

Original Article

A novel polymer-lipid hybrid nanoparticle for efficient nonviral gene delivery

Jian LI¹, Ying-zi HE¹, Wen LI², Yun-zhen SHEN², Yu-ru LI^{3,*}, Yun-feng WANG^{2,*}

¹Institutes of Biomedical Sciences of Fudan University, Shanghai 200032, China; ²Department of Otolaryngology, Affiliated Eye and ENT Hospital of Fudan University, Shanghai 200031, China; ³Department of Otolaryngology, the First Hospital of Harbin Medical University, Harbin 150001, China

Aim: To develop a novel non-viral vector with high transfection efficiency and low cytotoxicity.

Methods: Poly (ethylene glycol)-distearoylphosphatidylethanolamine (PEG-DSPE) was incorporated into polymer-lipid hybrid nanoparticles (PLN) to construct a PEG-DSPE modified long circulating PLN (L-PLN). The L-PLN was prepared by the emulsifying-solvent evaporation method, L-PLN and L-PLN/DNA complexes were characterized. Both HEK293 and MDA-MB-231 cells transfected by L-PLN/DNA complexes were observed under a fluorescence microscope. The transfection efficiency of the complexes to HEK293 cells was further evaluated by flow cytometry.

Results: The GFP fluorescence intensity in HEK293 cells transfected by the L-PLN/DNA complexes (N/P=10) was about 37.2%, which was higher than those transfected by PLN alone or commercial Lipofectamine™ 2000. The L-PLN exhibited minimal toxicity at a low N/P ratio compared with other vectors.

Conclusion: L-PLN as a novel gene delivery system, has higher transfection efficiency and acceptable cytotoxicity compared to the corresponding PLN, which is beneficial for the development of non-viral gene transfer vectors and may offer an alternative strategy for the future gene therapy.

Keywords: polymer-lipid hybrid nanoparticles; non-viral vectors; polyethylenimine; poly(ethylene glycol)-distearoylphosphatidylethanolamine; triolein; gene delivery

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Introduction

In the past decades, numerous gene delivery systems have been developed to transfect various cells *in vitro* and *in vivo*. However, a safe and efficient gene delivery remains the most challenge of human gene therapy^[1]. Generally, gene transfer systems are basically classified into viral and non-viral vectors. The non-viral vector is a promising alternative to recombinant viral vector in gene delivery due to their potential advantages in low immunogenicity and ease of preparation^[2–4].

Polyethylene glycol (PEG) derivative supplement at the carrier surface can obtain the long-circulating colloids. Poly(ethylene glycol)-distearoylphosphatidylethanolamine (PEG-DSPE) has been widely used in the development of drug delivery systems such as stealth liposome, micelles and long circulating lipid nanoparticles^[5, 6]. However, the PEG-DSPE has not been applied in non-viral gene transfer vectors. Fur-

thermore, previous study has developed polymer-lipid hybrid nanoparticles^[7, 8] as a non-viral gene transfer vector that possessed the advantages of a polymeric carrier and a lipid formulation. Therefore, we hypothesized that PEG-DSPE could enhance the transfection efficiency of non-viral gene transfer vectors. In our study, PEG-DSPE was incorporated into the polymer-lipid hybrid nanoparticles (PLN) formulation as PEG-DSPE modified PLN (L-PLN) to develop a novel non-viral vector with high transfection efficiency and low cytotoxicity.

Materials and methods

Cell culture

Human embryonic kidney (HEK) 293 cells and human breast cancer MDA-MB-231 cells were kindly provided by the center lab of Affiliated Eye and ENT Hospital of Fudan University (Shanghai, China). The two kinds of cells were maintained at 37 °C in a humidified atmosphere with 5% CO₂ at different cell culture dishes. These cells were cultured in RPMI 1640 and DMEM (Gibco BRL, USA) supplemented with 10% fetal bovine serum (FBS, Hyclone, USA), 100 U/mL penicillin and 100 µg/mL streptomycin.

* To whom correspondence should be addressed.

E-mail wyf80612@gmail.com (Yun-feng WANG);

yuruli612@gmail.com (Yu-ru LI)

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L-PLN preparation and measurement

In this study, L-PLN was made according to early reports using emulsion-solvent evaporation method^[8]. Different combination of triolein, polyethylenimine (PEI), egg yolk phosphatidylcholine (EPC) and PEG-DSPE was applied to obtain the optimal PEI/EPC ratio. The triolein/EPC/PEG-DSPE ratio was 1:1:0.05 and different ratio of PEI/EPC was tried, namely 0.05, 0.08, 0.1, 0.2, and 0.4. When PEI/EPC ratio was 0.1, HEK293 transfected cells expressed GFP mostly with highest fluorescence intensity. L-PLN which made up by cetylated PEI (600 Da), triolein, EPC, and PEG-DSPE with molar ratio 0.1:1:1:0.05 was selected. In which cetylated PEI (600 Da) was obtained according to previous published method^[9], triolein (Shanghai National Medicine Co, Ltd, China), EPC (Taiwei Co, Ltd, China) and PEG-DSPE (molar ratio=0.1:1:1:0.05, Germany) were dissolved in dichloromethane (CH₂Cl₂). Then, the mixture was agitated with double-distilled water, and further emulsified by sonication. The evaporated pressure was reduced at 35 °C until the emulsion was clear. Similarly, the corresponding PLN consisting of cetylated PEI, triolein and EPC (molar ratio=0.1:1:1) was prepared. Finally, both L-PLN and PLN solutions were diluted in ddH₂O and their particle size and zeta potential were measured using NICOMPTMZLS 380 at 25 °C.

Preparation of L-PLN/DNA complex

The L-PLN was added to solutions with appropriate dilutions of plasmid DNA at appropriate nitrogen (PEI)/phosphate (DNA) (N/P) ratios and mixed gently^[9]. Then, the mixture solution was incubated for 20 min at 37 °C, and the size and zeta potential were measured. Then, electrophoresis was performed with a voltage of 100 V for 20 min in TAE buffer [40 mmol/L Tris-HCl, 1% (v/v) acetic acid, 1 mmol/L EDTA]. The retardations of complexes at various N/P ratios were stained with ethidium bromide and observed^[10].

In vitro transfection

HEK293 cells were seeded into 24-well plates, 1×10⁵ cells each well, cultured in 0.5 mL of complete medium. The medium was changed into 1 mL of fresh serum-free medium 24 h before transfection^[9]. The L-PLN/DNA (or PLN/DNA) complexes equivalent to 1 μg DNA plasmid encoding the enhanced green fluorescent protein (pEGFP-N2) which was amplified using *Escherichia coli* DH5α (Invitrogen Corporation, USA) and purified at desired N/P ratios were added to each well, and then incubated for 4 h at 37 °C. The medium was then replaced with 0.5 mL fresh complete medium followed by incubation for another 48 h at 37 °C. While those cells transfected with LipofectamineTM 2000 (Invitrogen Corporation, USA)/DNA complexes were treated as positive controls. LipofectamineTM 2000 (2 μL) was gently mixed, added with 1 μg of plasmid DNA and incubated for 20 min at room temperature. The complexes were then added to HEK293 cells. The medium was gently mixed by rocking plate back and forth followed by incubation at 37 °C for 4 h. Subsequently, 0.5 mL fresh complete medium was used instead for 48 h at

37 °C. The whole procedure was repeated for three times. MDA-MB-231 cells were treated in the same way, except cells amount were 5×10⁴ cells/well.

HEK293 cells and MDA-MB-231 cells in 24-well plates were rinsed with PBS, and lysed with 1% reduced Triton X-100 for 30 min, then centrifuged at 3000 round per minute for 10 min. Cells were observed under a fluorescence microscope (Zeiss, Germany), the GFP fluorescence intensity of which was determined by fluorescence spectrum^[9, 11]. Meanwhile, the fluorescence intensity of the supernatant was measured with an excitation wavelength of 493 nm and an emission of 510 nm separately.

Flow cytometry

The plasmid pEGFP-N2 was transfected into HEK293 and MDA-MB-231 cells with the mediation of L-PLN or PLN formulation. After 48 h of incubation, cells were rinsed with cold PBS and harvested, then, fixed in 2% paraformaldehyde solution at 4 °C for 30 min. The fixed cells were rinsed with PBS twice and stored in 0.1% paraformaldehyde solution for further experiment. The GFP positive cells were calculated using FACS flow cytometer (BD Biosciences, USA) by a standard gating technique^[12].

Cell cytotoxicity assay

Cells (1×10⁴) were seeded in each well of 96-well plate for 24 h before cytotoxicity assay. MTT colorimetric assay (Amersco Corporation, USA) was used to detect the toxicity of L-PLN and PLN formulation in HEK293 cells. Cells were transfected at a dose of 100 ng/200 μL with PLN/DNA complexes or L-PLN/DNA complexes at various N/P ratios per well. MTT assay was performed 24 h after transfection^[8]. The cell viability was measured with a microplate reader (BioTek ELx800, USA) at 570 nm of absorbance wavelength for each well.

Statistical analysis

Results are expressed as mean±standard deviation (SD). Statistical evaluation of the data was performed with one-way ANOVA. Pair-wise comparisons were conducted by a Student Newman-Keuls test. Two-tailed *P*<0.05 was considered statistically significant. All analyses were performed using SPSS software version 13.0 (SPSS Inc, Chicago, IL, USA).

Results

Characterization of L-PLN and the L-PLN/DNA complexes

PLN was composed of cetylated PEI (600 Da), triolein and EPC (molar ratio: 0.1:1:1). PEG-DSPE was introduced into the optimized PLN formulation to prepare L-PLN and the molar ratio of PEG-DSPE to EPC was 0.05. The mean particle size of PLN was 128 nm (Figure 1A), which increased slightly after introduction of PEG-DSPE into PLN formulation. The zeta potential of L-PLN formulation was +35.2 mV and did not profoundly change compared to PLN formulation (Figure 1A), which could facilitate the formation of L-PLN/DNA complexes. The distribution of particle size and zeta potential of L-PLN/DNA complexes were determined by NICOMPTMZLS

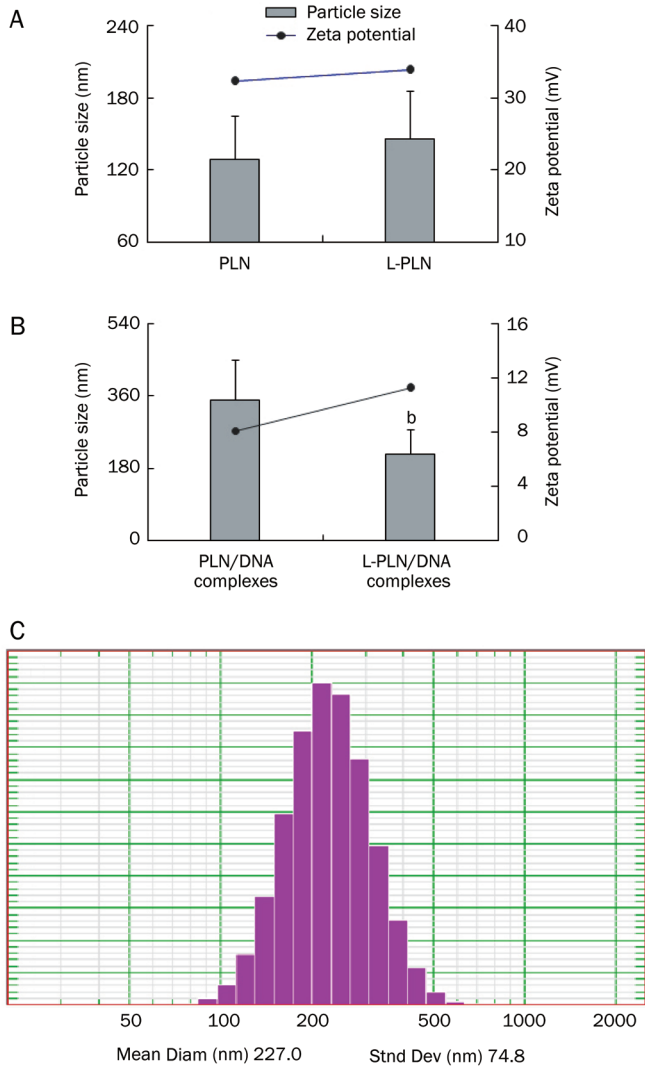


Figure 1. Characterization of the L-PLN and the L-PLN/DNA complexes. (A) Particle size and zeta potential of L-PLN and the corresponding PLN formulation; (B) Particle size and zeta potential of the L-PLN/DNA complexes and PLN/DNA complexes (N/P ratio=10). ^b $P < 0.05$ vs PLN/DNA complexes group; (C) The size distribution of the L-PLN/DNA complexes (N/P ratio=10).

380 (Figure 1B), and gel retardation assay for complex formation at various N/P ratios was also performed to analyze the DNA condensation capacity of PLN and L-PLN formulation. The particle size distribution of L-PLN/DNA complexes (N/P=10) (Figure 1C) was measured by NICOMP™ ZLS 380. The mean particle size of complexes was about 240 nm, which was smaller than PLN/DNA complexes (N/P=10). The complete retardation of complexes could be observed when the N/P ratio was over 10, suggesting that plasmid DNA was completely condensed by PLN (Figure 2A) or L-PLN (Figure 2B) formulations.

In vitro transfection of nanoparticle complex

The nanoparticle complexes were transfected into HEK293 and MDA-MB-231 cells, and the GFP fluorescence intensity

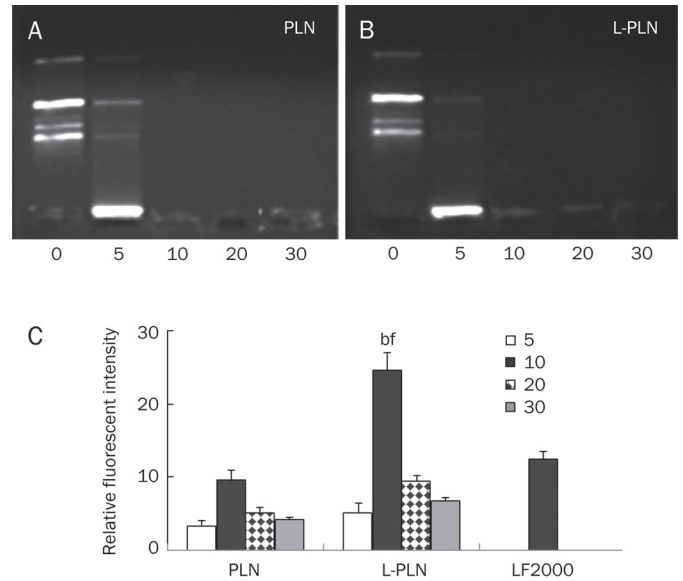


Figure 2. (A) Gel retardation of the PLN/DNA complexes at various N/P ratios. (B) Gel retardation of the L-PLN/DNA complexes at various N/P ratios. (C) Fluorescence intensity of the expressed GFP in HEK293 cells transfected by the L-PLN/DNA complexes and PLN/DNA complexes ($n=3$). The L-PLN or PLN formulation was complexed with 1 μ g plasmid DNA at various N/P ratios. At 48 h after transfection of HEK293 cells, the expressed GFP fluorescence intensity was determined fluorometrically. Transfection of Lipofectamine™ 2000 (LF2000) was performed as positive control. ^b $P < 0.05$ vs LF2000, ^f $P < 0.01$ vs PLN.

of which after 48 h of incubation were determined by fluorescence spectrum. The relative fluorescence strength was the mean fluorescence intensity of GFP by enzyme-labeled meter after transfection under each N/P condition ($n=3$, Figure 2C). Results indicated that the fluorescence intensity of HEK293 and MDA-MB-231 cells transfected by PLN or L-PLN formulation reached a maximum when the N/P ratio of complexes was 10, and thereafter decreased with the increase of N/P ratio. When PEG-DSPE was not introduced into PLN formulation, the transfection efficiency was comparable to that of commercial Lipofectamine™ 2000. Nevertheless, after introduction of PEG-DSPE into optimized PLN formulation, the GFP fluorescence intensity of HEK293 and MDA-MB-231 cells transfected with L-PLN/DNA complexes (N/P=10) was significantly higher, indicating that the transfection with L-PLN was more efficient than that with PLN formulation or Lipofectamine™ 2000.

Transfection efficiency of L-PLN in HEK293 cells and MDA-MB-231 cells

The transfection efficiency of PEG-DSPE modified PLN formulation was assessed in two strains of cells (HEK293 cells and MDA-MB-231 cells) and compared with that of the positive controls. The GFP expressions in both cells transfected by the optimal L-PLN/DNA (N/P=10) or PLN/DNA complexes (N/P=10) were detected. The GFP fluorescence intensity in HEK293 and MDA-MB-231 cells were observed respectively at

24 h after transfection, and the expression reached a maximum at d 2 and 4. GFP expression in the two kinds of cells transfected by the Lipofectamine™ 2000/DNA complexes, PLN/DNA complexes (N/P ratio=10) and L-PLN/DNA complexes (N/P ratio=10) were observed under fluorescence microscopy (Figure 3). The number of GFP positive HEK293 cells was counted by a FACScan flow cytometer with standard gating technique (Figure 4). MDA-MB-231 cells were treated in the same way (data not shown). At a dose of 1 μg of DNA/well, the percentage of HEK293 and MDA-MB-231 cells transfected by PLN/DNA complexes (N/P ratio=10) was about 21.9% and 20.1%, which was comparable to that of cells transfected by Lipofectamine™ 2000/DNA complexes (about 19.3% and 18.5%). However, after introduction of PEG-DSPE into PLN formulation, the number of HEK293 and MDA-MB-231 cells transfected by L-PLN/DNA complexes (N/P=10) was about 37.2% and 34.3%, which was significantly higher than that of PLN formulation and commercial Lipofectamine™ 2000.

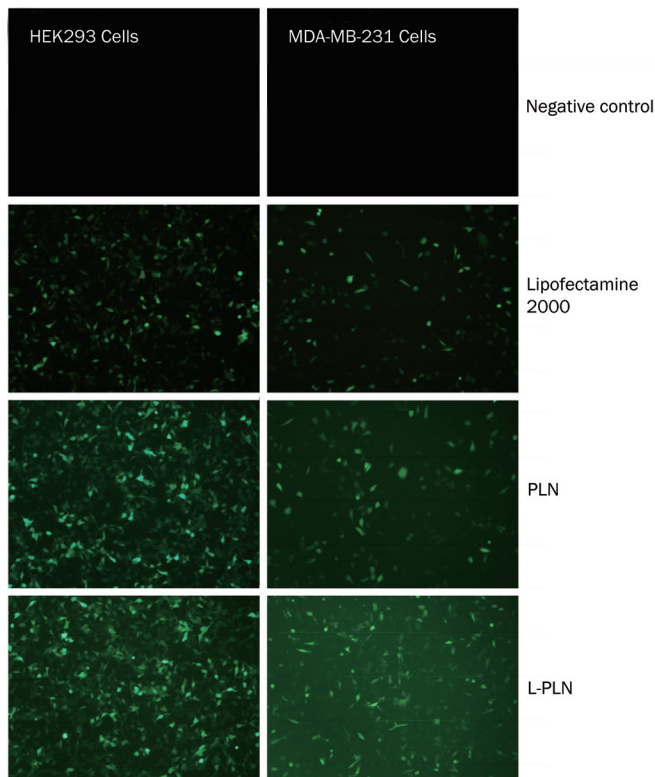


Figure 3. Fluorescent images of HEK293 cells and MDA-MB-231 cells after 48 h transfection of the Lipofectamine2000/DNA complexes, the optimized PLN/DNA complexes (N/P ratio=10) and the L-PLN/DNA complexes (N/P ratio=10).

Cytotoxicity assay

The cell viability was tested in the presence of L-PLN/DNA complexes at various N/P ratios (Figure 5) and cell without transfection were used as controls, which viability was set as 100%. Results found that both L-PLN and PLN formulation

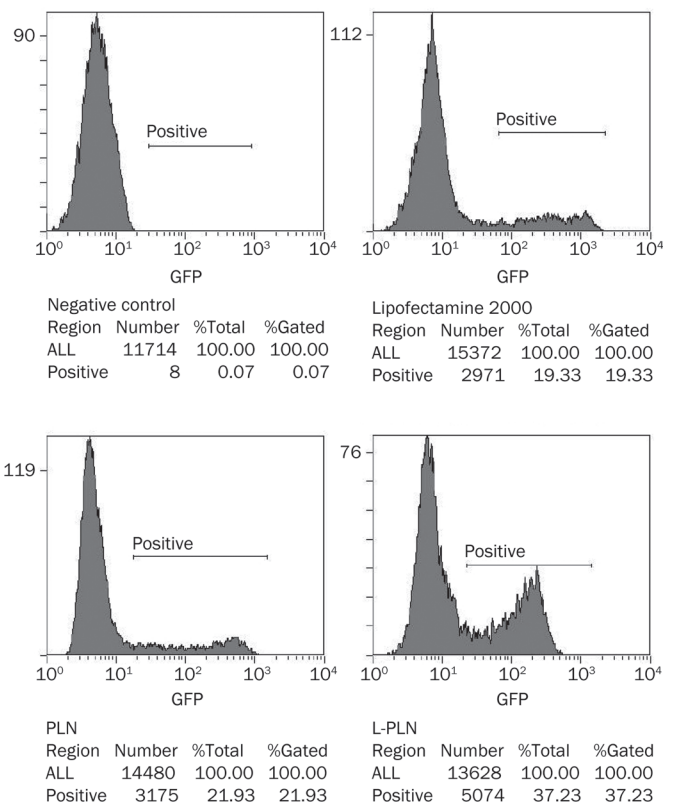


Figure 4. Transfection efficiency of the Lipofectamine2000/DNA complexes, the optimized PLN/DNA complexes (N/P ratio=10) and the L-PLN/DNA complexes (N/P ratio=10) in the absence of serum at 48 h after transfection. The transfection efficiency in HEK293 cells was quantified by FACS analysis.

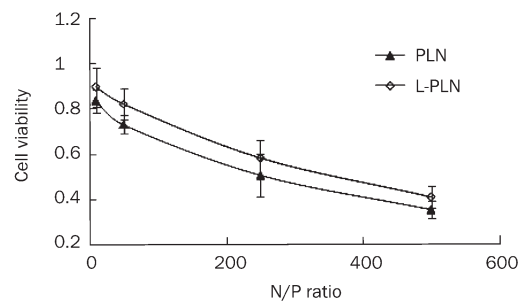


Figure 5. Cytotoxicity of the L-PLN/DNA complexes and PLN/DNA complexes in HEK293 cells at various N/P ratios ($n=6$).

exhibited minimal toxicity at low N/P ratios and the cells had relatively high viability, in which L-PLN formulation had less toxic effect.

Discussion

In non-viral gene delivery systems, synthetic polycations and cationic liposomes have been widely investigated^[13-15]. Synthetic polycations or cationic lipid in cationic liposomes can form a complex with anionic DNA. After endocytosis of complexes, these systems can deliver DNA through endosomes.

The polyethylenimine/DNA complexes are taken up into mammalian cells via the endosomal pathway and released into the cytoplasm from endosomes due to the proton-sponge effect^[16]. On the other hand, in the cationic liposomes composed of cationic lipids and dioleoyl phosphatidylethanolamine (DOPE), it is well known that DOPE can form the inverted hexagonal (HII) phase at a low pH to destabilize the endosomal membrane and enhance transfection efficiency^[17]. Recently, LPD II vectors^[18, 19] and polycation liposomes (PCL)^[20] have been developed with the advantages of both cationic liposomes and polycations.

Efficient transfection has been observed in cultured mammalian cells with the mixtures of univalent cationic lipid dioleoyl trimethylammonium propane (DOTAP) and neutral helper lipid DOPE, but not with those of DOTAP and dioleoylphosphatidylcholine (DOPC)^[21, 22]. Moreover, DOPE is essential in LPD II vectors which are composed of polycationic condensed plasmid DNA trapped in anionic pH-sensitive liposomes. Transfection of LPD II vectors formulated with DOPE has been found to be effective. However, a totally transfection inactivity occurs when LPD II vectors are formulated with non-fusogenic lipid DOPC^[18, 19, 23]. Furthermore, polycationic liposomes (PCL)^[9, 20, 24] composed of cetylated PEI and DOPE were more efficient than that consisting of cetylated PEI and EPC. The impact of DOPE on gene transfection may attribute to the formation of inverted hexagonal (HII) phase^[17, 25, 26] destabilizing endosomal membrane and facilitating the release of plasmid DNA from lysosomes to protect it from degradation.

Polycations have been widely used in gene delivery because they can enable the compaction of DNA to form polyplexes. After uptake of polyplexes via endocytosis, polycation facilitates the escape of polyplexes from endocytosis due to the proton-sponge effect^[16]. Recently, LPD II vectors^[18, 19, 23] and polycation liposomes^[9, 20, 24] have been developed with advantages of both cationic liposomes and polycations, which offers an effective strategy to integrate the effects of inverted hexagonal (HII) phase and proton-sponge effect. Previous studies developed a novel non-viral gene transfer vector PLN consisting of cetylated PEI, triolein, and EPC, which was as effective as the commercial LipofectamineTM 2000. PEG-DSPE can be incorporated into PLN formulation to develop the L-PLN formulation which may combine the effects of inverted hexagonal phase and proton-sponge effect. The results from *in vitro* transfection showed transfection with L-PLN formulation was more efficient and less toxic when compared with PLN formulation. We assumed that it might be due to no significant changes of positive charge of L-PLN after adding DSPE, the ability to enter cell has not decreased. PEG had protective layer, and can reduce the degradation of plasmid DNA by lysosomal enzyme after entering into lysosome. We postulated that PLN formulation incorporated with PEG-DSPE can enhance transfection efficiency, which needs further *in vivo* study.

In conclusion, L-PLN can be developed by modifying PLN formulation with PEG-DSPE and prepared by the emulsifying-solvent evaporation method. It possesses the advantages of

nanoparticles, cetylated PEI and PEG-DSPE, and the transfection of which is more efficient and less cytotoxic than PLN thus might be beneficial for the development of novel non-viral gene transfer vectors.

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Author contribution

Jian LI, Yun-feng WANG, and Yu-ru LI designed the experiments. Jian LI, Wen LI, Yun-zhen SHEN and Yun-feng WANG conducted the experiments. Jian LI, Yun-feng WANG, and Ying-zi HE analyzed the data and prepared the manuscript.

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