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Full Length Research Paper

Cationic liposomes as carriers for gene delivery: Physico-chemical characterization and mechanism of cell transfection

Bing Wang[1,2], Jiti Zhou[1], Shaohui Cui[2], Baoling Yang[2], Yinan Zhao[2], Budiao Zhao[2], Yan Duan[3] and Shubiao Zhang[2]*

[1]College of Environmental and Biological Science, Dalian University of Technology, Dalian 116024, Liaoning, China.
[2]SEAC-ME Key Laboratory of Biotechnology and Bioresources Utilization, Dalian Nationalities University, Dalian 116600, Liaoning, China.
[3]Department of Pharmacy, National University of Singapore, Singapore 117543.

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The inter-correlations among the parameters that influence the transfection efficiency of liposome-DNA complexes (lipoplexes) as carriers in gene therapy, optimum procedures for transfection and mechanism of cell transfection were investigated. The morphology, size and ζ-potential of liposomes and lipoplexes were measured by atomic force microscopy (AFM) and dynamic light scattering techniques. The lipoplexes formation was tested by using gel electrophoresis. Transfection efficiency was assessed using luciferase and green fluorescence protein reporter plasmids, respectively. It was found that transfection efficiency markedly depended on liposome to DNA weight ratio, lipoplexes morphology and size. The intracellular trafficking of exogenous DNAs was observed by confocal laser microscopy. After 4 h incubation, DNAs delivered by Lipofectamine 2000 reached the nucleus with a much higher frequency than 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP). It was concluded that lipoplexes with filaments contributing more than globular obtained higher transfection efficiency with the maximum expression of 500000 RLU/mg luciferase in HeLa cells, for they are easy to release pDNA after endocytosis. These results implied that by further modifying the chemical features of the lipid vectors and controlling their biological behaviors, more efficient lipid delivery vectors may be achieved.

Key words: Cationic liposomes, gene delivery, transfection efficiency, AFM, intracellular trafficking.

INTRODUCTION

As a promising strategy for the treatment of many inherited and acquired diseases, gene therapy has attracted many researchers during the past several decades (Ma et al., 2007). The aim of gene therapy is to deliver healthy exogenous genetic drugs such as plasmid DNA and single-strand oligonucleotides to replace a missing gene so as to cure genetic diseases, for instance, cystic fibrosis, malignant melanoma, and Gaucher's disease (Zhang et al., 2004). Based on these factors, reliable and efficient vectors delivering exogenous genes into target cells are urgently awaited (Zhang et al., 2007). Though viral systems usually give high transfection efficiency, safety concerns from potential mutation, recombination, oncogenic effect, and high cost, however, greatly limit their therapeutic applications. In contrast, non-viral transfection agents, mainly including cationic lipids and cationic polymers, are believed to cause less safety problems although non-specific cytotoxicity associated with cationic liposomes has been observed (Matsui et al., 2006), thereby making it easier to manufacture in larger scale and to deliver large pieces of DNA (Laporte et al., 2006), and also to modulate chemically for improvement of transfection efficiency (Wasungu and Hoekstra, 2006).

There are great deals of structural variations in

*Corresponding author. E-mail: zsb@dlnu.edu.cn. Tel: +86 411 876 56141. Fax: +86 411 876 44496.

lipid molecules that are able to mediate transfection. Since the cationic liposome vector was first introduced by Felgner et al. (1987) who used N-[1-(2,3-dioleyloxy) propyl]-N,N,N-trimethylammonium chloride (DOTMA) to carry out a DNA-transfection protocol, many cationic lipids and polycations used for DNA transfer have been widely investigated (Martin et al., 2005). For transfection application, cationic lipids are often mixed with helper lipids like dioleoylphosphatidylethanolamine (DOPE) or cholesterol, potentially promoting conversion of the lamellar lipoplex phase into a non-lamellar structure, which presumably rationalizes their ability to improve cationic lipid mediated transfection efficiency (Wasungu and Hoekstra, 2006). Moreover, optimization of transfection efficiency may be attempted by using a trial and error approach consisting of synthesizing and testing a large number of derivatives. On the other hand, rational design of highly efficient cationic lipids and polymers requires a deeper understanding of the interaction between vectors and DNAs, as well as the cellular pathways and mechanisms involved in DNA entry into cells and ultimately nucleus (Niculescu-Duvaz et al., 2003; Elouahabi and Ruysschaert, 2005; Bally et al., 1999).

However, despite the recent clinical success, the basic knowledge of particle structure–activity relationship is still unsatisfactory and the optimization of these systems remains largely a result of trial and error (Masotti et al., 2009). In order to better understand the role of different parameters in improving the clinical performances of these systems, a systematic *in vitro* study of the physico-chemical and biological properties of both liposomes and lipoplexes is crucial. Liposome-mediated gene delivery transfection processes, which ultimately govern the transfection efficiency, are influenced by a variety of physico-chemical parameters such as the cationic liposome/DNA charge ratio, the particle size of lipoplexes, the superficial charge and the morphology. The details of the intracellular trafficking of exogenous DNA should be very important for effective transgene expression. In the current, generally accepted model, exogenous DNA is incorporated by cells mainly via an endocytosis pathway (Wrobel and Collins, 1995; Huth et al., 2006). The enhanced released DNA as “naked” DNA into the cytoplasm (Kamiya et al., 2002) and entry into the nucleus is a very important obstacle to high transgene expression (Hashimoto et al., 2006).

Lipofectamine 2000 can efficiently transfect post-mitotic neurons (Farrow et al., 2006) and rat primary hepatocytes as well (Green et al., 2006), thus suggesting that it may promote penetration of DNA (Okumura et al., 2009) through intact nuclear envelopes (Lonez et al., 2008) in these cell types, further demonstrating its utility. 1,2-Dioleoyl-3-trimethylammonium-propane (DOTAP) liposome was used to transfect cells with DNA and siRNA (Kleivel et al., 2008) and to quantify intracellular DNA release and elimination kinetics after transfection (Ruponen et al., 2009). They were used as models in order to correlate physico-chemical parameters affecting transfection efficiency in this paper. The aim of this present study was to examine the correlation of lipoplex morphology, size, cell density, DNA amount and liposome/pDNA weight ratio, lipoplex formation and transfection time, serum and cell lines with transfection efficiency and toxicity. To study the mechanism of cationic lipid-mediated gene delivery with higher transfection efficiency, we visualized intracellular trafficking with fluorescently labeled DNA molecules.

MATERIALS AND METHODS

Reagents and materials

Commercial reagents, Lipofectamine 2000 and DOTAP, were purchased from Invitrogen and Roche-Diagnostics, respectively as aqueous suspensions at concentrations of 1 mg/ml. The pGL3-control plasmid (Promega) contained a modified coding region for firefly (*Photinus pyralis*) luciferase and the pGFP-N2 plasmid (Clontech) containing a coding region for green fluorescence protein. Plasmid DNAs were extracted and purified using the Endo-Free Plasmid Purification Kit (Qiagen). λ DNA/EcoRI+ HindIII Marker was purchased from Fermentas. Cells were purchased from Institute of Biochemistry and Cell Biology, SIBS, CAS, China. Growth media, RPMI1640 and DMEM, were purchased from Gibco, and the other chemicals used were of reagent-grade.

Chemical structure and composition

The commercial liposomal formulations have different chemical compositions and were generally mixtures of neutral and cationic lipids. The chemical structures of the lipids are shown in Figure 1. DOTAP liposome contains DOTAP (N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium chloride) and DOPE at 1:1 ratio.
Lipofectamine 2000 may be mixtures of lipofectin and lipofectamine contain DOSPA (2,3-dioleyloxy-N-[2(sperminecarboxamido)ethyl]-N,dimethyl-1-propanaminium trifluoroacetate), DOTMA and DOPE. The difference between DOTAP and Lipofectamine 2000 may reside on the different head groups that in the latter case, consist of a polyamine moiety.

Preparation of plasmid DNA

The pGL3-control and pGFP-N2 plasmids were amplified in *Escherichia coli* (strain JM109 and DH5-α, respectively) cells and purified by column chromatography (Sigma). The purity of the plasmid was measured by OD260/OD280 (1.85-1.90; OD, optical density), as well as by electrophoresis in a 0.8% agarose gel. Plasmid DNA was labeled with Rhodamine (Rh) using Label It ® CX-Rhodamine Labeling Kit (Mirus).

Electrophoresis

Cationic liposome/pDNA complexes were formed using pGL3-control at a final DNA concentration of 0.5 μg/μL and at weight ratios from 0.5 to 8. The complexes were then loaded onto 0.8% agarose gels containing ethidium bromide (0.5 mg/ml). The gel running buffer was composed of 40 mM Tris acetate and 1 mM EDTA. Electrophoresis was performed at 100 V for 30 min.

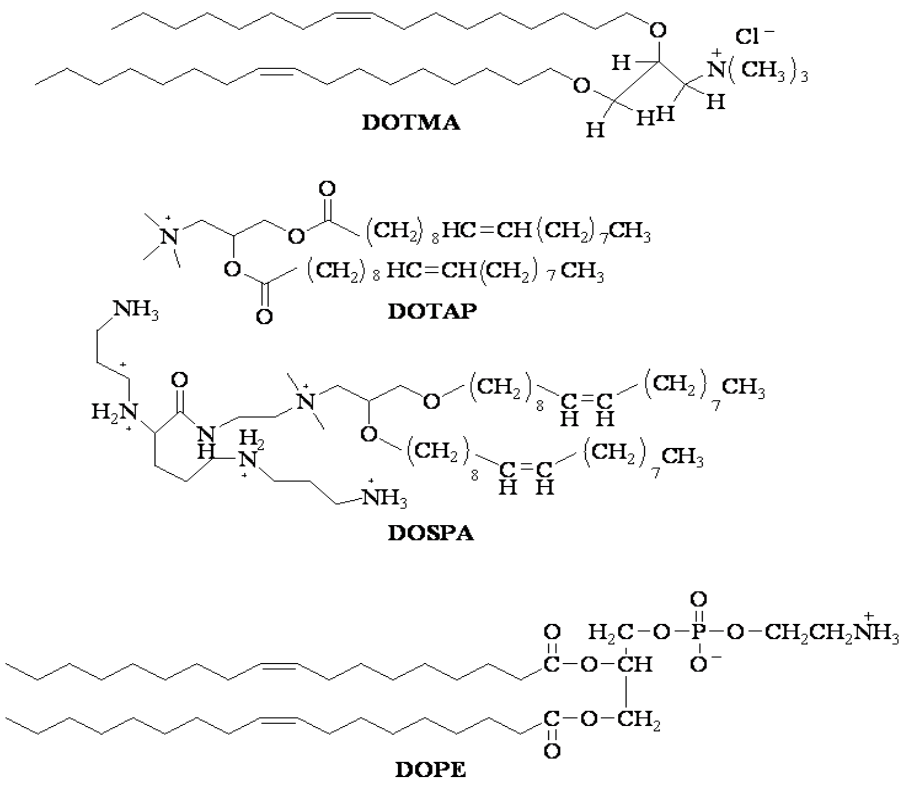


Figure 1. Chemical structures of glycerol-based efficient cationic lipids and co-lipid investigated.

Fluorescence of the intercalated dye was measured using a gel imaging system (SYNGENE).

AFM of liposomes and lipoplexes

For each sample, 10 µL aliquot was adsorbed for 10 min on freshly cleaved muscovite mica. It was dried under undefiled condition. Atomic force microscopy (AFM) imaging was performed in tapping model at a scanning speed of 2 Hz/s with a Veeco Dimension3100 using high frequency (320 kHz) silicon cantilevers with a tip radius of 2 to 10 nm (Veeco, USA).

Dynamic light scattering (DLS) measurement

The mean particle size and zeta potential of cationic liposomes and lipoplexes were measured by photon correlation spectroscopy (Nano ZS 90, Malvern, UK) using a helium–neon laser with a wavelength of 630 nm. The particle size and zeta potential of liposomes and lipoplexes in Opti-MEM I medium (Invitrogen) were determined. The determination was repeated three times per sample.

Cell culture

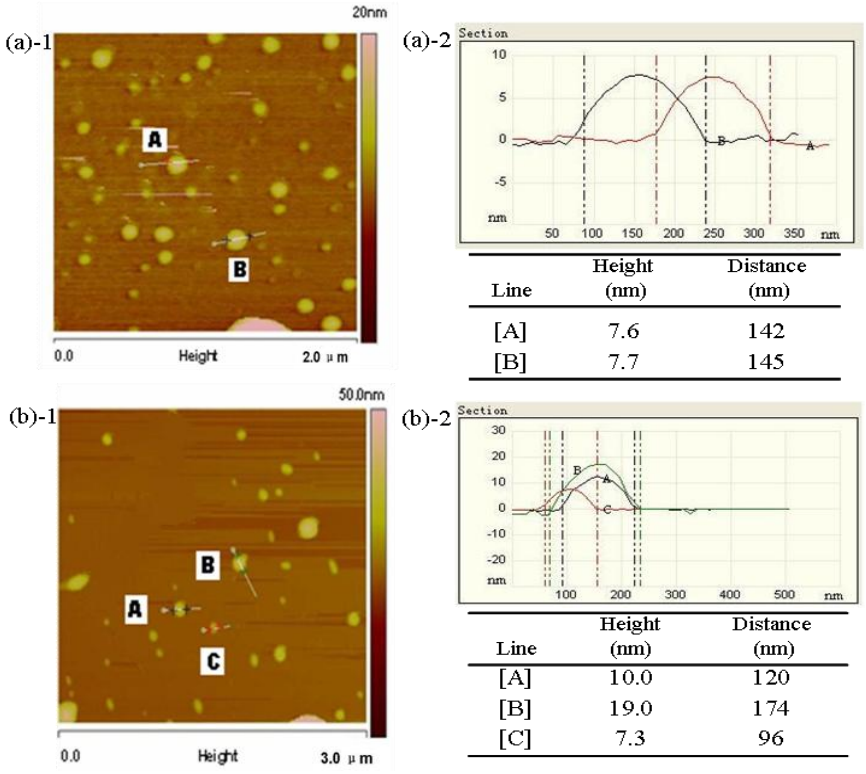
HeLa, Hep-2, SMMC-7721, HT-29, SW480 and MCF-7 cells were grown in 100 ml culture flask in RPMI1640 supplemented with 10% fetal bovine serum and antibiotics (100 U/ml penicillin and 100 mg/ml streptomycin) at 37°C in a humidified atmosphere containing 5% CO_2.

Transfection procedure

A day before transfection, 0.5 to 2×10^4 cells were seeded per well (96-well plate) in 100 µL of growth medium (RPMI1640) without antibiotics until the cell obtained 80% confluence at the time of transfection. Liposomes labeled with 5 mol% of NBD-PE (N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-1,2-dihexadecanoyl-snglycero-3-phosphoethanolamine triethylammonium salt, Avanti Polar Lipids Inc.) and 0.3 (or 0.5) µg pDNA were diluted in 25 µL DMEM without serum, respectively and mixed gently. The weight ratios of liposome to pDNA were 2, 3, 4, 5 and 7 according to the demands. Five minutes after dilution, the diluted liposome was added to the diluted DNA. The mixture was mixed gently and stood for 20 min at room temperature. Cells were washed once with PBS, and then lipoplexes (50 µL) were added to each well and mixed gently by rocking the plate back and forth. The cells were incubated at 37°C with 5% CO_2 for 4 to 6 h, then washed by PBS or DMEM once and the medium was replaced by a complete medium and further cultured for 24 to 48 h, prior to analysis according to reporter genes.

Luciferase and GFP assay

Relative luciferase activity was assessed using the Bright-Glo™ Luciferase Assay System (Promega) and Synergy™ 2 Multi-Detection Microplate Reader (BioTek). The growth medium was removed and luciferase activity was measured after lysis buffer was added and incubated for 5 min at room temperature. The protein concentrations of wells were determined using BCA protein assay reagent. Transfection activity were expressed as relative light units (RLU) per milligram of protein. The expression of green



Line	Height (nm)	Distance (nm)
[A]	7.6	142
[B]	7.7	145

Line	Height (nm)	Distance (nm)
[A]	10.0	120
[B]	19.0	174
[C]	7.3	96

Figure 2. AFM images of liposomes. The AFM profiles were obtained from the height images and section analysis. (a) Height images of Lipofectamine 2000 and (b) DOTAP with section analysis.

fluorescence protein was imaged by inverted fluorescence microscope (Olympus). The number of GFP-expressing cells versus the total cell quantity in the microscope was defined as the transfection efficiency. Cell counting was performed randomly in microscopic observation scope under 10 × 16 magnifications with 3 repeats.

Cytotoxicity (MTT) assay

Cytotoxicity was evaluated by the MTT assay. Cells were seeded at a density of 1×10^4 cells/well in 96-well plates, and incubated 12 h before treatment. The liposome and 50 µL of DMEM medium were added to each well and incubated for 24 h at 37°C. After incubation, 50 µL of 5 mg/ml MTT solution in PBS was added and incubated for an additional 4 h. MTT-containing medium was aspirated off, and 150 µL of DMSO was added to dissolve the formazan crystal formed by live cells. The absorbance was measured at 575 nm to determine cell viability as percentage of control (without liposome).

Confocal microscopy

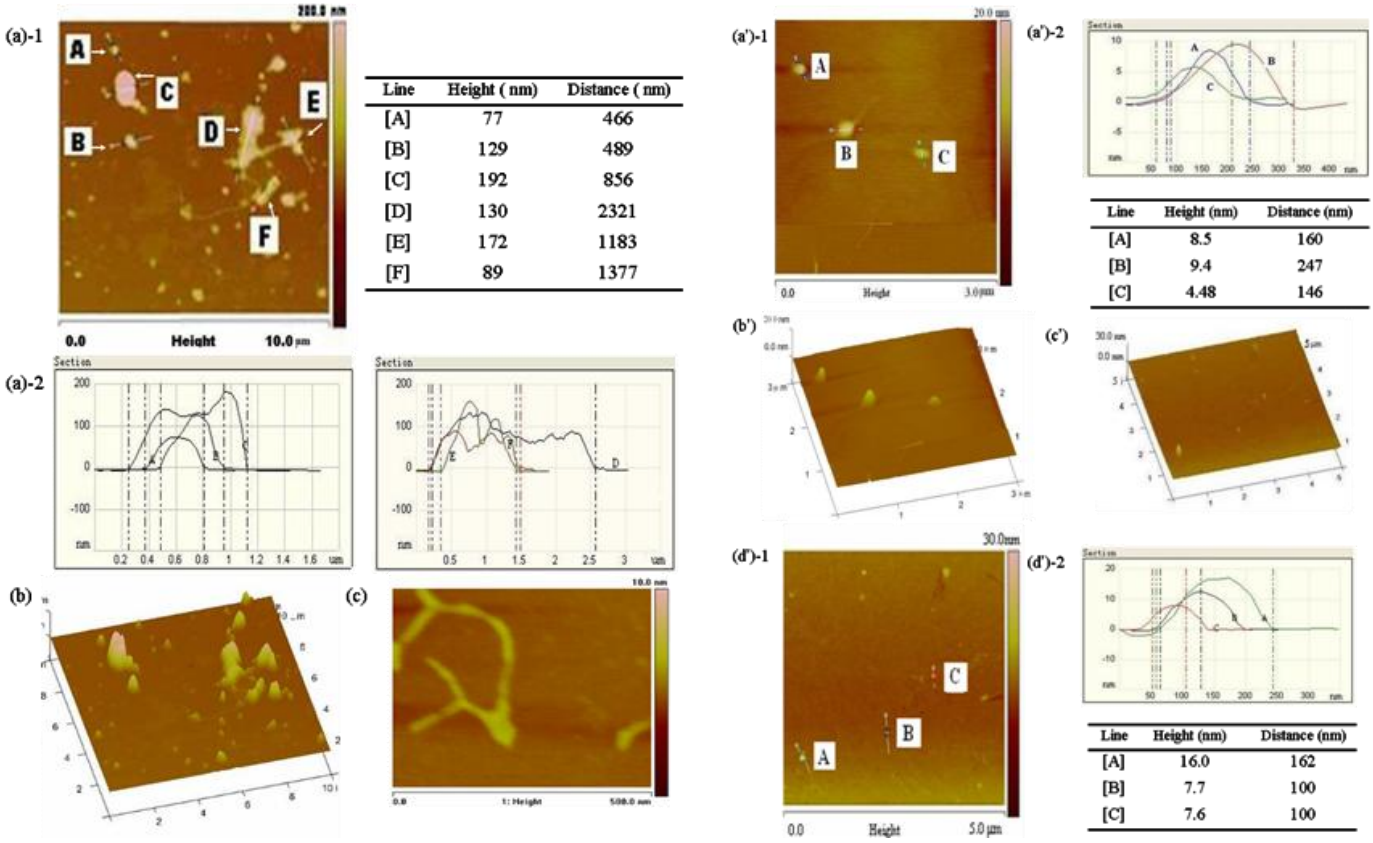
To observe the intracellular distribution of lipoplexes, Hep-2 cells, were transfected as described above with Rh-pGL3-control complexed with NBD-Lipofectamin2000 or NBD-DOTAP. At 2, 4 and 8 h after transfection, cells were washed twice with phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde in PBS for 20 min at room temperature. After the cells had been washed with PBS, they were incubated with 10 mg/ml 4′,6-diamidino-2-phenylindole (DAPI) for 15 min. The fluorescence was examined with a confocal laser scanning microscope (SP2, Leica). An argon laser (488 nm excitation) was used to induce the green fluorescence of NBD-PE; a helium-neon laser (543 nm) was used to excite the red fluorescence of Rhodamine; while a UV laser (364 nm) was used to obtain the blue fluorescence.

RESULTS

Physico-chemical characterization of liposomes

Morphologies and ζ-potential

AFM images of liposomes, Lipofectamine 2000 and DOTAP (Figure 2a and b), showed an asymmetrical and flattened spherical shape and polydisperse nature described as planar vesicles. Lipoplex structures of Lipofectamine 2000 were observed ranging from granules (Figure 3a-A to C) to filaments (Figure 3a-D to F) at the weight ratio of 3, while more compacted globular structures of the lipoplexes of DOTAP at the weight ratios of 6 and 8 (Figure 3a' and d') were observed. The diameters were shown from the height image and the graphic section analysis in Figure 2a-2 and b-2, while the diameter of Lipofectamine 2000 was 142 or 145 nm as particle A and B in Figure 2a-1, whereas DOTAP was 96, 120 and 174 nm as particle A, B and C in Figure 2b-1.



Line	Height (nm)	Distance (nm)
[A]	77	466
[B]	129	489
[C]	192	856
[D]	130	2321
[E]	172	1183
[F]	89	1377

Line	Height (nm)	Distance (nm)
[A]	8.5	160
[B]	9.4	247
[C]	4.48	146

Line	Height (nm)	Distance (nm)
[A]	16.0	162
[B]	7.7	100
[C]	7.6	100

Figure 3. AFM images of Lipofectamine 2000/pDNA lipoplexes (weight ratio: 3/1, left) and DOTAP/pDNA lipoplexes (right). The AFM profiles were obtained from the height images and section analysis. Height image of (a) Lipofectamine 2000/pDNA complexes with section analysis; (b) a 3D-view of AFM topography; (c) pDNA; Height image of (a') DOTAP/pDNA lipoplexes at weight ratio 6 with section analysis and (b') a 3D-view of AFM topography. (c') a 3D-view of AFM topography of DOTAP/pDNA lipoplexes at weight ratio 8 and (d') height image with section analysis.

Particle A to F (Figure 3a) showed that the diameter of Lipofectamine 2000 lipoplexes changed from 466 to 1377 nm. Smaller lipoplexes of DOTAP were observed from 100 to 247 nm as particle A, B and C (Figure 3a', d') compared with those of Lipofectamine 2000 (Figure 3a-D to F).

Particle size and ζ-potential of the cationic liposomes were also determined using a light scattering particle sizer (ZS90, Malvern) (Figure 4b). The average sizes of Lipofectamine 2000 and DOTAP were 144 and 160 nm, while ζ-potentials were 46.7 and 48.5 mV, respectively. Size and potential distribution showed that particles of liposomes ranged from 100 to 200 nm, and ζ-potential from 35 to 50 mV. The size of DOTAP obtained by DLS showed little difference than the average size showed by AFM. The size of the complexes of cationic liposomes and pDNA at different molar charge ratios without serum has also been measured by DLS. The size of Lipofectamine 2000 /pDNA lipoplexes increased as the function of liposome/pDNA weight ratio, from 150 to 475 nm within 60 min according to size distribution intensity in Figure 4b. Lipofectamine 2000/ pDNA lipoplex suspensions presented two distinct populations, approximately 50 to 100 nm and 300 to 500 nm in diameter. The ζ-potentials of Lipofectamine 2000/pDNA lipoplex suspensions were -22.4, -16.3 and -12.4 mV at weight ratios of 1, 3 to 5, respectively. This was consistent with gel retarding results, and the lipoplexes of Lipofectamine 2000 were negatively charged, which could migrate from the starting wells at lane 3 to 9 when liposome/pDNA weight ratio was below 6 (Figure 4a).

Gel retarding

Vectors with high transfection could bind DNA sufficiently and rapidly, which can be measured by agarose gel electrophoresis. Plasmid DNA in lane 2 as a mixture of both supercoiled and circular forms was shown (Figure 4a). When DOTAP and Lipofectamine 2000 were mixed with pDNA, the amount of uncomplexed (free) DNA decreased gradually with progressively increasing proportions of liposomes. Plasmid DNA was completely retained at liposome/pDNA weight ratio 8 for Lipofectamine 2000, but DOTAP/pDNA complexes were negative. It was obvious that Lipofectamine 2000 could

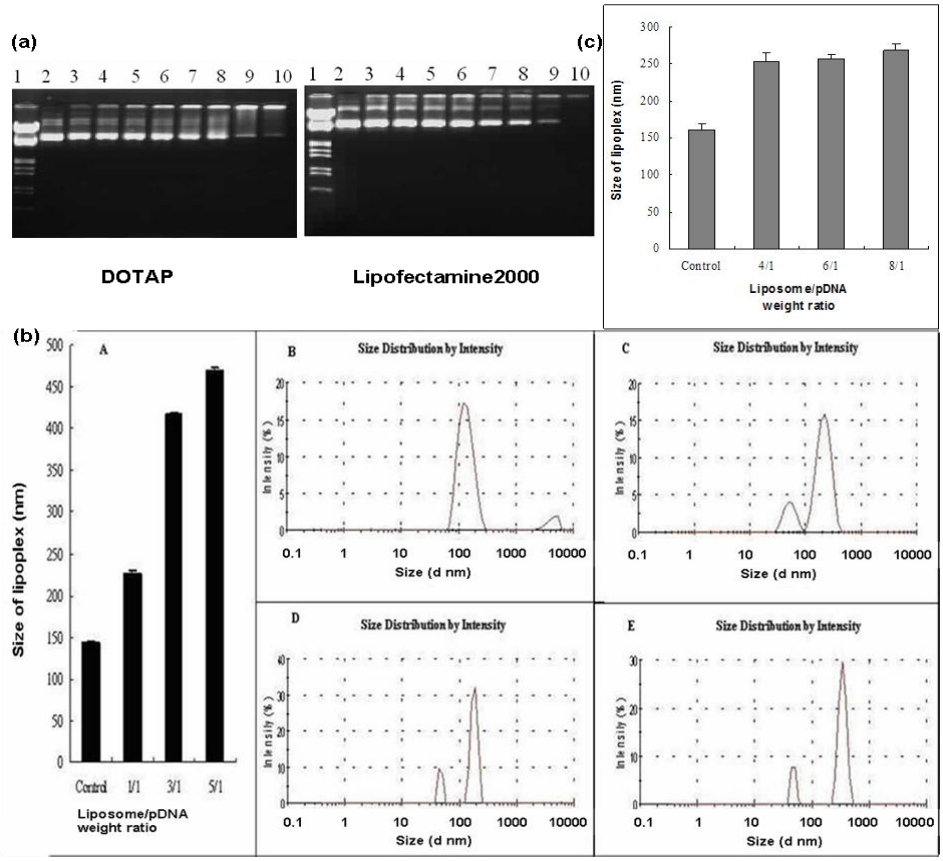


Figure 4. (a) Gel retardation of liposome/pDNA complexes. Lane 1, marker (λ DNA/EcoR I + Hind III Markers); lane 2, naked pDNA and lanes 3 to 10, pDNA (lane 3, 0.5; lane 4, 1; lane 5, 1.5; lane 6, 2; lane 7, 3; lane 8, 4; lane 9, 6; lane 10, 8), (b) Size and size distribution of liposome and liposome/pDNA complexes by DLS measurement. (A) Size of Lipofectamine 2000/pDNA lipoplex at different weight ratios; size distribution by intensity against size of (B) Lipofectamine 2000; lipoplex of Lipofectamine 2000/pDNA at weight ratio (C) 1; (D) 3 and (E) 5; (c) DOTAP

condense pDNA more efficiently than DOTAP.

In vitro transfection

Cell density

Transfection in HeLa cells with various densities are recorded in Figure 5A. The luciferase expression was not proportional to the cell density, and the optimum cell density of Lipofectamine 2000 was from 1×10^4 to 2×10^4 cells per well at weight ratios of liposome to pDNA totaling to 2 to 4.

DNA amount and liposome/pDNA weight ratio

The effects of Lipofectamine 2000/pDNA weight ratios on transfection are shown in Figure 5B and C. When the amount of pDNA was held constant at 0.3 µg per well, an optimal result of at least 3 to 4 fold excess of transfection at liposome/pDNA ratio of 4 over other ratios was obtained. However, when the amount of pDNA was 0.5 µg per well, the maximum luciferase activity was more than 2 to 3 fold excess at liposome/plasmid weight ratios of 2 and 3 over the other ratios. The influence of liposome/pDNA weight ratios and DNA amount on transfection was significantly different for liposome-based lipoplexes. The maximum value of transfection was obtained at weight ratio of 6 for DOTAP, at 2 to 4 for Lipofectamine 2000.

Lipoplex formation and transfection time

The effects of lipoplex formation time and transfection time were investigated on transfection, as they were not considered much in literatures. Luciferase expression was significantly improved as the liposome/pDNA complexation time about 30 min (Figure 5D). The

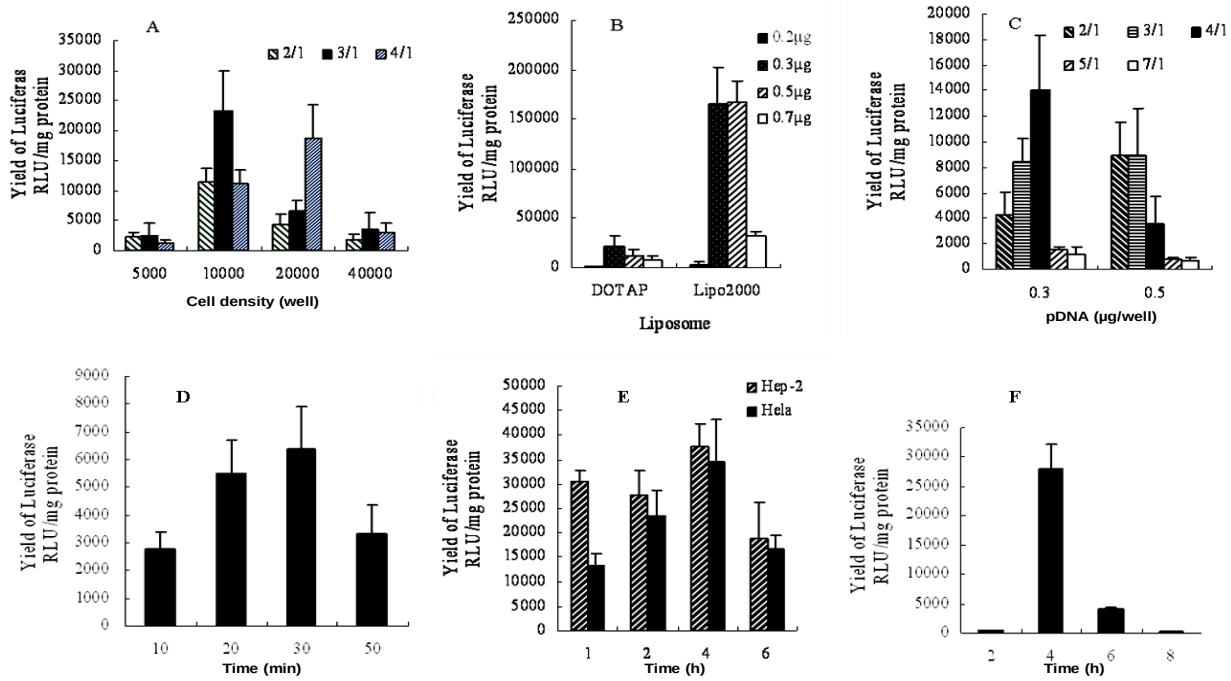


Figure 5. Effect of (A) cell density, (B) the amount of pDNA, (C) liposome/pDNA weight ratio, (D) liposome/pDNA complex formation time and (E, F) transfection time on transfection efficiency. Cell density was 1-1.5×10^4 per well. (A): Cell line, HeLa; pDNA: 0.3 μg per well; Liposome: Lipofectamine 2000. (B): the amount of pDNA from 0.2 to 0.7 μg at optimum ratios of liposome/pDNA, in which DOTAP at 6, Lipofectamine 2000 at 3 (C): HeLa cells and Lipofectamine 2000 were used. (D) HeLa cells transfected with 0.3 μg of luciferase reporter plasmid and 0.9 μg of Lipofectamine 2000. (E) HeLa and Hep-2 cells were transfected with 0.3 μg of pGL3-control and 0.9 μg of Lipofectamine 2000. (F) HeLa cells were transfected with 0.5 μg of pGL3-control plasmid and 9 μg of DOTAP.

influence of lipoplex transfection time with cells on transfection efficiency was tested as shown in Figure 5E and F, which demonstrated that 4 h incubation was optimum.

Serum and cell lines

There was no significant difference of transfection after addition of serum, thus indicating that serum did not significantly affect the transfection efficiency of Lipofectamine 2000 (Figure 6A). However, extremely large differences in the gene expression level were observed among the cells. HeLa and Hep-2 cells showed higher gene expression (Figure 6B and C) in contrast to HT29 and SMC7721 cells. The transfection efficiency of Lipofectamine 2000 was significantly higher than DOTAP in HeLa and MCF-7 cells. HT29 cells were difficult to be transfected by these two liposomes.

Cytotoxicity

Under the optimal transfection conditions, Lipofectamine 2000 displayed lower toxicity (< 10%) and no significant differences were observed (Figure 6D). Also DOTAP displayed a similar behavior, even if with a slightly higher toxicity (< 20%). In five cell lines, B16 and Hep-2 obtained higher cell toxicity.

Intracellular trafficking

Intracellular distribution of lipoplexes was visualized using Rh-pDNA (red), NBD-liposome (green), and DAPI (blue) in Figure 7. Figure 7A and F shows phase contrast pictures at 2, 4 and 8 h after transfection of DOTAP-pDNA and Lipofectamine2000-pDNA, respectively. Fluorescent pictures were for DAPI in Figure 7B and G, for NBD-liposome in Figure 7C and H, and for Ph-pDNA in Figure 7D and I. The tone of each image was adjusted and overlapped to a merge picture by digital processing (Figure 7E and J).

As shown in Figure 7 E and J, the fluorescence of NBD-DOTAP (green), NBD-Lipofectamine2000 and Rh-DNA (red) was observed at the same locations on the surface and in the cytoplasm of cells at 2 h after the addition of the complex, indicating that the complex bound to the cells and became internalized. Surprisingly, the labeled DNAs in their nuclei at 2 h were observed (Figure 7J). Four to eight hours later, more Rh-DNA fluorescence delivered by Lipofectamine 2000 was

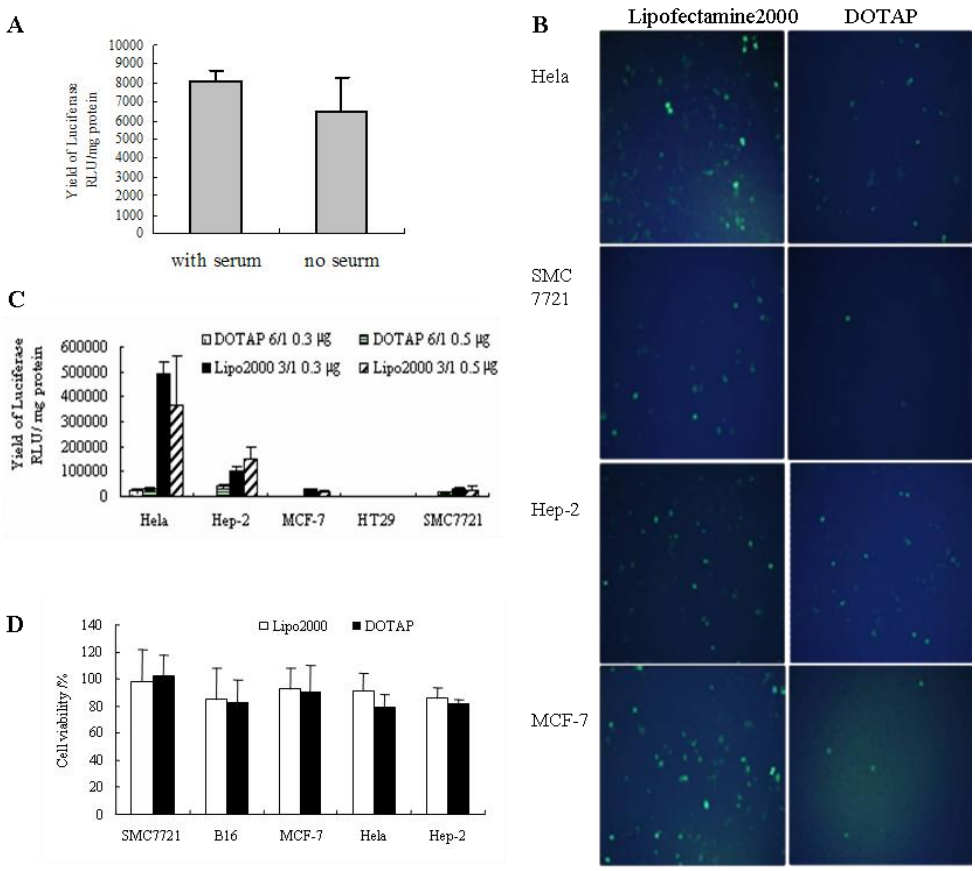


Figure 6. Effect of serum (A) and cell types (B and C) on transfection and (D) cell toxicity by MTT analysis. (A) HeLa cell was transfected with 0.3 μg of plasmid and 0.9 μg of Lipofectamine 2000 per well. (B) The GFP and (C) Luciferase expression at liposome/pDNA ratio of 3 and 6 for Lipofectamine 2000 and DOTAP, respectively. Cell density was 1.5×10^4 per well and transfected with 0.5 μg of plasmid per well for GFP expression, 0.3 and 0.5μg of plasmid per well for luciferase expression. (D) The amount of pDNA used was 0.3 μg per well for Lipofectamine2000, and 0.5 μg per well for DOTAP.

observed in the nuclei (Figure 7 J' and J'') that were stained with DAPI (blue), indicating entry of the gene into the nucleus. Lipofectamine 2000 therefore showed higher pDNA transport efficiency to nucleus than DOTAP. Interestingly, no green fluorescence was observed in the nuclei from the merged pictures and NBD fluorescence was observed in the cytoplasm (merged-images at 4 and 8 h in Figure 7), suggesting that the liposome had detached from the complex and remained in the cytoplasm, maybe in the endosomes.

DISCUSSION

At present, liposome plays a significant role as drug delivery vehicles being considered very promising for gene therapeutics. Many parameters influence liposome-mediated gene transfection of adherent cells, hence the application of these drug delivery systems widely depends on their physico-chemical characteristics such as the shape, zeta potential, the surface morphology and the size distribution (Dass, 2002).

Atomic force microscopy (AFM) is being used as a tool to evaluate the formation and the geometry of liposome/pDNA complexes in the development of gene therapy (Barbara et al., 2007; Hansma and Pietrasanta, 1998). The homogeneity of the flattened spherioidal surface was better described in AFM image by section analysis (Figure 2a and b). AFM image of lipoplexes Lipofectamine 2000 showed filaments (Figure 3a) compared with compact globular structures (Figure 3a' and d') of DOTAP at ratios of 6 and 8. Lipofectamine 2000 with higher transfection efficiency (Figure 5B) suggested that lipoplexes have filament structures that easily release pDNA after endocytosis. Our results are in conformity with Ruponen et al. (2009), who discussed that "spaghetti-like" structures were metastable particles in which the DNA was coated with lipid and such structures were thought to be of higher transfection efficiency than thermodynamically stable complexes of

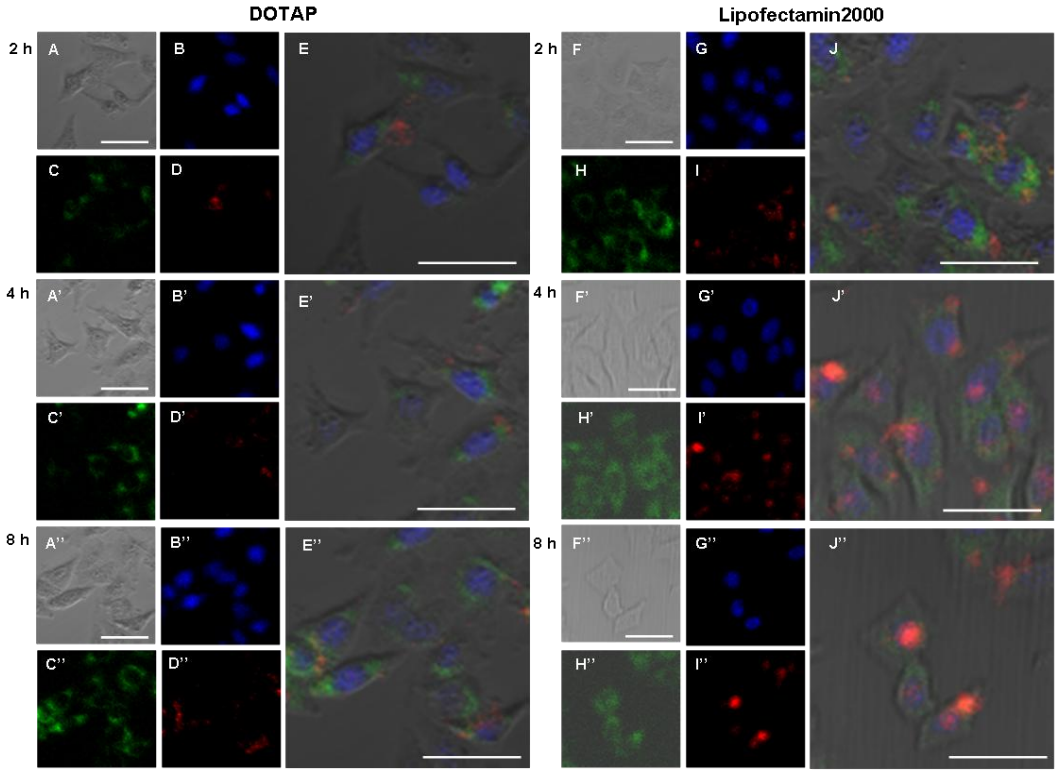


Figure 7. Intracellular distributions of Rh-plasmid/NBD-liposome lipoplexes observed with a confocal florescence microscope in Hep-2 cells. Cells were treated with DOTAP and Lipofectamin2000 lipoplexes for 2, 4 and 8 h. The NBD-labeled liposome is shown in green; and the Rhodamine-labeled plasmid DNA, in red. The cells were fixed, and the nuclei were stained with DAPI in blue. Phase contrast images (A and F), nuclei staining (B and G), distribution of NBD-DOTAP and NBD-Lipofectamine2000 (C and H), distribution of Rh-DOTAP and Rh-Lipofectamine2000 (D and I) and merged images (E and J) of the above (A, B, C and D merged into E; F, G, H and I merged into J) were indicated. Scale bar indicates 50 μM and is shown on only phase contrast and merged images for the sake of image clarity.

the same composition. However, it has been suggested that spherical condensates were more likely to have high stability due to minimal surface energies.

Transfection efficiency depends on the size of lipoplexes and an increasing trend was observed when the average size increased from 226 to 417 nm, but declined at 469 nm (Figure 4b-A). We observed that liposome/pDNA weight ratio of 3 or 4 maximized the transfection (Figure 5A and B) and the average size of lipoplexes at this ratio was in the range of 400 to 450 nm (Figure 4b-A). Almofti et al. (2003) pointed out that lipoplex size was a major determinant factor for lipofection efficiency of DOTAP-based liposomes. They reported DOTAP-based lipoplexes had a size ranging from 200 to 2000 nm and large lipoplex particles seemed to determine a higher lipofection than small particles did. Larger particles (1 to 2 μM) show a higher transfection efficiency than smaller particles, which has been attributed to an enhanced sedimentation of the former onto the cells. Larger particles of Lipofectamine 2000 lipoplexes above 500 nm (Figures 4b-A and 6B) did not lead to higher transfection efficiency at liposome/pDNA weight ratios of 5 and 7. However, Hansma and Pietrasanta (1998) had shown that lipopolyplexes of medium size particles with diameters of 140 to 220 nm may transfect cells as effectively as 1 μM aggregated lipopolyplexes. Nakanishi and Noguchi (2001) also pointed that the lipoplex with moderate vesicles of 0.4 to 1.4 μM gave the highest transfection efficiency. Whether this difference reflects differences in complex stability and/or size-driven differences in cellular processing remains to be determined, but better insight into such parameters is essential for the rational development of non-viral vectors and improvement of their transfection efficiency.

Surface charge density of transfection complexes can be related to the transfection efficiency. Here, the highest transfection was obtained with the negatively charged complexes in HeLa, Hep-2 and MCF-7 cells (Figure 5B and C) at liposome/pDNA weight ratios of 3 and 4, while other authors observed that the highest values of transfection occurred for the positively charged complexes (Farrow et al., 2006). Though positive complexes are invariably found to be essential for efficient

transfection such as avoiding DNA enzyme degradation, lipoplexes with positive potential do not always guarantee higher transfection (Wasungu and Hoekstra, 2006). Efficient gene delivery requires the uptake of the plasmid DNA (pDNA) in the target cell, release of the pDNA into the cytoplasm and transport to the nucleus where transcription takes place. At a weight ratio of Lipofectamine 2000/pDNA larger than 5, we observed for the system investigated, a dramatic decrease of transfection efficiency, probably due to the difficulty of the complexes to undergo dissociation, thus hampering the plasmid release inside cells. It seems that the restricted release at a higher ratio could be an important factor in determining the optimal liposome/pDNA ratio for transfection. The zeta potential of lipoplexes related the transfection efficiency to the property has been reported. Moreover, where the charge ratio of lipid/DNA was 1, the complexes are neutral, resulting in very unstable aggregates. At charge ratios greater than 1, the DNA was completely protected resulting in stable cationic lipoplexes (Davis, 2002; Nakanishi and Noguchi, 2001). At various stages of interactions of the lipoplexes with the cell components, the surface charge gained different importance of binding with cell membrane or dissociation with liposomes.

Increasing pDNA amount should be done with caution because of saturation effect and decreased transfection efficiency (Figure 5C), though sufficient pDNA must be used to allow high level of transgene expression. This suggested that the efficient transfection was achieved when liposomes bound relatively high amount of pDNA, but not to completely neutralize the positive charge of the liposome surface. Moreover, it was found that a cell density that is too high can result in inhibition of cell growth and metabolism following transfection. Conversely, if the cell density is too low, recovery of the culture from the effects of transfection can be poor. The optimum cell density of Lipofectamine 2000 was from 1×10^4 to 2×10^4 cells per well for luciferase gene expression in HeLa cells (Figure 5A), but the optimum cell density was 2×10^5 cells per well for β-galactosidase activities in 293-H cells (Masotti et al., 2009).

Optimal transfection was observed when the diluted liposomes were stood for 20 to 30 min with optimal complex formation for 30 min. When the complex formation time is prolonged, rigid assemblies of cationic lipids can preclude efficient recruitment of cationic lipid by pDNA, causing pDNA aggregation of larger particles to the sizes of several microns, resulting in lower transfection efficiency, presumably caused by a reduced internalization of complexes by cells. Serum did not significantly affect transfection efficiency of Lipofectamine 2000. It was proven that gene carriers that are efficient *in vitro* often fail to show the same efficiency when applied *in vivo*. The main reason for the poor efficacy *in vivo* is the sensitivity to serum (Nakanishi and Noguchi, 2001). These results imply that by controlling these factors, an efficient lipid delivery system may be achieved for *in vitro* and *in vivo* gene therapy. It will also be interesting to note whether alternative lipid formulations can improve transfection while reducing lipid-mediated toxicity. Cationic-liposome based reagents were cell dependent with the different cell internalization pathway. The general health of cells prior to transfection is known to be an important source of variability from one transfection to another.

The toxicity is still an obstacle to the application of non-viral vectors to gene therapy. Cationic lipids and cationic polymers for gene delivery may cause toxic effect. For example, lipoplexes caused several changes to cells, which included cell shrinking, reduced number of mitoses and vacuolization of the cytoplasm (Salvati et al., 2006). The toxicity of cationic lipids is mainly determined by their cationic nature. The quaternary ammonium head-group is more toxic than tertiary amine. The import of a heterocyclic ring as a substitution of the linear amine head-group, such as pyridinium and guanidine, can spread the positive charge of the cationic head, and then toxicity is decreased significantly (Lv et al., 2006). Results (Figure 6D) show that toxicity was cell-specific. Cationic lipids with ester bonds such as DOTAP in the linker zone are more biodegradable and associated with less cytotoxicity in cultured cells. The toxicity difference of DOTAP and Lipofectamine 2000 was therefore considered as not significant.

Furthermore, we observed the intracellular distribution of fluorescent-labeled lipid and Rh-pDNA. They were located at the same locations on the surface and in the cytoplasm of the cells at 2 h after addition of the complex, indicating that the complex bound to the cells and became internalized by the endocytotic pathway. In addition, no green fluorescence was observed in the nuclei from the merged pictures, suggesting that the liposomal lipid had detached from the complex. These results may indicate the importance of pDNA release from endosome, lipoplex and pDNA delivery into the nucleus for Lipofectamine 2000 successful gene expression.

Conclusion

In summary, lipid structure, liposome/pDNA weight ratios, lipoplex morphology seemed to be the most important ones among parameters influencing transfection efficiency. Moreover, liposome transfection properties were the result of interplays between them and were highly modulated by the target cell type. It was noted that the release of plasmid DNA from the complex was one of the most crucial steps determining the optimal ratio for cationic liposome-mediated transfection. We believe that the methods outlined herein will enable others to address important biological questions to further modify the chemical features of the lipid vectors, and thereby better control their biological behavior, thus rendering them as useful candidates for therapeutic gene delivery.

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