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Article

Optimization of a cationic liposomal gene delivery system and study of its endocytic pathway

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A cationic liposomal gene delivery system comprising DOTAP, DOPE, and cholesterol was prepared and optimized. The results showed that the liposome/DNA (LP/DNA) system had spherical morphology, with a particle size of around 150 nm and zeta potential of approximately 30 mV. Cytotoxicity experiments showed that cells treated with all of the liposome carriers—with the exception of LP1—had more than 80% viability even at a weight ratio of 30. The in vitro transfection efficiency was measured using a Promega™ Luciferase Assay System. Of the tested lipoplexes, LP2/DNA showed the highest cell transfection efficiency (at a weight ratio of 10)—which was similar to or slightly lower than that of Lipofectamine® 2000 in HeLa, A549, and SPC-A1 cell lines. After freeze-drying, the cell transfection efficiency decreased slightly ($P>0.05$). The cell uptake mechanism study showed that LP/DNA lipoplexes mainly entered cells via clathrin-mediated and caveolin-mediated endocytic pathways. The results confirmed that LP2 has potential for use as an effective gene carrier, and provides experimental evidence to support its further development as a safe and effective gene delivery system.

Keywords: Cationic liposomes. Gene delivery. Lyophilization. Uptake mechanism.

INTRODUCTION

The continual development of molecular biology has led to the rapid advancement of gene therapy, which now shows great potential for cancer treatment. To date, a lack of effective transmission systems has been a major problem limiting the development of gene therapy (Guo, Huang, 2012). Therefore, finding efficient and safe gene delivery systems had become one of the core issues in gene therapy research.

Since the concept of liposomes was first proposed by Bangham in 1965, they have been widely studied as drug delivery systems. A liposome with at least one bilayer is a spherical vesicle with an aqueous solution core surrounded by a hydrophobic membrane. It has been shown that liposomes can be used to deliver drugs/genes for the treatment of cancer and other diseases (Rasoulianboroujeni *et al.*, 2017). The term lipoplex refers to a liposome and DNA complex that is used to deliver genes (Torchilin, 2006). Recently, cationic liposomes have been intensively studied as gene vectors owing to their high transfection efficiency (compared with traditional liposomes), biological safety, design flexibility, and biocompatibility (Huang *et al.*, 2012; Vitor *et al.*, 2013; Valero *et al.*, 2018). Cationic liposomes are generally composed of cationic lipid and ancillary phospholipid. Cationic lipids such as 2, 3-dioleyloxy-N-[2(sperminecarboxamido)-ethyl]-N, N-dimethyl-1-propanaminium trifluoro acetate (DOSPA), 1, 2-dioleoyl-3-trimethylammonium-propane (DOTAP), and DOGS can provide positive charge on the liposome surface under physiological pH conditions. This allows the liposomes

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to readily combine with DNA through electrostatic adsorption to form nanoscale liposomes, which can be deposited on the cell surface. Ancillary phospholipids such as 1, 2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), 1, 2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), dipalmitoyl phosphatidylcholine (DPPC), and phosphatidylcholines (PC) can stabilize lipid bilayers and promote the intracellular release of DNA, enhancing the transfection efficiency of genes (Hattori *et al.*, 2019; Soema *et al.*, 2015; Nascimento *et al.*, 2015; Ewe *et al.,*2017).

High instability—for example as a result of lipid oxidation, hydrolysis, and liposome aggregation during storage—is a major problem of liposomes, limiting their clinical application (Lin *et al.*, 2015). Lyophilization has been proposed as an effective method of generating dehydrated formulations that are resistant to shipping stresses and offer the potential for prolonged storage at room temperature (Stark, Pabst, Prassl, 2010; Wieber, Selzer, Kreuter, 2012; Di Tommaso *et al.*, 2010).

There have been numerous clinical trials of gene delivery systems based on the use of lipoplexes without sufficient understanding of all of the physico-chemical characteristics responsible for their action (Rasoulianboroujeni *et al.*, 2017). Therefore, gaining a greater understanding of non-viral vectors is expected to aid the development of liposomal gene delivery systems.

In this study, DOTAP, DOPE, and Chol were selected for the preparation of a cationic liposomal gene delivery system and the formulation was optimized for cell cytotoxicity and transfection efficiency. Lyophilization techniques were used to improve the stability of the liposomes, and the cell transfection efficiencies before and after lyophilization were evaluated and compared. In addition, the endocytic pathway of the lipoplexes was studied.

MATERIAL AND METHODS

Reagents

DOTAP, DOPE, and Chol were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL, USA). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and trypsin-EDTA solution were purchased from Beyotime (Shanghai China). Roswell Park Memorial Institute 1640 (RPMI) and Dulbecco's modified Eagle medium (DMEM) were purchased from Hyclone (Logan, UT, USA). Lipofectamine® 2000 reagent was purchased from Invitrogen (Carlsbad, CA, USA). Luciferase Reporter 1000 Assay System for the in vitro transfection assay and Cell Culture Lysis Reagent were purchased from Promega (Madison, WI, USA). Plasmid was amplified with a competent Escherichia coli bacterial strain, DH5α, and purified using a QIAGEN (Chatsworth, CA, USA) kit. Chlorpromazine, wortmannin, and methyl-β-cyclodextrin were purchased from Solarbio® life sciences (Beijing China). Spectra/Pro dialysis bag was purchased from SPECTRUM, Inc. (Rancho Dominguez, CA, USA).

Cell culture

SPC-A1, A549, and HeLa cells were cultured in Roswell Park Memorial Institute 1640 medium (RPMI) or Dulbecco's modified Eagle medium (DMEM) and incubated at 37°C in a humidified incubator with 5% CO_2. 10% fetal bovine serum (FBS), 100 μg/mL streptomycin, and 100 U/mL penicillin were added to the cell culture medium.

Preparation of liposomes

Liposomes (LP) composed of DOTAP, DOPE, and Chol were prepared using the ethanol dilution method (Li *et al.*, 2016). Briefly, DOTAP, DOPE, and Chol were dissolved separately in ethanol and mixed at different molar ratios (Table I). The mixtures were then injected into HEPES buffer (pH=7.4, 5% glucose). After standing for 20 min at room temperature, the solution was transferred to a dialysis bag and dialyzed against pH 7.4 HEPES buffer for 24 h.

TABLE I - The formulation of the cationic liposomes

	DOTAP (mol)	DOPE (mol)	Cholesterol (mol)
LP1	70	15	15
LP2	50	25	25
LP3	40	50	10
LP4	40	30	30
LP5	40	10	50

DOTAP is 1, 2-dioleoyl-3-trimethylammonium-propane, DOPE is 1, 2-dioleoyl-sn-glycero-3-phosphoethanolamine.

Gel retardation assay

The ability of the liposomes to bind DNA was determined using an agarose gel electrophoresis experiment. Briefly, lipoplexes were freshly prepared using different weight ratios of LP to pEGFP-N2, and then 6× agarose loading dye was added to the lipoplexes. The mixtures were incubated for 10 min at room temperature, and then loaded onto 0.8% agarose gels with EtBr (10 mg/mL), and run with tris boric acid EDTA (TBE) buffer at 100 V for 40 min. The gel was observed on a UV illuminator.

Characterization of LP/DNA (pGL3) lipoplexes

The particle size and zeta potential of the lipoplexes were measured at 25°C using a NanoBrook instrument (Brookhaven, USA). The samples were diluted to an appropriate concentration with ultrapure water before testing.

The morphologies of the lipoplexes (WR=10) were observed by transmission electron microscopy (TEM).

Cell viability assays

In vitro cytotoxicity was measured using an MTT proliferation kit. SPC-A1, A549, and HeLa Cells were separately seeded in 96-well plates at an initial density of 1×10^4 cells/well in 200 μL of growth medium and incubated in an atmosphere of CO_2 for 18–20 h to reach 80% confluency. The growth medium was then replaced with 100 μL of fresh serum-free media containing various weight ratios of LP/pGL3 lipoplexes. After incubating for 24 h, 0.5 mg/mL MTT solution was added to each well and cells were further incubated at 37°C for 4 h in an atmosphere of 5% CO_2. The absorbance at 490 nm was then measured using a microplate reader (Thermo scientific, MULTISKAN GO, Shanghai, China).

Cell transfection efficiency measurement

In vitro transfection efficiency was measured using a Promega™ Luciferase Assay System. SPC-A1, A549, and HeLa Cells were seeded in a 24-well plate and incubated for 18–20 h to reach 80% confluency at the time of transfection. The media was then replaced with 500 μL of serum-free media with various weight ratios of LP/pGL3 and further incubated at 37°C in 5% CO_2 for 6 h. The media was then changed for cell culture media and incubation was continued for 24 h. The luciferase assay was performed in accordance with the manufacture's protocol. A chemiluminescence microplate reader (Thermos Scientific, LUMINOSKAN ASCENT, Shanghai, China) was used to detect the relative light units (RLUs). Protein quantification was carried out using a BCA assay, and the RLUs were normalized to the amount of protein in the cell extracts (Shah *et al.*, 2017). The transfection experiments were executed in triplicate, and RLUs were used to express transfection activity.

For microscope observation, HeLa cells were seeded and incubated with LP2/pEGFP-N2 lipoplexes (0.1 μg pEGFP-N2) at a weight ratio of 10 (LP2/pEGFP-N2), and lipofectamine® 2000 was used as a control. The expression of enhanced green fluorescent protein (EGFP) was observed using an inverted fluorescence microscope with 100× magnification.

To investigate techniques for improving long-term sample stability, liposomes were lyophilized immediately after preparation. Two per cent lactose was added as a lyoprotectant. The lyophilized sample was reconstituted with ultrapure water, ultrasonicated, and the particle size and zeta potential were measured. The cell transfection

efficiencies before and after lyophilization were evaluated and compared.

Cellular endocytosis mechanism study

To explore why LP2 had the best gene transfection efficiency, a cell uptake study was conducted. SPC-A1 cells grown to the logarithmic phase were seeded on a six-well plate (4×10^5 cells/well) and cultured at 37°C for 18–22 hours. The medium was then aspirated and LP2/Cy5SE-DNA, LP4/Cy5SE-DNA, and LP5/Cy5SE-DNA containing 5 μg Cy5SE-DNA were added to each well. After incubating for 6 h in the dark, each well was aspirated to remove the supernatant, washed three times with PBS buffer, and observed using a fluorescence microscope.

The cellular endocytosis mechanism of LP was also investigated in this study. Based on previous studies, chlorpromazine, wortmannin, and methyl-β-cyclodextrin were used as the specific endocytic pathway inhibitors (Korang-Yeboah *et al.*, 2015; Kou *et al.*, 2013). SPC-A1 cells were incubated with or without endocytosis inhibitors for 30 min at 37°C, 5% CO_2 atmosphere, then transfected with LP2/pGL3 polyplexes. The transfection efficiencies of the two groups were measured and compared. To exclude the effects of inhibitor cytotoxicity on the transfection efficiency data, the cytotoxicity of the inhibitors was also determined.

Statistical analysis

All analysis was performed using SPSS Statistics 17.0. The results are presented as mean ± standard deviation (SD). One-way analysis of variance (ANOVA) was performed for the comparison among groups. A *P* value less than 0.05 was considered statistically significant.

RESULTS

Preparation of lipoplexes and gel retardation assay

Five liposomes (LP1, LP2, LP3, LP4, and LP5) comprising different amounts of DOTAP, DOPE, and cholesterol (Table I) were prepared using the ethanol dilution method. pEGFP-N2 plasmid coding for the luciferase gene was selected to form the lipoplexes. The interactions between DNA and the LP for different formulations and different weight ratios were assayed by agarose gel electrophoresis.Under the influence of an electric field DNA in an agarose gel can migrate freely from the cathode to the anode because of its negatively charged phosphate groups (Mochizuki *et al.*, 2013). When lipoplexes form, the DNA migration is retarded depending on the condensation ability of the liposomes. As shown in Figure 1, LP2, LP4, and LP5 completely prevented DNA migration at a weight ratio of 10. Because LP3 was unable to condense the DNA, it was excluded from subsequent experiments.

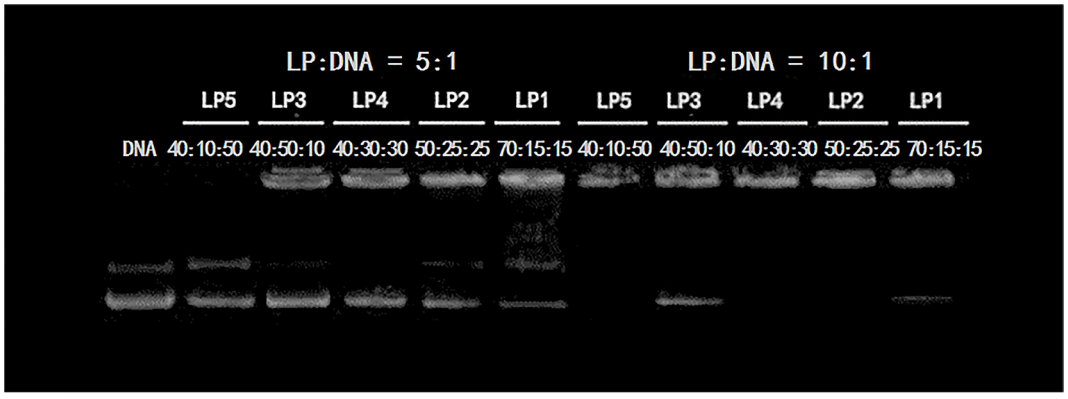

FIGURE 1 - Agarose gel electrophoresis of LP/pGL3 lipoplexes with different formulations and weight ratios.

Characterization of LP/DNA

The ratio of the LP components—DOTAP, DOPE, and Chol—was expected to have a significant influence on the gene transfection efficiency. Therefore, the component ratio was optimized in terms of the resulting particle size, cytotoxicity, and cell transfection efficiency. The particle size and zeta potential of the LP/DNA lipoplexes with different formulations were measured at a weight ratio of 10. As shown in Figure 2, the particle sizes of all of the samples were around 150 nm (Figure 2A), with LP2 being the smallest. The zeta potential of LP1 was −2.53±0.46 mV, while those of the other liposomes were around 30 mV (Figure 2B). The morphology of the LP/DNA lipoplexes was observed by TEM. As shown in Figure 2C, the LP/DNA lipoplexes were spherical in shape and around 150–200 nm in diameter, which was consistent with the particle size data.

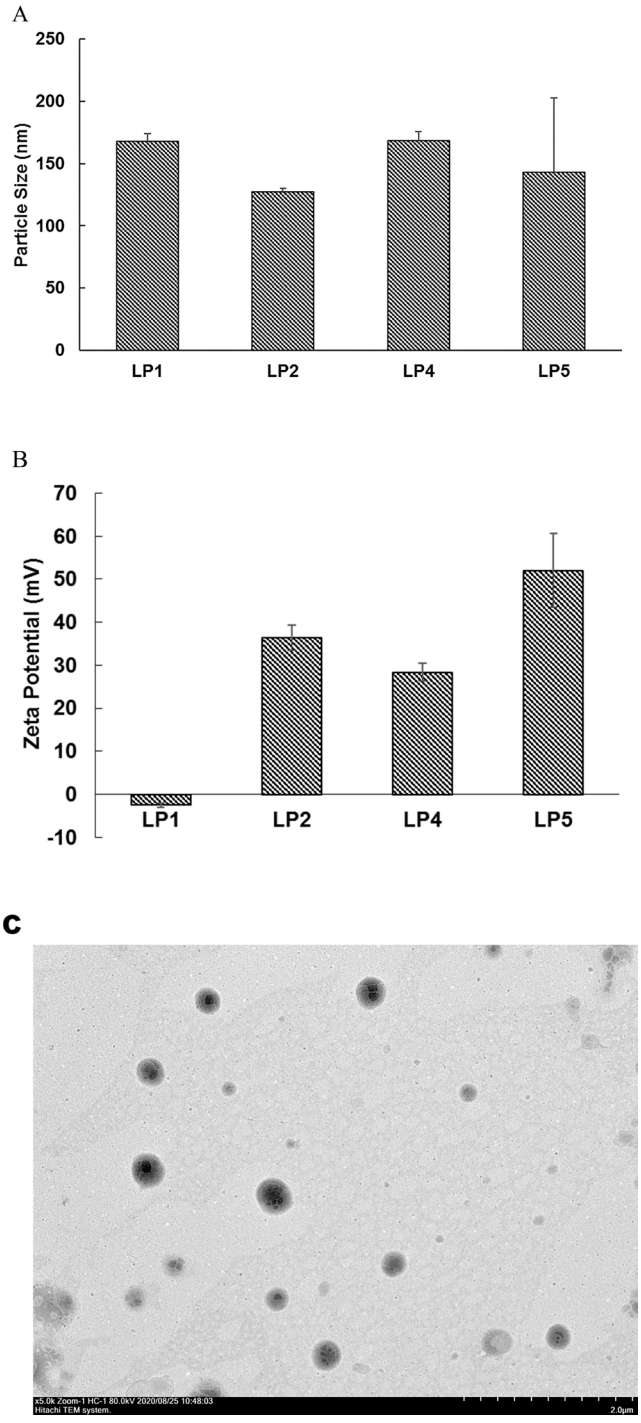

FIGURE 2 - Characterization of LP/DNA lipoplexes (A)-Particle size of LP/DNA at a weight ratio of 10; (B)-Zeta potential of LP/DNA at a weight ratio of 10; (C)-TEM image of LP2/DNA at a weight ratio of 10.

Cell viability

To evaluate the cytotoxicity of LP/DNA, cell viability was measured by MTT assay. As shown in Figure 3—at the same weight ratio—the viability of cells treated with LP1/DNA was the lowest of all treated cells, and it decreased with the increasing weight ratio of LP1 to DNA in the HeLa, SPC-A1, and A549 cell lines. The cytotoxicity and transfection efficiency of gene vectors vary from cell to cell. The cytotoxicity of LP1 with different weight ratios towards SPC-A1 cells was significantly different to that towards HeLa cells. In addition, the cytotoxicity of LP2 towards HeLa cells was only significantly different between the 5:1 and 30:1 groups. The viabilities of the cells treated with LP2/DNA lipoplexes and LP4/DNA lipoplexes were greater than 80% for all of the tested weight ratios. These findings indicate that LP2 and LP4 did not have significant cytotoxicity against the test cell lines, even at relatively high doses (Wang *et al*., 2014). The lower cytotoxicity of the LP2/DNA lipoplexes might be one of the reasons for their higher transfection efficiencies.

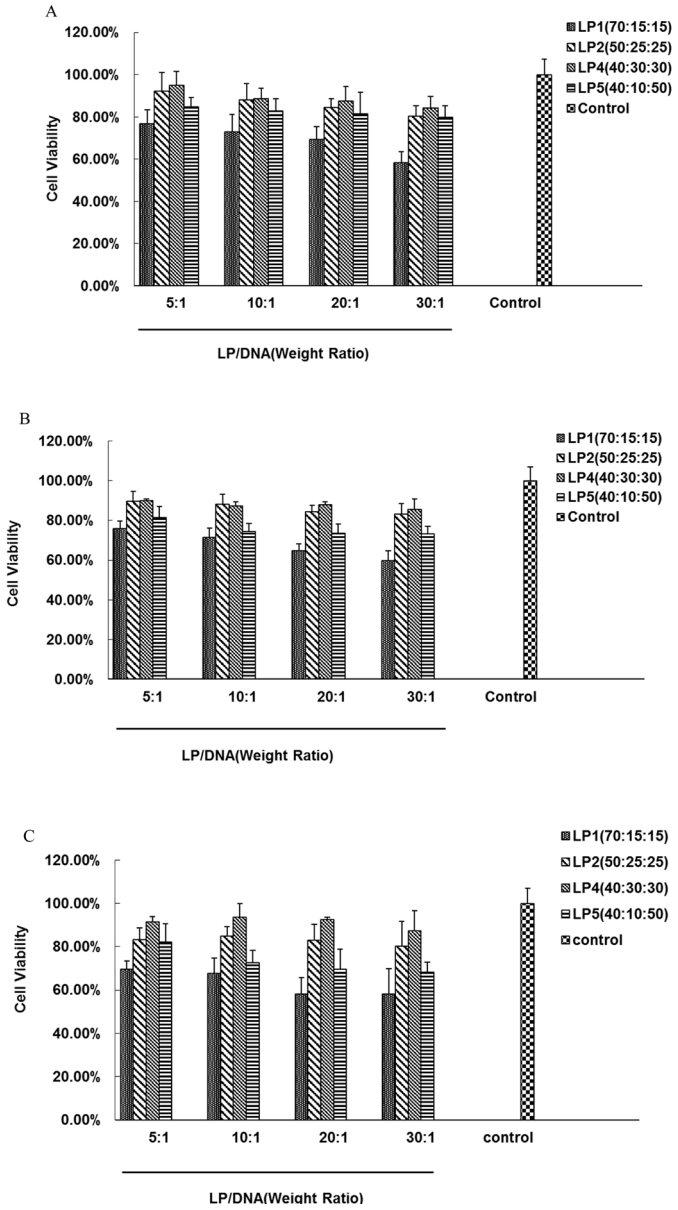

FIGURE 3 - Cell viability of LP/DNA (pEGFP) at various weight ratios in different cell lines (mean ±SD): A-HeLa; B-SPC-A1; C-A549.

Cell transfection efficiency measurement

The in vitro transfection efficiencies of the LP/pGL3-N2 lipoplexes were measured in HeLa, SPC-A1, and A549 cells using the Promega™ Luciferase Assay System. The transfection efficiencies of LP/pGL3-N2 showed an initial increase followed by a decrease with the increasing weight ratio of LP to pGL3-N2. The highest transfection efficiency occurred at a weight ratio of 10 in all tested cell lines. The data also showed that the ratio of DOTAP, DOPE, and Chol significantly influenced the gene transfection efficiency. LP2/pGL3-N2 showed the highest transfection efficacy among the LP/pGL3-N2 lipoplexes. The transfection efficiency of LP2/pGL3-N2 was similar or slightly lower than that of lipofectamine® 2000/pGL3-N2 in the tested cell lines (Figure 4).

The transfection efficiency of LP2/pGL3-N2 with a weight ratio of 10 was further observed using an inverted fluorescence microscope. Compared with lipofectamine® 2000, LP2/pEGFP-N2 lipoplexes showed similar expression of EGFP in SPC-A1 cells (Figure 5), which further supports the effective gene delivery properties of LP2.

High instability in liquid formulations is a major problem for non-viral gene delivery systems (Morais *et al.*, 2016; Anchordoquy, Koe, 2000; Abdelwahed, *et al.,* 2006). Freeze-drying is considered an effective way of solving this problem and facilitating prolonged storage at room temperature. LP2 was lyophilized immediately after preparation and the lyophilized powder was ultrasonicated for 5 min at 95 W intensity. The cell transfection efficiency was measured and compared with that of a freshly prepared sample. As shown in Figure 6, although the transfection efficiency of LP2 decreased slightly after lyophilization, the difference between the two groups was not significant ($P>0.05$). The particle size and zeta potential of the lyophilized liposomes were also measured. The zeta potential was 32.67 mV and the particle size was 215 nm, which is slightly greater than before lyophilization. However, the transfection efficiency results (Figure 6) showed that although the particle size of the liposomes increased as a result of the freeze-drying process, it did not significantly affect the cell transfection efficiency.

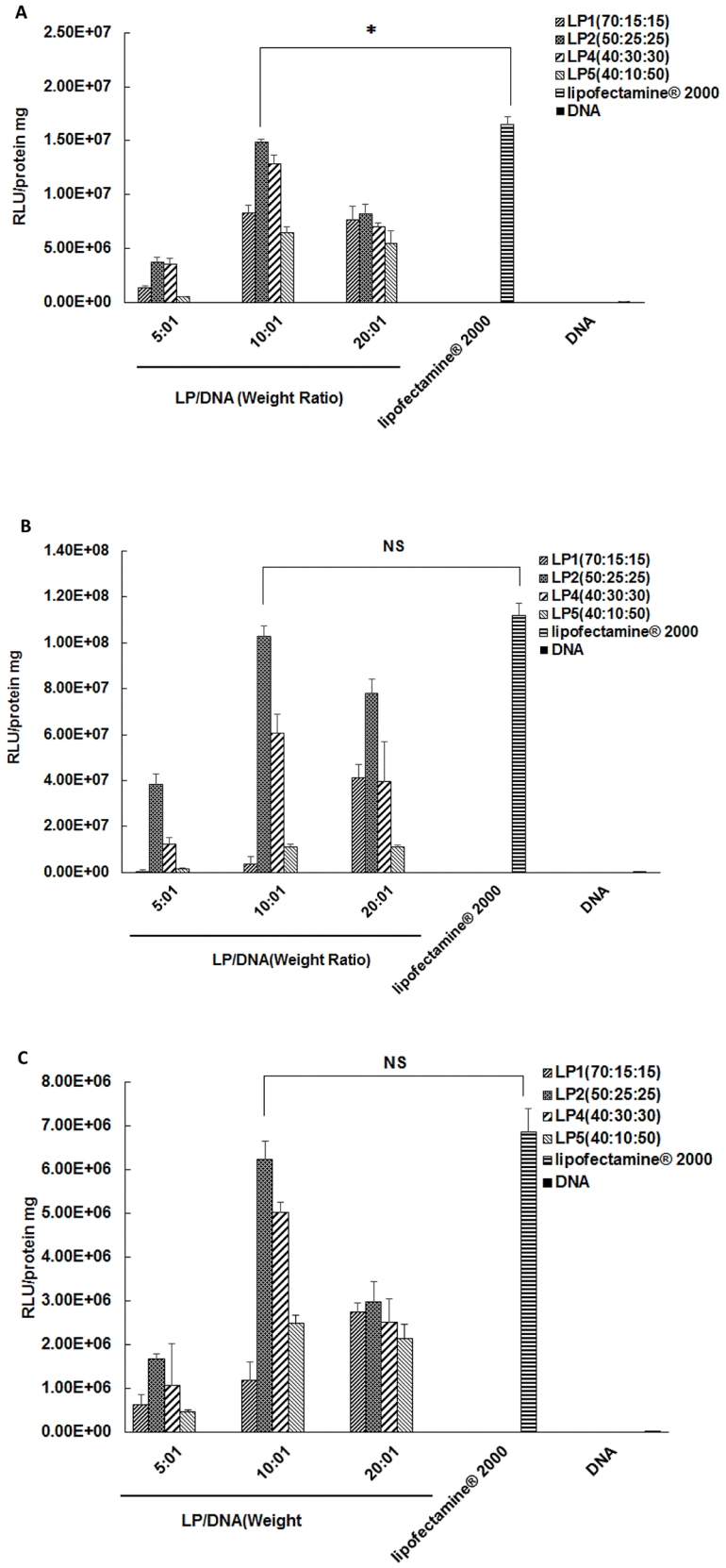

FIGURE 4 - Transfection efficiency of LP/DNA (pGL3) at various weight ratios for different cell lines (mean ±SD *n=3*; *$p<0.05$): A-HeLa; B-SPC-A1; C-A549.

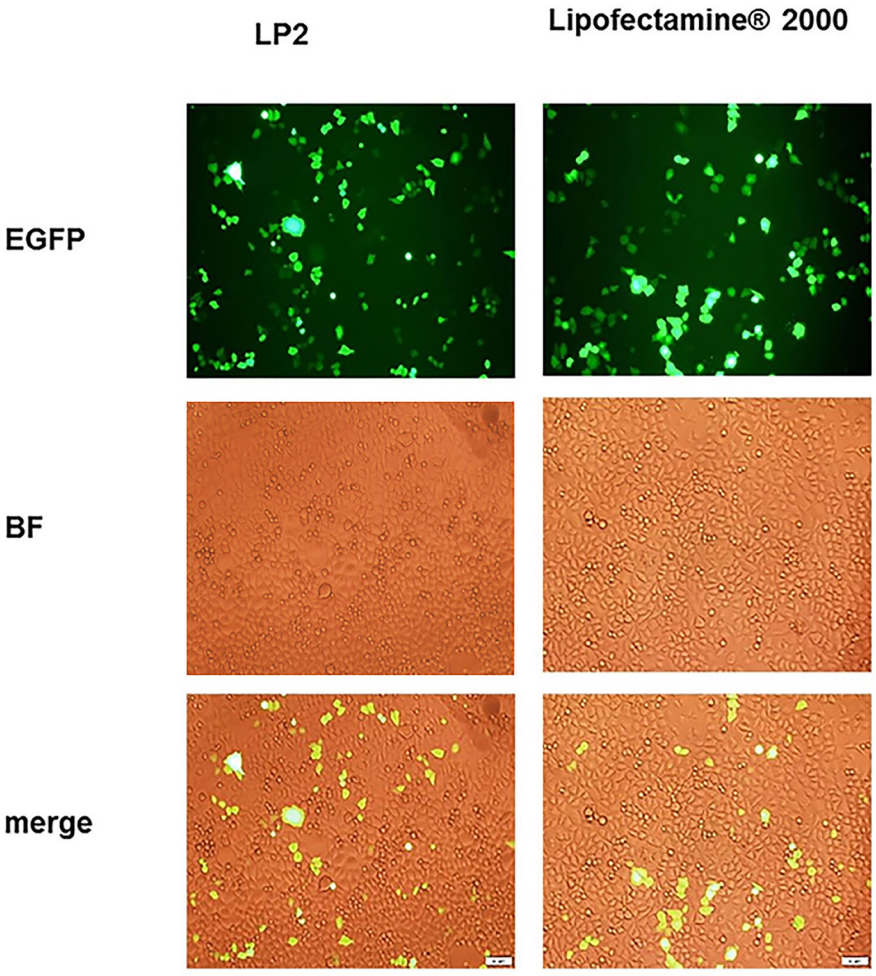

FIGURE 5 - EGFP expression in HeLa cells transfected with LP2/pEGFP lipoplexes at a weight ratio of 10 and lipofectamine® 2000 by inverted fluorescence microscopy at 100× magnification.

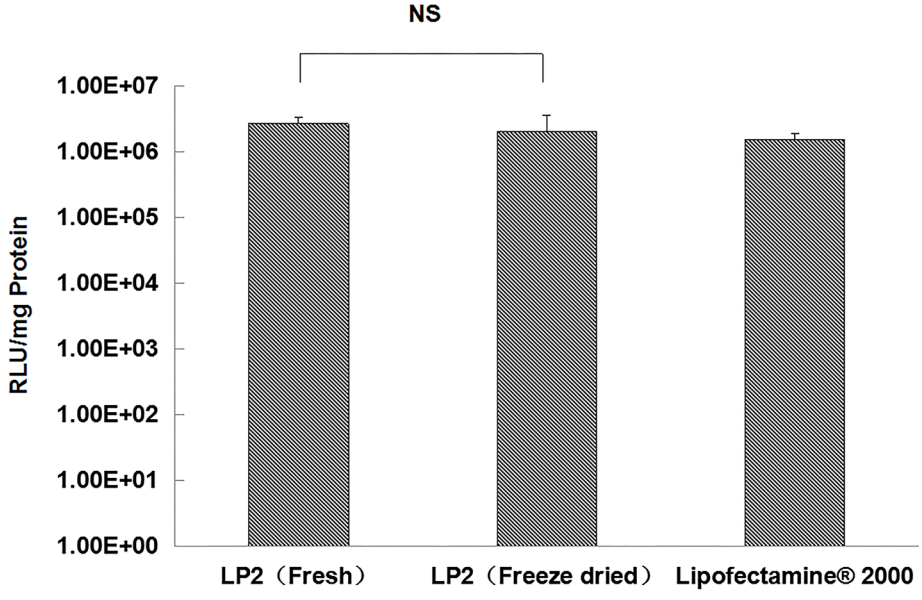

FIGURE 6 - Transfection efficiency of LP/DNA (pGL3) before and after freeze-drying.

Cellular uptake mechanism study

The cell uptake results (Figure 7) show that SPC-A1 cells exhibited the best uptake efficiency for LP2/Cy5SE-DNA, while the LP5/Cy5SE-DNA group had the lowest cell uptake efficiency under the same conditions. The observation that the LP2 group most efficiently underwent endocytosis may be one reason for it having the highest transfection efficiency. The LP composition ratio has therefore been shown to affect the endocytosis process, whether it affects the escape of particles from endosomes must be studied in future work.

To investigate the mechanism of liposome entry into the cytoplasm, we studied the transfection efficiency of the liposomes. Chlorpromazine (10 μg/mL), wortmannin (30 nM), and methyl-β-cyclodextrin (1 mM) were used as inhibitors of clathrin-mediated endocytosis, phagocytosis-mediated endocytosis, and caveolae-mediated endocytosis pathways, respectively. The cytotoxicity of the inhibitors is shown in Figure 8A. The cell viability in the presence of all of the inhibitors was around 100%, indicating that the inhibitors had no toxicity towards the cells at any of the measured concentrations.

As shown in Figure 8B, the transfection efficiencies of the LP2/pGL3-N2 polyplexes decreased when SPC-A1 cells were treated with chlorpromazine and methyl-β-cyclodextrin. However, wortmannin had no effect on the transfection efficiency of LP2/pGL3-N2 polyplexes, indicating that LP2/pGL3-N2 polyplexes were mainly taken up via clathrin-mediated and caveolae-mediated endocytosis (Love *et al.*, 2010; Zhang *et al.*, 2012; Van Deutekom, Van Ommen, 2003). It has been reported that caveolae-mediated endocytosis may circumvent normal lysosomal degradation; therefore, the cellular uptake mechanism of LP/pGL3-N2 may have facilitated the more efficient escape of DNA from the endosomes/lysosomes (Jin *et al.*, 2014; Kamimura *et al.*, 2011; Cui *et al.*, 2019).

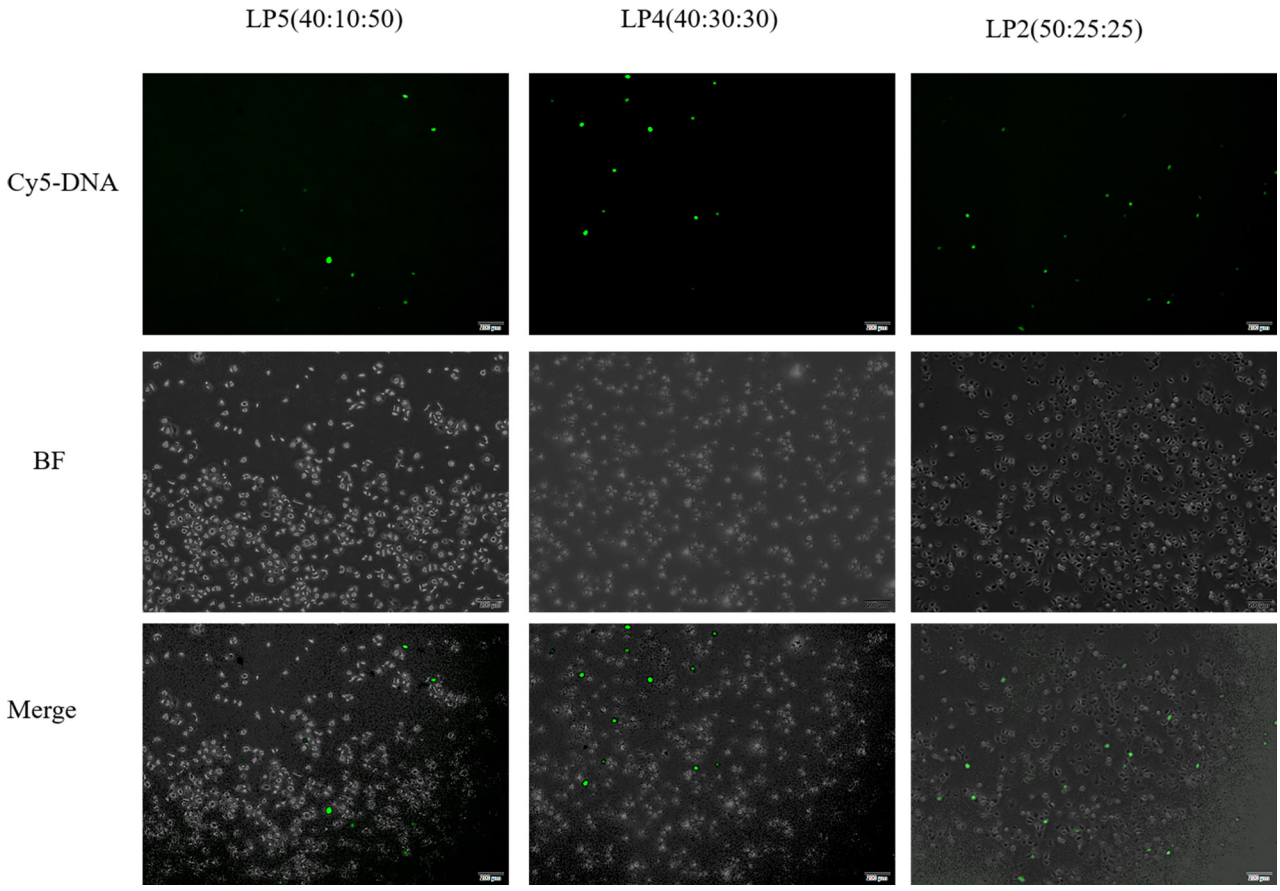

FIGURE 7 - Cellular uptake of LP/Cy5SE-DNA with different composition ratios by SPC-A1 cells at 6 h.

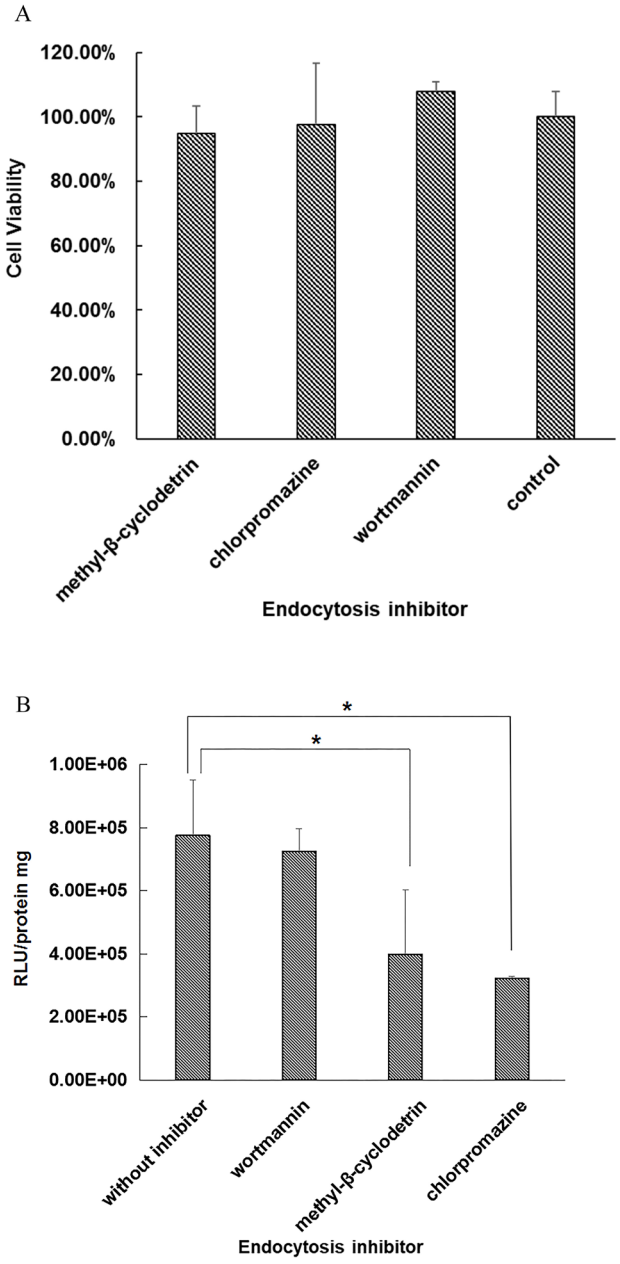

FIGURE 8 - The effect of cell endocytosis inhibitors on cell transfection efficiency A-cell viability; B-Compared with the group "without endocytosis inhibitors", (mean ±SD $n=3$, *$p<0.05$).

DISCUSSION

Increasing numbers of researchers are becoming interested in the biophysical and biochemical factors involved in cationic liposome-mediated gene delivery; such as structural features, cellular association, and intracellular trafficking. However, the specific relationships between these factors and the mechanisms of liposome internalization by cells have not been fully established (Sheng *et al.*, 2018). It has been reported that the strength of the interaction between liposomes and nucleic acids is related to structural properties such as size, shape, and surface charge (Jia *et al.*, 2016). In this study, DOTAP, DOPE, and Chol were used at different molar ratios to prepare a range of liposomes. DOTAP was the cationic head, the neutral lipid DOPE was used as a helper lipid, and Chol provided a hydrophobic region to favor self-assembly. The results of LP characterization showed that the optimal LP composition was a weight ratio (LP/DNA) of 10 and a molar ratio (DOTAP: DOPE: Chol) of 50:25:25. This was confirmed by the cell viability and transfection efficiency experiments. It was also found that the transfection efficiency of the liposome was affected by not only the amount of DOTAP, but also the molar ratio of DOPE and Chol. This is consistent with previous reports (Zhang *et al.*, 2010; Wang *et al.*, 2018). The zeta potentials of LP/DNA liposomes with a weight ratio (LP/DNA) of 10 were positive, with the exception of that of LP1/DNA. This may be because the liposomes were not fully bound to the DNA, as demonstrated by the DNA bands observed in the LP1 group in the gel retardation assay.

Because non-viral vectors are less efficient than the viral ones, understanding the mechanism by which they traverse the membrane may contribute to overcoming the hurdles in gene delivery (Cui *et al.*, 2014). It has been shown that gene carriers/pDNA complexes mainly enter the cell via several endocytosis pathways, including clathrin-mediated endocytosis (inhibitor: chlorpromazine), caveolae-mediated endocytosis (inhibitor: methyl-β-cyclodextrin), and micropinocytosis (inhibitor: wortmannin) (Luu *et al.*, 2012; Zhang *et al.*, 2012; Der Aa *et al.*, 2007). In this study, the transfection efficiency of SPC-A1 cells decreased markedly after chlorpromazine and methyl-β-cyclodextrin treatment, which indicated that LP/DNA may be taken up via clathrin-mediated endocytosis and caveolae-mediated endocytosis pathways.

Based on the findings in Figure 4, we know that LP2/PGL3-N2 exhibits similar transfection efficiency to Lipofectamine® 2000/pGL3-N2, which indicates that

our synthesized cationic liposomes have potential for use as an effective carrier for gene delivery. We believe that high transfection efficiencies and clinically relevant therapeutic outcomes can be achieved, and intend to use LP as the basis for exploring the versatility and tunability of the platform. In future work, we will modify LP with targeting groups to introduce active targeting, and encapsulate chemotherapeutic and genetic drugs concurrently to increase the anti-cancer efficacy.

CONCLUSION

In this study, DOTAP, DOPE, and Chol were used to prepare cationic liposomes, and the mole ratio of the components was optimized. The findings show that LP2 exhibits good gene delivery properties, and may be taken up via clathrin-mediated and caveolae-mediated endocytic pathways. We hope that this study will provide a basis for further exploration of safe and effective gene delivery systems and offer additional insight into the characters of non-viral vectors.

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DISCLOSURE

All authors declare no competing financial interests.

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