In Vitro Gene Transfection in Human Glioma Cells Using a Novel and Less Cytotoxic Artificial Lipoprotein Delivery System

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Purpose. To develop and evaluate a novel artificial lipoprotein delivery system for *in vitro* gene transfection in human glioma cells.
Method. Nanoemulsion was formulated with similar lipid compositions present in natural lipoproteins. The oil phase of nanoemulsion was composed of triolein (70%), egg phosphatidylcholine (22.7%), lysophosphatidylcholine (2.3%), cholesterol oleate (3.0%), and cholesterol (2.0%). To replace the surface protein as in natural lipoprotein, poly-L-lysine was modified to add palmitoyl chains at a basic condition and was incorporated onto the nanoemulsion particles through hydrophobic interaction. A model plasmid DNA, pSV-β-Gal containing a reporter gene for β-galactosidase was carried by the nanoemulsion/poly-L-lysine particles. The charge variation of so-formed complex was examined by agarose gel electrophoresis and zeta potential measurement. *In vitro* transfection was conducted on human SF-767 glioma cell line using this new system. After standard X-Gal staining, transfected cells were observed under light microscope. The effect of chloroquine on the transfection was examined and, finally, the cytotoxicity of this new system was evaluated in comparison with commercial Lipofectamine™ gene transfection system.
Results. The plasmid DNA was effectively carried by this artificial lipoprotein delivery system and the reporter gene was expressed in the glioma cells. Transfection efficiency was significantly increased by the treatment of chloroquine, indicating that endocytosis possibly was the major cellular uptake pathway. Compared to Lipofectamine™ system, this new delivery system demonstrated similar transfection efficiency but a much lower cytotoxicity. In the experiment, the cell viability showed up to 75% using this system compared to only 24% using Lipofectamine™ system.
Conclusion. A new artificial lipoprotein delivery system was developed for *in vitro* gene transfection in tumor cells. The new system showed similar transfection efficiency but a much lower cytotoxicity compared with commercial Lipofectamine system.

KEY WORDS: Gene delivery; transfection; glioma; palmitoyl poly-L-lysine; nanoemulsion.

INTRODUCTION

Gene transfection can be defined as the delivery to and subsequent expression of functional genetic material in specific cells to manipulate their intrinsic genetic profiles. During the last decade, researches involving gene transfection have been expanding rapidly and many gene delivery systems have been developed to efficiently transfect various cells in *in vitro* and *in vivo* experimental conditions. As an effective gene delivery system, it must be able to carry sufficient amounts of genetic material and express the genetic information in specific cells resulting in significant changes in genetic profiles. In general, genetic materials can be carried and expressed in specific cells by either viral vector systems or non-viral vector systems. The viral vector systems, including retrovirus, adenovirus, adeno-associated virus, herpes simplex virus, and lentivirus have been extensively investigated because of their high transfection efficiency. However, their applications are limited by their complicated handling procedures for *in vitro* experiments and poor safety profiles for *in vivo* studies (1–2). Compared to the viral vector systems, the non-viral vector systems are easy to handle and have better safety profiles. Consequently, development of effective non-viral gene delivery systems has become the center pieces in many research laboratories (3–5).

Because lipids are the main components of cell membrane, most non-viral vectors are lipid-based such that the vectors can be effectively incorporated into cell membrane and facilitate the delivery of genetic materials into specific cells. Among these non-viral vectors, cationic liposomes, which carry positive charge and electrostatically interact with negatively charged DNA to form complexes, are most widely studied (6–11). However, the success of using cationic liposomes for gene transfection is partly hampered by the cytotoxicity of the cationic lipids. Polymer-based non-viral vectors have also been widely investigated, including poly-L-lysine, polyethenimine, polyamidoamine dendrimer, and chitosan (12–20). One main disadvantage of these systems is the low efficiency of transfection. Recently, Kim *et al.* developed a new gene delivery system called Terplex system, which is based on a complex formed by natural low-density lipoprotein (LDL) and stearyl-poly-L-lysine (21–22). Through hydrophobic interaction, stearyl-poly-L-lysine can be incorporated into the LDL particles. The assembled complex possesses positive charge and was able to carry negatively charged DNA and successfully deliver the DNA into vascular smooth muscle cells.

In this article, we report the development and evaluation of a novel artificial lipoprotein delivery system that can carry DNA materials for effective *in vitro* gene transfection in tumor cells. Similar to the structure of natural lipoproteins, this artificial lipoprotein delivery system consists of nanoemulsion cores made of natural lipids and surface lipidized poly-L-lysine, which replaces the surface protein as in natural lipoproteins. With proper weight ratio of poly-L-lysine to the lipids in nanoemulsion, the artificial lipoprotein delivery system efficiently carries plasmid DNA containing β-galactosidase gene and transfected human SF-767 glioma tumor cells. Our experiments showed that because the lipids used in the system are all natural substances, the cytotoxicity of this delivery system could be lower than the commercial gene trans-

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ABBREVIATIONS: PLL, poly-L-lysine; p-PLL, palmitoyl poly-L-lysine; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; ONPG, o-nitrophenyl-β-D-galactopyranoside; X-Gal, 5-bromo-4-chloro-3-indoyl-β-D-galactoside; LDL, low-density lipoprotein; PBS, phosphate-buffered saline.

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fection systems using cationic liposomes. The benefit associated with the low cytotoxicity makes it especially useful as an alternative to Lipofectamine™ or other commercial gene transfection systems. Another advantage of this system is that it can be readily assembled using commercial available materials including phospholipids, cholesterol, and poly-L-lysine. The chemical composition, particle size and type of surface poly-peptide or surface protein can be controlled and optimized allowing widely-diversified gene or drug delivery applications.

MATERIALS AND METHODS

Materials

Triolein (99%), egg yolk phosphatidylcholine (99%), cholesterol (99%), poly-L-lysine hydrobromide (MW 57900 Dalton based on viscosity), chloroquine (99%), o-nitrophenyl-β-D-galactopyranoside (ONPG), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were purchased from Sigma (St. Louis, MO, USA). L-α-lysophosphatidylcholine (99%) was purchased from Avanti (Alabaster, AL, USA). Cholesterol oleate (99%) was obtained from Acros (Pittsburgh, PA, USA). 5-bromo-4-chloro-3-indoyl-β-D-galactoside (X-Gal) was from Life Technologies (Rockville, MD, USA). Electrophoretic grade agrose was purchased from FMC Bioproducts (Rockland, ME, USA). All other chemicals were of analytical grade obtained from Sigma or J. T. Baker (Phillipsburg, NJ, USA).

Preparation of Nanoemulsion

The oil phase of emulsion was composed of triolein (70%), egg phosphatidylcholine (22.7%), lysophosphatidylcholine (2.3%), cholesterol oleate (3.0%), and cholesterol (2.0%). The lipid components were dissolved in chloroform individually and then mixed thoroughly. The chloroform was then removed completely by a stream of nitrogen gas. In each 100 mg of lipid mixture, 10 ml of 2.4 M NaCl solution was added. The mixture was sonicated under nitrogen flow for 30 min using Model 450 Sonifier® (Branson Ultrasonics Corporation, Danbury, CT, USA) with a duty cycle dial setting of 90% at output of 40 watts. The temperature of the mixture was maintained at 55°C during sonication. The prepared emulsion was then passed through an Emulsiflex B3 device (Avestin, Ontario, Canada) at a pressure of 70 psi for 10 times to reduce the particle size to nanometer level. The emulsion was dialyzed against phosphate-buffered saline (PBS) using Spectra/Por® 2 molecularporous membrane tubing with molecular weight cut-off of 6000–8000 d (Spectrum Medical Industries, Inc. Houston, Texas, USA). The emulsion particle size distribution was measured by Submicron Particle Sizer Autodiluter Model 370 (NICOM Particle Sizing System, San Barbara, CA, USA). In addition, the nanoemulsion was stored at room temperature and the particle size distribution was measured in 2, 4, 8, and 16 weeks, respectively, to examine the stability of the nanoemulsion particles.

Lipidization of Poly-L-Lysine

Lipidization of poly-L-lysine was performed as described by Kim *et al.* (21) with slight modification. Briefly, poly-L-lysine hydrobromide (30 mg) was dissolved in 2 ml DMSO in a 50 ml round-bottom flask. After triethylamine (10 μl) was added, palmitoyl chloride (20 mg) was added to the mixture to react with the amino group of the lysine residues in poly-L-lysine. The mixture was allowed to react at room temperature for 2 h and filtered. Acetone was added to the filtrate to precipitate the lipidized polymer, palmitoyl poly-L-lysine or abbreviated as p-PLL. The product was dissolved in methanol, re-precipitated by acetone, and dried under vacuum overnight. The modified polymer was characterized by proton NMR.

Incorporation of p-PLL into Nanoemulsion Particles

In each 1.5 ml microcentrifuge tube, nanoemulsion (50 μl) was diluted with 0.2 ml PBS solution and incubated with various amounts of p-PLL at 37°C based on the weight ratio of p-PLL to triolein in nanoemulsion. The weight ratios of p-PLL to triolein in the mixture were 0.125:1, 0.25:1, 0.5:1, and 1:1, respectively. After incubation for 1 h, the mobility of the nanoemulsion particles in electric field was examined by agarose gel electrophoresis using Nile Red as the fluorescent dye. Agarose gel (0.4%) was prepared in TAE buffer (40 mM Tris-acetic acid, 1 mM EDTA, pH 8.0). Five microliters of Nile Red solution in acetone (100 μg/ml) was dried out in a test tube and redissolved in 30 μl of the incubation mixture as described above. In each sample, 6 μl of glycerine was added to increase the density of the sample and the sample (30 μl) was loaded in each sample well of the agarose gel. Electrophoresis was conducted for 1 h at 70 volts at room temperature using Horizontal Mini-gel System (CBS Scientific Company Inc. Del Mar, CA, USA). The mobility of the particles in electric field was visualized by Eagle Eye II Video System (Stratagene, CA, USA).

Amplification and Purification of Plasmid DNA

Plasmid DNA, pSV-β-Galactosidase Control Vector, was purchased from Promega (Madison, WI, USA) and was introduced into Epicurian Coli® XL1-Blue MRF' (Stratagene, CA, USA) by using standard transformation protocol. The transformed *E. coli* strain was maintained in Luria–Bertani (LB) medium containing 15% of glycerol at –80°C. To amplify the plasmid DNA, the *E. coli* strain was cultured in LB medium containing 100 U/ml of ampicillin at 37°C overnight and the cells were harvested by centrifugation. Plasmid DNA in the cells was extracted and purified using Wizard Plus SV Minipreps DNA Purification System (Promega, Madison, WI, USA). The purity of plasmid DNA was confirmed by determining the ratio of optical absorbance at 260 nm and 280 nm ($>=1.8$) and further by 0.6% agarose gel electrophoresis. The agarose gel was stained with ethidium bromide (0.5 μg /ml) for 15 min and destained with deionized water for 10 min. DNA bands in the agarose gel were visualized by Eagle Eye II Video System. The concentration of plasmid DNA was determined by spectrophotometer at wavelength of 260 nm (1 $OD_{260} \approx 50$ μg/ml).

Assembly of the Complex of Nanoemulsion, p-PLL and Plasmid DNA

Nanoemulsion (50 μl) in 0.2 ml PBS was mixed with various amounts of p-PLL in the same way as described above. The weight ratios of p-PLL to triolein in the nano-

emulsion were 0.0625:1, 0.125:1, 0.25:1, 0.5:1, and 1:1, respectively. After incubation at 37°C for 1 h, DNA (2 μg) was added and incubated at room temperature for 15 min. Samples were then loaded into 0.4% agarose gel and the electrophoresis was performed as described above. Zeta potential and mobility of the assembled particles are measured by Submicron Particle Size Analyzer 90Plus (Brookhaven Instrument Corporation, Holtsville, NY, USA). Before they were measured for zeta potential, the samples were diluted with sodium nitrate solution (1 mM, pH 7.4) until the count of particles in the sample reached 100–300 kilo-counts per second (KCPS). Water and solutions used in zeta potential measurement were filtered with 0.1 μm Supor Acrodisc (Gelman Sciences, Ann Arbor, MI, USA). The particle size, zeta potential and mobility was recorded by the built-in PC computer system.

Gene Transfection Experiment

Human glioma cell line SF-767 was obtained from the tissue bank of Brain Tumor Research Center (University of California-San Francisco, San Francisco, CA, USA) and used in our transfection experiment because of its characteristics of aggressive growth. The cells were grown at 37°C in 5% CO_2 with Eagle's Minimal Essential Medium (EMEM) medium supplemented with 10% fetal bovine serum (BioCell Laboratories, Rancho Dominguez, CA, USA), 100 U/ml penicillin, and 100 μg/ml streptomycin. Culture was passaged twice a week to maintain the cells in exponential growth. The transfection was conducted in 6-well (35-mm in diameter) culture plates. SF-767 cells were seeded with 3×10^5 cells in each well 24 h before the transfection. During the day of transfection, nanoemulsion (50 μl) in 0.2 ml PBS was mixed with p-PLL in the ratios as described above, and incubated at 37°C. After 1 h of incubation, 2 μg of plasmid DNA was added and incubated at room temperature for 15 min to obtain the complex of nanoemulsion/p-PLL/DNA. Cells were washed with PBS buffer three times and 1 ml of EMEM (without serum and antibiotics) was added in each well. The nanoemulsion/p-PLL/DNA complex was added to each well and mixed with the medium completely by swirling. The cells were then incubated at 37°C in 5% CO_2 for 12 h before 1 ml EMEM medium containing 20% fetal bovine serum was supplemented. The cells were incubated for an additional 24 h. Both the nanoemulsion/p-PLL complex and the naked DNA were used, respectively, as the negative controls. As the positive control, Lipofectamine™ reagent purchased from Invitrogen (Carlsbad, CA, USA) was incubated with the DNA and the transfection experiment was performed at the same condition.

Detection of β-Galactosidase by X-Gal Staining and Enzymatic Assay

After 24 h of transfection incubation, the cells in each well were washed twice with PBS buffer and then fixed with 2 ml fixing solution (2% formaldehyde and 0.2% glutarldehyde in PBS buffer) for 15 min at room temperature. After the cells were washed three times with PBS, 1.5 ml of staining solution (5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 1 mM $MgSO_4$, and 1 mg/ml X-Gal from 20 mg/ml stock in dimethyl formamide) was added. The cells were incubated at 37°C in 5% CO_2 for 30 h before the transfection was evaluated by light microscope. β-Galactosidase activity of cells was determined by β-Galactosidase Enzyme Assay System (Promega, Madison, WI, USA). In brief, the cells from each well of the plate were trypsinized and collected by centrifugation. They were disrupted by mixing with 200 μl of Lysis Buffer and incubating for 30 min at room temperature. The protein concentration of the cell lysate was determined by Bradford method (23). Cell lysate (100 μl) was mixed with 100 μl of ONPG solution in 2× Assay Buffer (1.33 mg/ml) and incubated in water bath at 37°C for 5 h. The enzymatic reaction was terminated by adding 300 μl of 1 M sodium carbonate solution. After the reaction mixture was diluted, the absorbance at 420 nm was read in a spectrophotometer (Baush & Lomb Spectronic 2000, Rochester, NY, USA). The enzymatic activity unit was defined in a similar way as described by Kim *et al.* (21).

Effect of Chloroquine on the Transfection Efficiency

To demonstrate the effect of chloroquine, which is a lysomotropic agent, on the transfection efficiency, chloroquine solution in PBS was added to the cell culture (80% confluent) with a final concentration of 100 μM. After 30 min of incubation at 37°C and 5% CO_2, the culture medium was removed and the cells were washed with PBS three times. Fresh medium was supplemented before the transfection experiment was started. The complex of nanoemulsion (50 μl in 0.2 ml PBS) with p-PLL (p-PLL:triolein = 0.25:1) was used as the carrier for 2 μg of plasmid DNA in the transfection experiment. After transfection incubation, the cells were trypsinized and collected by centrifugation. The β-galactosidase activity was measured by the method as described above.

Cytotoxicity Comparison of Nanoemulsion/p-PLL Complex and Lipofectamine

Cellular toxicity of the nanoemulsion system was tested according to the MTT method reported by Mosmann (24). A complex of nanoemulsion (50 μl in 0.2 ml PBS) with p-PLL (p-PLL:triolein = 0.25:1) was prepared in a way similar to described earlier. In 96-well microplate, SF-767 cells were seeded at 2×10^4 cells in each well containing 0.1 ml of EMEM medium. After 24 h of incubation at 37°C and in 5% CO_2, the nanoemulsion/p-PLL complex or Lipofectamine reagent was added to the cell culture. The amount of nanoemulsion/p-PLL complex or Lipofectamine reagent added to the cell culture was determined such that similar transfection efficiency could be obtained based on the transfection experiments. The cell culture grown on EMEM medium without nanoemulsion/p-PLL complex or Lipofectamine reagent was used as the control. Because only living cells are able to cleave the tetrazolium ring to produce dark blue crystals, which can be measured colorimetrically, the viability of cells after additional 1, 2, 3, and 4 days of growth was determined by measuring the ability of the cells to degrade tetrazolium salt MTT. Briefly, 25 μl of MTT solution in PBS buffer (0.5 mg/ml) was added to each well of culture and incubated at the same condition for additional 4 h. The medium was then removed and 150 μl of DMSO was added to each well and mixed thoroughly until all the dark blue crystals were dissolved. The plate was read on an OPTImax Tunable Microplate Reader

(Molecular Devices Corporation, Sunnyvale, CA, USA) at wavelength of 550 nm. The cell viability was calculated and expressed as $(OD_{trt}/OD_{ctrl}) \times 100\%$, where OD_{trt} is the optical absorbance from the culture treated with either nanoemulsion/p-PLL complex or Lipofectamine reagent, and OD_{ctrl} is the optical absorbance from the culture of control.

RESULTS

Preparation of the Nanoemulsion and the Size Distribution

With the proper control of temperature (55°C) during sonication, the obtained emulsion seemed to be homogeneous. The number weighted mean size of the emulsion particle was 110.2 ± 42.9 nm. Because the emulsification was conduced by metal probe sonication, chelating agent (0.1 mM EDTA) was added to remove the free iron ion before it is dialyzed against PBS overnight (PBS changed every 6 h). Followed by 10 cycles of size reduction by Emusiflex B3 device (Avestin, Ottawa, Canada), the number weighted mean particle size was reduced to 48.9 ± 19.8 nm.

The physical stability of the nanoemulsion can be investigated by measuring the change in size distribution of the particles. The size distribution of the nanoemulsion particles was measured at 0, 2, 4, 8, and 16 weeks after the preparation and results are shown in Fig. 1. The size distribution did not change significantly on storage at room temperature. After 16 weeks of storage at room temperature, the size of the emulsion particles was 78.9 ± 14.6 nm, indicating that the nanoemulsion particles were stable.

Incorporation of p-PLL into Nanoemulsion Particles

Because p-PLL is positively charged, the incorporation of p-PLL molecules into nanoemulsion particles will result in a change in surface charge of the particles. The change can be seen in the picture of agarose electrophoresis (Fig. 2). Nanoemulsion particles moved to anode because they were negatively charged (Lane 1). The negative surface charge of the nanoemulsion particles was also confirmed by the zeta potential and mobility, which was −42.28 ± 2.3 mV and −3 ± 0.06 (m/s)/(V/cm), respectively. The incorporation of p-PLL neutralized the surface charge (Lane 2 to Lane 4) and resulted in the retardation of move in the electric field. When they were incubated with sufficient amounts of p-PLL, the surface charge of particles was reversed to be positive and moved toward the opposite direction in the electric field (Lane 5). The results indicated that p-PLL could be incorporated into the nanoemulsion particles.

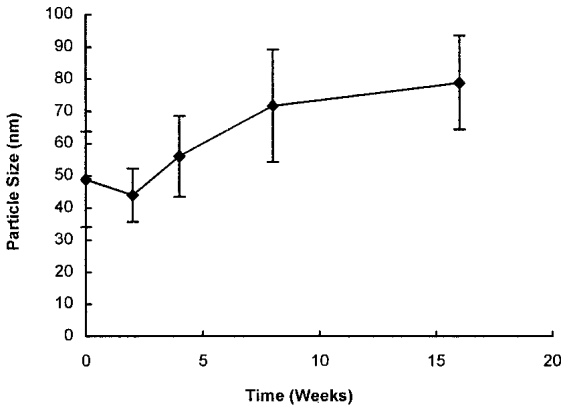

Fig. 1. The size change of nanoemulsion particles upon storage at room temperature (mean SD, n > 6).

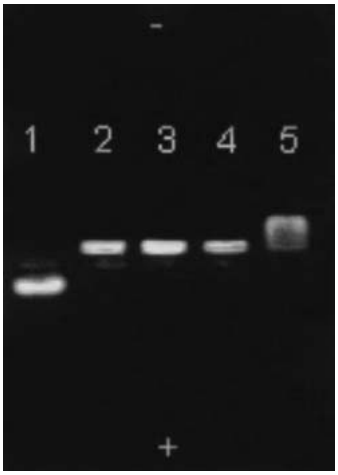

Fig. 2. Agarose gel electrophoresis of nanoemulsion particles and their complexes with p-PLL stained with Nile Red (Lane 1: nanoemulsion; Lane 2 to Lane 5 were the complex of nanoemulsion and p-PLL with the ratio of p-PLL to triolein to be 0.125:1, 0.25:1, 0.5:1, and 1:1 respectively).

Interaction of p-PLL Associated Nanoemulsion with DNA

After incorporation of sufficient amount of p-PLL molecules into nanoemulsion particles, the complex carried a positive charge and can electrostatically interact with negatively charged DNA molecules. As indicated in Fig. 3, plasmid DNA (Lane 1) migrated toward the positive anode. When DNA plasmid was incubated with p-PLL (Lane 2), no DNA migration was observed, possibly because DNA molecules were bound by p-PLL, and the ethidium bromide molecules could not intercalate into the DNA molecules resulting in no fluorescence emission. Lane 3 to Lane 7 showed the change in DNA carrying capability of the complex resulted from different ratios of p-PLL to nanoemulsion (i.e. the p-

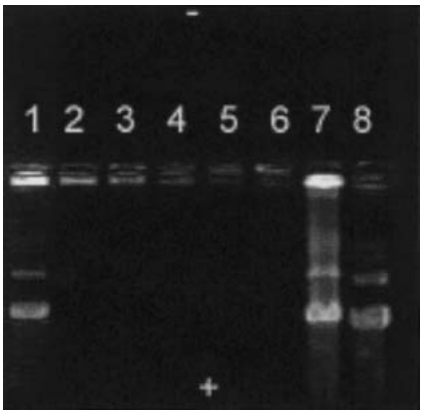

Fig. 3. Agarose gel electrophoresis of the complex of nanoemulsion and p-PLL with plasmid DNA stained with ethidium bromide (Lane 1 and Lane 8: Pure DNA; Lane 2: DNA/p-PLL; Lane 3 to Lane 7 were complexes of nanoemulsion with different amount of p-PLL and DNA. The ratio of p-PLL to triolein was 1:1, 1:0.5, 1:0.25, 1:0.125, and 1:0.0625 respectively).

PLL to triolein ratio). At a high ratio of p-PLL to nanoemulsion, DNA was tightly held by the complex and thus no DNA migration band seemed (Lane 3 to Lane 6). When the ratio of p-PLL to nanoemulsion became sufficiently low (0.0625:1 as the p-PLL to triolein ratio), plasmid DNA started to escape from the complex and free DNA band (Lane 7) seemed in the agarose gel.

Because the surface charge of the nanoemulsion/p-PLL/DNA complex is important to transfection, the zeta potential and mobility of these complexes were measured and the results are shown in Fig. 4 and Fig. 5. With fixed amount of plasmid DNA (2 μg), the increased amount of p-PLL led to an increase in zeta potential of the particles.

Transfection of Glioma Cell Line SF-767 by the Complex of Nanoemulsion/p-PLL/DNA

Most of the positively charged nanoemulsion/p-PLL/DNA complexes (with varying ratios of nanoemulsion/p-PLL/DNA) used in the experiments were found to transfect the glioma SF-767 cells, but with different transfection efficiency. The complex containing nanoemulsion and p-PLL (p-PLL:triolein = 0.25:1) and 2 μg DNA, which had a zeta potential of 8.47 ± 1.85 mV and a loading capacity of 1 μg DNA per 0.25 mg of lipid (or per 50 μl of formulation), had the highest transfection efficiency. Its efficiency was comparable to that by Lipofectamine™ reagent (Fig. 6). Under microscope, those cells that expressed active β-galactosidase seemed to be blue-green (as dark spots in Fig. 6) by X-Gal staining and the extent of transfection seemed to be comparable for the nanoemulsion complex (Fig. 6, C) to that by Lipofectamine™ reagent (Fig. 6, A). However, Lipofectamine™ reagent seemed to be much more toxic than the nanoemulsion complex, as indicated by the significant difference in cell counts.

Effect of Chloroquine on the Transfection

Cellular uptake of particles by way of endocytosis will result in the particles being processed by endosomal-lysosomal pathway. This pathway will lead to the degradation of the carried plasmid DNA and greatly lower the transfection efficiency. Chloroquine is a weak basic, lysomotropic drug, which will interfere with the endosomal acidification and cause the bursting of endosomes. To examine whether the nanoemulsion vector is delivered by way of endosomal-lysosomal pathway, cells were treated with 200 μM of chloroquine at 37°C for 30 min before the transfection procedures were conducted. The β-galactosidase activity of the cells with or without the treatment of chloroquine was shown in Fig. 7, based on two quantities of the nanoemulsion/p-PLL/DNA complexes. The treatment of the cells by chloroquine solution greatly increased the transfection efficiency and the effect was obvious at both complex quantities. This result suggested that endocytosis be the main mechanism of the cellular uptake of the nanoemulsion/p-PLL/DNA complex by glioma cells.

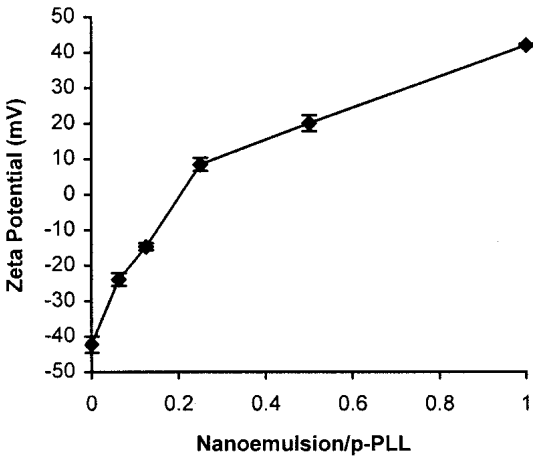

Fig. 4. Zeta potential of the nanoemulsion particles and their complexes with different amount of p-PLL and DNA (2 μg) (mean SE, n = 3).

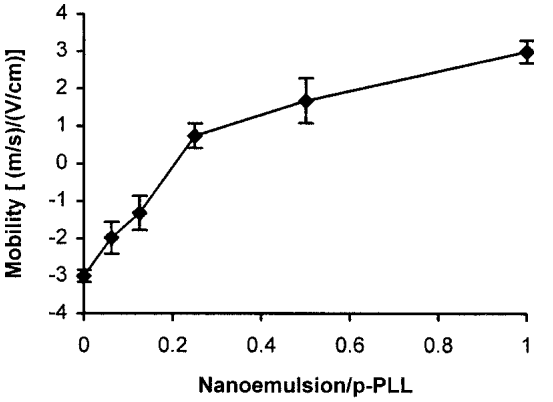

Fig. 5. The mobility of the nanoemulsion particles and their complexes with different amount of p-PLL and DNA (2 μg) (mean SE, n = 3).

Cellular Toxicity Evaluation of Nanoemulsion/p-PLL Complex

Cellular toxicity is one of the main concerns in the development of gene delivery system. It has been commonly shown that positively charged gene delivery systems, such as cationic liposomes, are cytotoxic. Other cationic polymers, e.g., poly-L-lysine, hydrophobized poly-L-lysine, were also found cytotoxic (21,25). Because the nanoemulsion particles in this research were negatively charged, the incorporation of positively charged and lipidized poly-L-lysine resulted in a neutralization of the charges on the surface. The cytotoxicity of the nanoemulsion/p-PLL complex, in comparison with that of the commercial Lipofectamine™ reagent, was shown in Fig. 8. The cytotoxicity was evaluated based on the relative

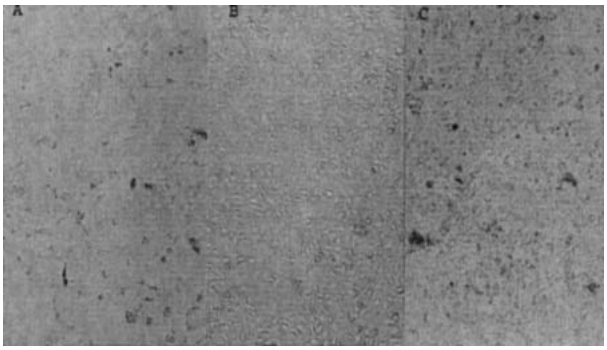

Fig. 6. X-Gal staining of glioma cells (A: Cells transfected using Lipofectamine™ reagent; B: Control; C: Cells transfected using nanoemulsion/p-PLL/DNA complex).

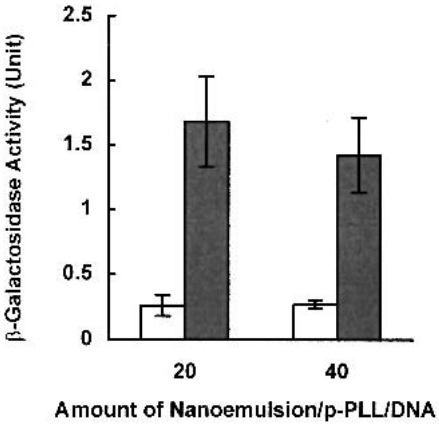

Fig. 7. The effect of chloroquine on the transfection by the nanoemulsion gene delivery system (white bar—untreated with chloroquine; gray bar—treated with chloroquine) (mean SD, n = 3).

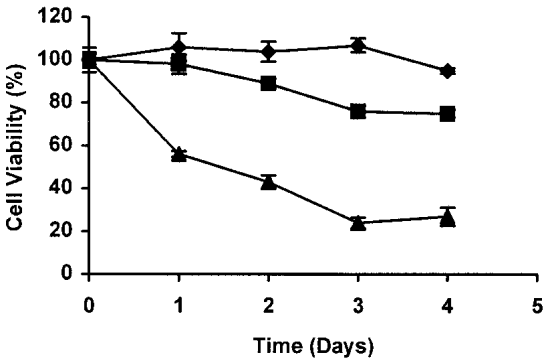

Fig. 8. Cytotoxicity comparison between nanoemulsion/p-PLL and Lipofectamine: control (♦, top), nanoemulsion/p-PLL (■, middle) and Lipofectamine (▲, bottom) (mean SD, n = 3).

viability of cells grown on the EMEM medium with and without the delivery systems. Four days after transfection, cell culture supplemented with the nanoemulsion/p-PLL complex had 75% cellular viability whereas that supplemented by Lipofectamine™ reagent (which is cationic liposome) had only 24% cell viability.

DISCUSSION

Effective gene transfection depends on the ability of the carrier system to deliver gene to and transfect in specific cells with high transfection efficiency and low cytotoxicity. Many synthetic carrier systems have been investigated with certain success and most of them belong to the category of cationic liposomes. However, DNA/liposome complex is in general cytotoxic and thus less cytotoxic but efficient gene carriers have been investigated. One of such approaches is to develop gene carriers that somewhat mimic the natural carriers in human body. Kim *et al.* (21,22) developed a novel terplex system based on the natural low-density lipoprotein associated with hydrophobized poly-L-lysine. The system is capable of condensing DNA and subsequently transfecting cells. Hara *et al.* (26) described the use of reconstituted chylomicrons remnants (RCR) as a non-viral vector for gene delivery. DNA was complexed with cationic lipid and solubilized in the core of these RCR particles. Both of these lipoprotein-based systems seemed to offer advantages over the conventional cationic liposome systems. Recently, the authors attempted to develop in their labs, an artificial lipoprotein system for controlled drug delivery. The artificial lipoprotein, similar to natural lipoprotein, consists of phospholipid nanoemulsion particles with functional proteins attached on the particle surfaces. A schematic drawing of the artificial lipoprotein system can be seen (Fig. 9) in comparison with natural human lipoproteins. Based on our earlier work in lipoprotein-resembling nanoemulsion for controlled delivery of an anti-tumor cholesteryl carborane compound (27), this article describes a new attempt to develop an artificial lipoprotein system that has poly-L-lysine attached on the particle surfaces for the purpose of gene delivery. The cytotoxicity of this system has been specifically investigated in comparison with commercial Lipofectamine™ reagent.

The lipoprotein-resembling particles were made of commercially available lipids and lipidized poly-L-lysine. The lipidization of poly-L-lysine was achieved through N-alkylation of the free ε-amino groups with palmitoyl chloride and confirmed by way of proton NMR (data not shown). The reaction condition was controlled to only lipidize about 25% of the lysine residues preserving sufficient amount of free ε-amino groups for maintaining the ability of poly-L-lysine to condense DNA. The nanoemulsion particles carried negative surface charge as shown by agarose gel electrophoresis in Fig. 2. When nanoemulsion particles are combined with unlipidized poly-L-lysine, immediate precipitation was observed indicating the formation of large aggregates caused by charge neutralization. When an appropriate amount of p-PLL was incubated with the particles, no precipitation or change in the turbidity was observed. These results indicate that with lipidized poly-L-lysine the interaction was not merely through electrostactic interaction, but also through hydrophobic interaction between the palmitoyl chains of p-PLL and the phospholipid of nanoemulsion particles. The charge of the nanoemulsion particles became reversed at the time enough p-PLL was added (Fig. 2).

The surface charge of the complex formed by nanoemulsion and p-PLL depends on their relative ratio. To carry

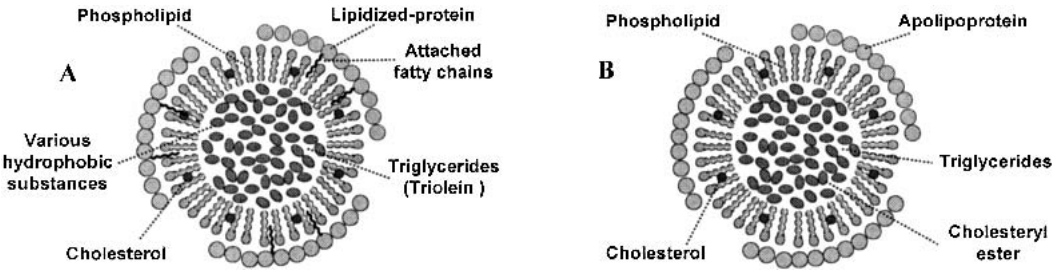

Fig. 9. (A) Artificial lipoproteins (20–100 nm) and (B) natural human lipoproteins (those in 20–100 nm range).

DNA molecules, which are negatively charged, the carrier needs to be positive. On the other hand, the surface charge of the complex after DNA is incorporated is also critical. Because cell surface is negatively charged, a positively charged nanoemulsion/p-PLL/DNA complex is essential for successful transfection. The surface charge of these particles can be monitored by agarose gel electrophoresis qualitatively or by zeta potential and mobility measurement quantitatively (Fig. 3, 4 and 5). Our studies showed that most of the positively charged complex formed by varying the ratios of nanoemulsion, p-PLL and DNA can transfect the glioma cells in certain extents. The complex containing nanoemulsion, p-PLL and DNA with the zeta potential of 8.47 ± 1.85 mV achieved the highest transfection efficiency indicating the proper charge balance among these components was important for transfection. This observation is consistent with that reported by Kim *et al.* (21) at the time the terplex carrier system involving natural LDL was used for gene delivery.

Chloroquine has been widely used to investigate the cellular uptake mechanism (28–29). It interacts with endosome inside the cell. A positive correlation of chloroquine level with the transfection efficiency indicates that the DNA is taken up through endocytosis and, furthermore, the endosomal-lysosomal pathway. Through chloroquine treatment, we have shown that the endocytosis seems the major cellular uptake pathway for the lipoprotein-resembling gene carrier.

Cytotoxicity is an important consideration for developing novel gene delivery systems. Using new gene carriers that mimic the nature substance such as human lipoproteins, the cytotoxicity associated with the delivery systems can significantly be reduced. It is known that poly-L-lysine is toxic to cells (Morgan, 1989). It is complex with the phospholipid nanoemulsion particles and plasmid DNA, however, cytotoxicity is low, as indicated by the experiments, in comparison with the Lipofectamine™ system. The nature of phospholipids, the neutralization of the positive charge of poly-L-lysine, and the proper balance among nanoemulsion, p-PLL and DNA apparently contribute to the reduced cytotoxicity for this new gene delivery system.

In conclusion, a novel artificial lipoprotein system has been developed for *in vitro* gene transfection to tumor cells. The system mimics the natural lipoprotein in composition but contains lipidized poly-L-lysine (instead of surface protein) to carry genetic materials. This system can be conveniently formulated from natural lipids, with the ability to control the size and surface charge. With proper ratios among its components, the new gene delivery system shows a similar transfection efficiency but a lower cytotoxicity compared with the commercial Lipofectamine™ gene transfection system, making it especially useful as an alternative to these commercial gene transfection systems.

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