensity. PEG-Mal shows one characteristic peak at 255 nm. In spectra of PLGA-PEG, the peak at 255 nm also exists only with decreased intensity, which also verified the successfully graft modification of $NH_2$-PEG-Mal to PLGA. With disappear of characteristic peak at 255 nm and appear of broaden peak at 280 nm in PLGA-PEG-Lf UV-vis spectra, it can be concluded that Lf with sulfhydryl group is succesufully grafted in PLGA-PEG-Mal. The results also reveal that the solubility ability of different materials in DMSO are in the following order: PEG-Mal＞PLGA-PEG＞PLGA-PEG-Lf＞Lf＞PLGA.

## 3.2 Evaluation of particle size, charge, and transfection efficiency

Table 1 shows a representative mean diameter and zeta potential of these PLGA NPs. The mean diameter of PLGA-PEG and PLGA-PEG-Lf were 139.8 ± 32.7 and 167.1 ± 37.6, respectively, and demonstrating a relatively narrow particle size distribution. It can be seen that the mean diameter of PLGA NPs have been decreased by adding polymeric surfactant of octadecyl quaternized carboxymethyl chitosans (OQCMC), which is coincident with reported[16]. Zeta potential of PLGA-PEG and PLGA-PEG-Lf were +5.1 ± 1.2 mV and +6.6 ± 1.5 mV. From table 1 it can be seen that zeta potentials of PLGA NPs also is increased by adding OQCMC and DOPE.

293T cells were selected by transfected with phosphate buffered saline (PBS), naked plasmid,

**Table 1 Mean diameter and Zeta potential analyses of nanoparticles**

| Sample | Mean diameter/nm | Polydispersity/($\mu/\Gamma^2$) | Zeta potential/mV |
|---|---|---|---|
| PLGA-PEG | 139.8 ± 32.7 | 0.191 | +5.1 ± 1.2 |
| PLGA-PEG-Lf | 167.1 ± 37.6 | 0.219 | +6.6 ± 1.5 |
| PLGA-PEG/DOPE | 121.3 ± 29.1 | 0.384 | +23.9 ± 5.3 |
| PLGA-PEG-Lf/DOPE | 142.2 ± 35.3 | 0.264 | +16.4 ± 3.6 |

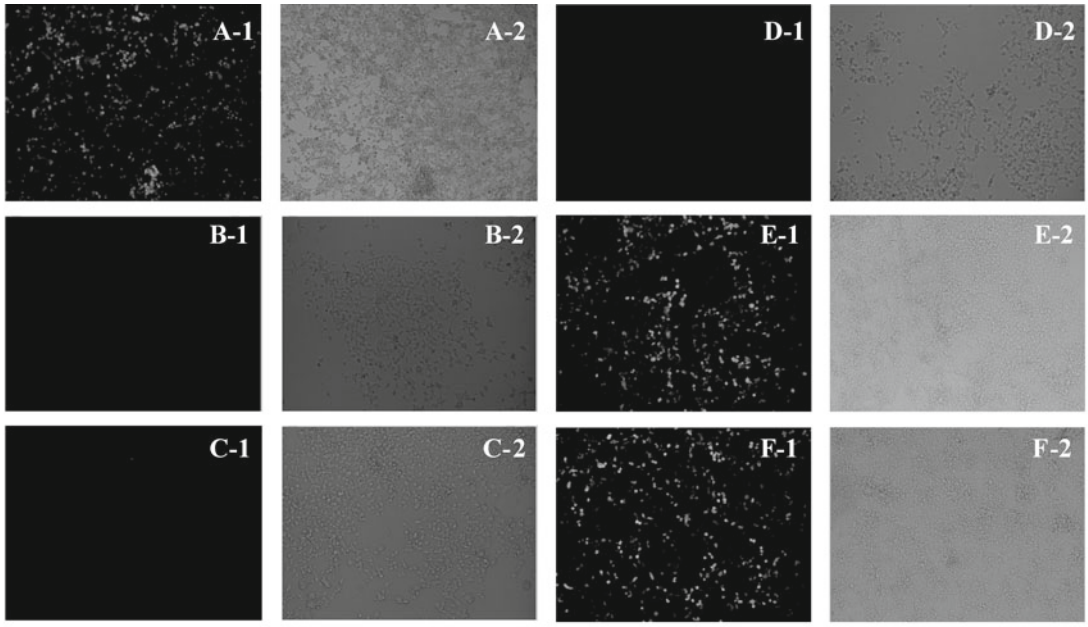


Fig.4 Comparison of fluorescent intensity in 293T cells. The cells were seeded in a 24-well plate and incubated in DMEM medium supplemented with 10% serum at 37 ℃. Different wells of cells were transfected with lipofectamine-2000 (A-1, A-2), PLGA NPs (B-1, B-2), PLGA-PEG NPs (C-1, C-2), PLGA-PEG-Lf NPs (D-1, D-2), PLGA/DOPE Nps (E-1, E-2) and PLGA-PEG-Lf/DOPE NPs (F-1, F-2) at varied weight ratios of total Materials/DNA of 5/1, 10/1 and 15/1 at a dose of 1 $\mu$g of DNA per well. EGFP plasmid was used. At 48 h fluorescent microscopy was performed

different PLGA NPs/DNA complex (at different CPL/DNA weight ratios of 5/1, 10/1 and 15/1) and liposome-2000/DNA complex. The results revealed that the transfection efficiency are in the following order: liposome-2000/DNA＞PLGA NPs with DOPE/DNA ＞PLGA NPs without DOPE/DNA＞naked plasmid＞ PBS. It can be concluded that transfection efficiency can be highly increased by adding polymeric surfactant OQCMC and DOPE. In this NPs system, OQCMC not only provided positive charge for nano-sized particles[20], but also acted as emulsifying agent. DOPE, with fusogenic property, can improve transfection capacity[21].

Although the transfection efficiency of PLGA NPs with DOPE was lower than liposome-2000, the results observed by inversion fluorescence microscope after transfection (Fig.4) still showed that PLGA NPs with DOPE/DNA complex may successfully transfer plasmid HO-1 DNA into 293T cells, and the gene can encode the green fluorescent protein.

### 3.3 Characterization of PLGA-PEG-Lf/DOPE NPs

The transmission electron micrograph of PLGA-PEG-Lf/DOPE NPs is illustrated in Fig.5. These PLGA NPs are generally spherical in shape with narrow particle size distribution. The hydrodynamic diameter of PLGA-PEG-Lf/DOPE NPs is 142.2 ± 35.3 nm, which is greater than the TEM diameter because of the hydration of polymeric liposomes bilayer in aqueous solution.

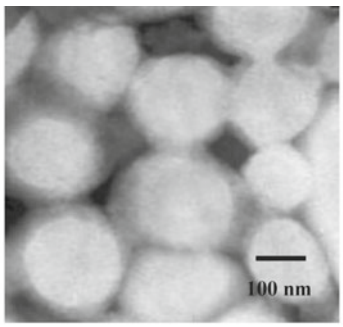


Fig.5 TEM image of PLGA-PEG-Lf/DOPE Nps

Agarose gel electrophoresis was performed to investigate the optimal ratio of NPs to DNA for the binding efficiency qualitatively and quantitatively. It was observed that naked plasmid DNA could migrate to the positive electrode under the electric field such as lane 1 and 8 in Fig.6. Once DNA was associated

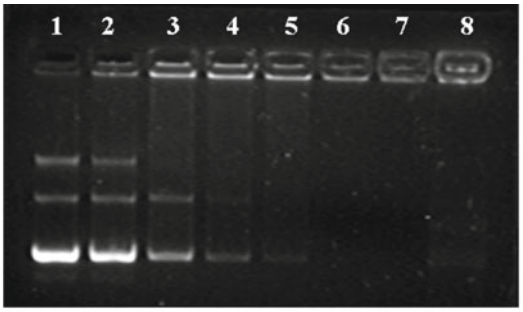


Fig.6 Agarose gel electrophoresis analysis of PLGA-PEG-Lf/DOPE combining with DNA at different mass ratio. Lane 1: DNA control; Lane 27: the mass ratio of NPs/DNA was 5:1, 15:1, 20:1, 25:1,30:1, 40:1, respectively; Lane 8: complex incubated with DNAse I, the mass ratio of NPs/DNA was 40:1

with the nanoparticles which was too large to diffuse through the agarose matrix, the mobility of DNA was hindered and it was detained in the well of the agarose gel. In addition, the part of DNA which did not bind with PLGA-PEG-Lf/DOPE NPs migrated to the positive electrode in the same manner with the control DNA. The results of agarose gel electrophoresis (Fig.6) showed that PLGA-PEG-Lf/DOPE NPs may bind with DNA to various degrees with different mass ratios of NPs to DNA in the complex. When the mass ratio of NPs to DNA reached 100:1 or above, almost all DNA was combined with NPs without free DNA bands in the lane visible.

# 4 Conclusion

Cationic PLGA-PEG NPs for gene delivery were successfully prepared by using PLGA-PEG, DOPE and OQCMC. A cell-targeting ligand Lf was constructed in the PLGA-PEG NPs via assembling PLGA-PEG-Lf and DOPE. Report gene, EGFP, was adsorbed onto the surface of the cationic PLA-PEG/DOPE NPs through electrostatic interactions. The obtained PLGA-PEG-Lf/DOPE NPs, with positive surface charge (+16.4 mV) and small particle size (142.2 nm), could successfully transfer plasmid EGFP into 293T cells compared with PLGA-PEG and PLGA-PEG-Lf NPs without adding DOPE and OQCMC. It can be anticipated that the established PLGA-PEG-Lf/DOPE NPs was a promising non-viral gene delivery system used in gene therapy.

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