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RESEARCH PAPER

Enhancement of Gene Transfer to Cervical Cancer Cells Using Transferrin-Conjugated Liposome

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ABSTRACT

Transferrin-conjugated cationic liposome (T_f-DDAB liposome) was developed as a targeted gene delivery system by using heterobifunctional cross-linking agent, N-succimidyl-3-(2-pyridyldithio)propionate (SPDP), and gradient metrizamide ultracentrifugation method. Physico-chemical properties of T_f-liposome were determined by scanning/transmission electron microscopy (SEM/TEM) and dynamic laser-light scattering method (DLS) with a mean diameter of 584±15 nm. Gel retardation assay was performed using various DDAB:DNA ratios, and proved that the 6:1 weight ratio formulation gave the most neutralized complex. In vitro transfection was done in human cervical cancer cells, HeLa, and the transfection efficiency of T_f-liposome was found to be fivefold higher than that of unconjugated (plain) DDAB liposome and twofold higher than that of Lipofectin™. In conclusion, a target-oriented T_f-DDAB liposome was made successfully and proved to be very efficient in DNA delivery into the cervical cancer cells in culture.

Key Words: *Cationic liposome; Gene delivery; Transferrin*

INTRODUCTION

The aim of gene therapy is to provide specific cells of a patient with the genetic information necessary to produce therapeutic proteins. It has become increasingly important as a novel therapeutic entity for human diseases.[1–3] The strategy for introducing genes includes viral and nonviral methods that

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possess unique characteristics. A virus-mediated method can offer high efficiency and stable integration of the exogenous gene into host chromosomes, but has long-term risks, such as possible recombination with endogenous viruses, oncogenic effects.[4,5] As an alternative nonviral DNA delivery carrier, cationic liposomes have recently been frequently used. Although cytotoxicity, low transfection efficiency, and precipitation problems were reported, they have been proved easy and safe for human clinical use.[6,7] In vitro transfection efficiency of cationic liposomes can be increased when complexed with transferrin (T_f), employing receptor-mediated endocytosis mechanisms.[8] Transferrin receptor (T_fR) levels are found to be elevated in various types of cancer cells, including squamous cell carcinomas such as oral tumors,[9] and correlate with the aggressive or proliferative ability of tumor cells.[10] Therefore, T_fR are considered to be useful as a prognostic tumor marker and as a potential target for drug delivery in the therapy of malignant cell growth.[11]

In this study, we employed a T_f-liposome/DNA complex system for gene transfection in vitro, and investigated the efficiency of T_f as a targeting ligand for DNA/cationic liposome complex. Liposomes were prepared by reverse-phase evaporation method using dimethyldioctadecyl ammoniumbromide (DDAB), cholesterol (Chol), and maleimide-derivatized phospholipid (MPB-PE). The T_f was conjugated to liposomes via the reaction of MPB-PE with a thiol-introduced protein by a heterobifunctional cross-linking agent, *N*-succimidyl-3-(2-pyridyldithio)-propionate (SPDP). Physico-chemical characterization of T_f-liposomes was done using scanning electron microscopy (SEM), transmission electron microscopy (TEM), and a zeta-sizer. The transfection efficiency of T_f-liposome measured by β-galactosidase expression from pCMV-β-gal in HeLa cells was compared to that of Lipofectin™.

EXPERIMENTAL

Materials

Dimethyldioctadecyl ammonium bromide, cholesterol, dithiothreitol (DTT), 4-(*p*-maleimidophenyl)-butyric acid *N*-hydroxy succinimide ester (SMPB), *N*-succimidyl-3-(2-pyridyldithio)propionate, and transferrin were purchased from Sigma Chemical Co., Ltd. (St. Louis, MO). Transphosphatidylated egg phosphatidylethanolamine (TPE) was purchased from Avanti Polar Lipids (Pelham, AL). Mouse anti-human transferrin receptor (CD71) was purchased from Zymed Co. (San Francisco, CA). Anti-mouse IgG-FITC was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). The pCMV-β-gal plasmid vector (7.2 kb) was from Gibco BRL (Grand Island, NY). Cervical cancer cell line, HeLa, was purchased from Korean Cell Line Bank (Seoul, Korea). All reagents for cell culture were purchased from Gibco BRL (Grand Island, NY). All other materials were reagent grade or better.

Cell Culture

Cervical cancer cells, HeLa, were grown in minimum essential medium supplemented with 10% fetal bovine serum (FBS), penicillin (100 units/mL), and streptomycin (100 μg/mL). Cells were grown in 25 cm^2 polystyrene tissue culture flasks in a humidified incubator (Sanyo Electric Co. Ltd., Japan) at 37°C with 5% CO_2 atmosphere.

Synthesis of Maleimidophenyl Butyl-Phosphatidyl Ethanolamine

Synthesis of MPB-PE was performed using the method of Martin and Papahadjopoulos.[12] Briefly, 200 μmol of transphosphatidylated egg phosphatidylethanolamine and 100 mg of 4-(*p*-maleimidophenyl)-butyric acid *N*-hydroxy succinimide ester were dissolved in 4 mL of freshly distilled lutidine and 10 mL of anhydrous methanol. After 2 hr of reaction with stirring under argon at room temperature, formation of MPB-PE was monitored by thin layer chromatography (TLC). The sample was developed by TLC using Merck silica gel 60F-254 (0.2 mm thickness) glass-baked TLC plates with a mixture of chloroform:methanol:H_2O (65:25:4, v/v) as the developing solution. The spots were visualized by spraying with ethanol solution of phosphomolybdic acid with heating at 200°C for 10 min. Evaporation of solvents (methanol and lutidine) and redissolving MPB-PE in chloroform were followed by column separation using silica gel (230–400 mesh, 60 Å) using a mixture of chloroform and methanol (chloroform:methanol = 40:1, 30:1, 20:1, 15:1, and 10:1, v/v) with increasing polarity to purify the product.

Preparation of DDAB Liposomes

Liposomes were prepared by the reverse-phase evaporation method.[13] Briefly, DDAB:Chol: MPB-PE at a molar ratio of 20:20:1 was dissolved in 1 mL of chloroform. After evaporation of solvent under argon at room temperature, dry lipid film was suspended in 1 mL of freshly hydrated diethyl ether, to which was added 0.7 mL of phosphate buffered saline (PBS, pH 7.4). The mixture was vortexed vigorously for 1 min and ether was then eliminated by rotary evaporator. Liposomes were downsized by extruding through 0.2 μm polycarbonate membranes 20 to 30 times using a Liposofast extrusion device (Avestin, Toronto, Canada). The whole extruder was autoclaved prior to use and all reagents and glassware used for liposome preparation were sterilized, too.

Preparation of Transferrin Conjugated Liposomes

Transferrin was modified to have reactive thiol groups by the method of Carlsson et al.[14] Briefly, 5 mg of transferrin was dissolved in 5 mL of 0.1 M PBS (pH 7.4) and the SPDP solution was freshly prepared at 20 mM in methanol. After 30 min reaction (SPDP:T_f = 25:1 molar ratio) with stirring, the pyridyl-dithiopropionate-derivatized transferrin (PDP-T_f) was separated from reactants by gel chromatography on a Sephadex G-75 column. The concentration of separated PDP-T_f was determined spectrophotometrically at 280 nm and 343 nm. Then 30 μL of 1 M DTT was added to 1 mL of PDP-T_f and stirred for 30 min at room temperature. Separation of SH-T_f from other reactants was done by gel chromatography on a Sephadex G-75 column. The concentration of separated SH-T_f was determined spectrophotometrically at 343 nm. The SH-T_f solution was sterilized by filtering through a 0.2-μm pore size Nalgene membrane and conjugation was initiated by mixing equal amounts of MPB-PE liposomes with SH-T_f overnight at room temperature. The T_f-conjugated liposomes were separated from unconjugated liposomes by metrizamide flotation method with a slight modification.[15] The structure of T_f-liposome is shown in Fig. 1.

Transmission Electron Microscopy

A copper grid was coated with carbon followed by collodionizing the grid. Then liposome solution was dropped onto the grid, to which was added 2% ammonium molybdate (pH 7.4). The grid was dried in a desiccator for 24 hr at room temperature and examined with a CM20 transmission electron microscope (Philips, 200 kV).

Gel Retardation Assay

Complexes of T_f-liposome/DNA were formed by mixing aqueous pCMV-β-gal plasmid solution with T_f-liposome in PBS (pH 7.4) at various weight ratios. After allowing 1 hr for the complex formation, samples were electrophoresed through a 1% agarose gel at 75 V for 1 hr and stained with ethidium bromide to visualize the DNA.

Dynamic Laser-Light Scattering and Zeta-Potential

The average droplet size and the zeta-potential of T_f-liposome/DNA complexes were determined by quasi-elastic laser-light scattering with a Malvern Zeta-sizer® (Malvern Instruments Ltd., Malvern, UK). The droplet size and the zeta-potential of lipo-

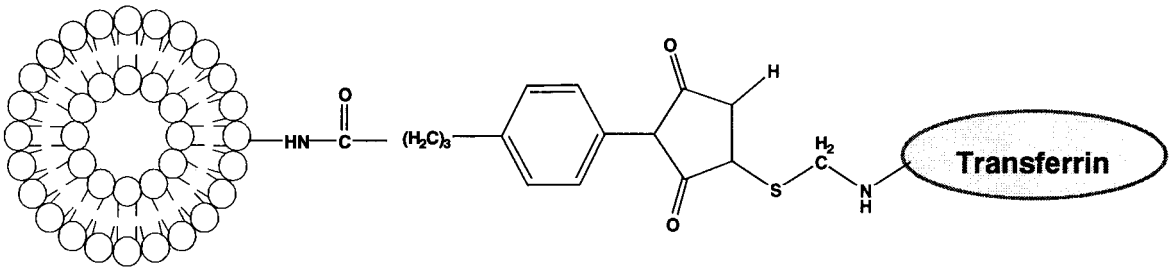


Figure 1. Structure of T_f-liposome.

somes were measured after a 300-fold dilution in water. The mixtures containing 1 μg of plasmid DNA and varying amounts of DDAB in the liposome to DNA weight ratios were used to measure the zeta-potential.

In Vitro Transfection and Cytotoxicity Test

The HeLa cells were seeded at a density of 3×10^4 cells/mL in a 96-well plate 24 hr prior to transfection. Complexes of pCMV-β-gal/liposome were prepared by mixing pCMV-β-gal and liposome in FBS-free cell culture medium at an appropriate charge ratio and incubated for 30 min at room temperature to achieve complex formation. The medium in the 96-well plate was replaced with a transfection mixture followed by 4 hr incubation at 37°C. The β-galactosidase activity in transfected cells was determined using a colorimetric ONPG (*o*-nitrophenyl-β-D-galactopyranoside) assay system (Promega, Madison, WI) at 420 nm after 48 hr.

For the cytotoxicity test, HeLa cells were treated with PBS, DDAB liposome, Lipofectin™, and T_f-liposomes in a 96-well plate, followed by 48 hr incubation at 37°C. Cytotoxicity was measured by MTT (3-(4,5-dimethylthiozol-2-yl)-2,5-bihenyltetrazolium bromide) assay[16] and the cell viability (%) was calculated according to the following equation:

$$\text{Cell viability (\%)} = (OD_{570(\text{sample})}/OD_{570(\text{control})}) \times 100$$

where $OD_{570(\text{sample})}$ represents the measurement from the wells treated with various liposome/DNA complexes and $OD_{570(\text{control})}$ represents the measurement from the wells treated with PBS only.

Preincubation of Transferrin

Competitive inhibition by free transferrin was evaluated by treating the cells with an excess amount of transferrin (10 mg/mL) in a 96-well plate, followed by 24 hr incubation at 37°C. The following procedures were the same as in the in vitro transfection method.

RESULTS AND DISCUSSION

Characterization of T_f-Liposome Conjugate

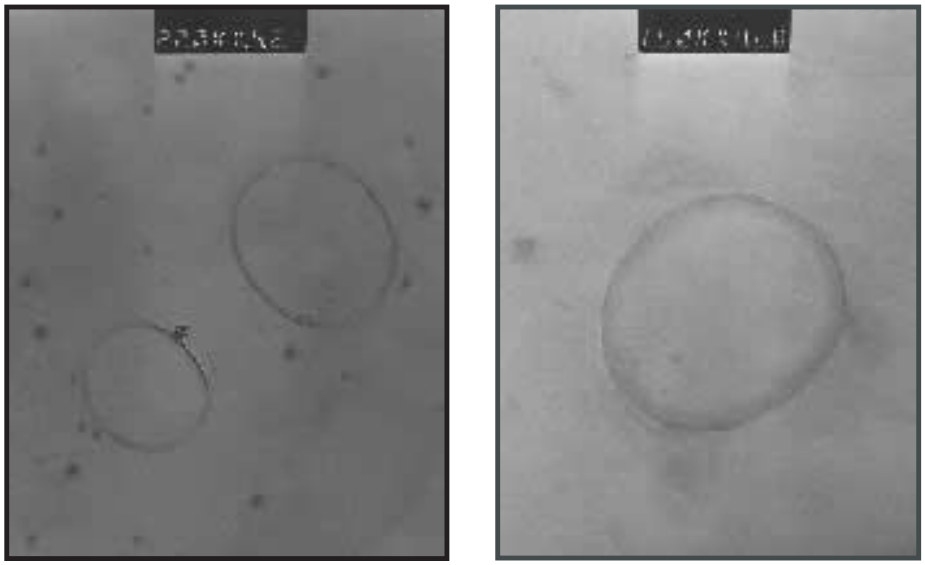
Transferrin was conjugated to DDAB:Chol:MPB-PE liposomes by thioether linkage (–S–) and the T_f-liposomes were then separated from unconjugated plain liposomes by a metrizamide flotation method. Several assumptions were made to calculate the amount (number) of conjugated transferrin to a liposome, such as the molecular weight of transferrin being 80,000 Da, each transferrin molecule weighing 1.33×10^{-19} g, and 1 μmol of DDAB (FW 631) giving 1.8×10^{12} liposome vesicles with 0.2 m pore size and 40 Å bilayer thickness. With these assumptions, it was found that the number of transferrin molecules attached to each liposome remained about 10–15 throughout the experiments. Physico-chemical properties of T_f-liposome were determined by SEM/TEM and DLS. Apparent differences in morphology were not observed between T_f-liposome and DDAB liposome (Fig. 2). The diameter of T_f-liposome determined by DLS was 584.2±8.8 nm and that of DDAB liposome was 466.2±13.3 nm (Table 1). So the T_f-conjugated liposome was slightly larger than the DDAB liposome. Though liposomes were filtered through a 0.2-μm membrane, the size of the liposomes was more larger than 400 nm, presumably due to the aggregation of liposomes during storage.

pCMV-β-gal/T_f-Liposome Complex

The cationic liposome forms a complex with the negatively charged pDNA through charge neutralization. Gel retardation assay was performed using various DDAB:DNA ratios for this matter. When the weight ratio reaches 6:1, the most charge-neutralized complex was formed (Fig. 3b) with a slight negative zeta-potential, −4.5 mV (Fig. 4). In addition, the formulation of 6:1 weight ratio showed the most effective transfection efficiency on HeLa cells (Fig. 5). These suggest that the effect of targeting ligand is more predominant than that of surface charge of liposome in receptor-mediated endocytosis of T_f-liposome/DNA complex.

In Vitro Transfection and Cytotoxicity Test

The transfection efficiency of T_f-liposome was investigated and compared with DNA only, DDAB liposome/DNA complex, and Lipofectin™/DNA complex on HeLa cells. The transfection efficiency was measured using a colorimetric β-galactosidase enzyme activity assay (ONPG assay). As shown in Fig. 6, transfection efficiency is presented as



(A) (B)

Figure 2. Transmission electron micrograph of liposomes. There was no significant difference in shape between T_f-liposome and DDAB liposome: (A) T_f-liposome (magnification 200,000×) and (B) DDAB liposome (magnification 150,000×).

Table 1

Size of Liposomes

Liposome formulation	DDAB liposome	T_f-liposome
Liposome diameter (nm)[a]	466.2±13.3	584.2±8.8

[a]Determined by quasi-elastic laser-light scattering.

β-galactosidase unit per milligram of cellular protein. The T_f-liposome showed up to a fivefold increase in β-galactosidase activity compared to the unconjugated (plain) DDAB liposome (0.87 unit vs. 0.18 unit) and a twofold increase compared to Lipofectin™ (0.87 unit vs. 0.45 unit), suggesting that the presence of transferrin facilitates the uptake of the T_f-liposome into HeLa cells, presumably by receptor-mediated endocytosis.

The T_f-liposome was also tested for its cytotoxicity and compared with Lipofectin™ for HeLa cells by MTT assay. As shown in Fig. 7, the cell viability from Lipofectin™ and DDAB liposome was 75% and 76%, respectively, at 20 μg/mL concentration. In contrast, the T_f-liposome showed 69% cell viability at the same concentration, indicating no significant cytotoxicity in HeLa cells compared with other known formulations.

Preincubation Study

The HeLa cells were preincubated with transferrin (10 mg/mL) in a 96-well plate prior to transfection, followed by 24 hr incubation at 37°C in 5% CO_2 incubator. As shown in Fig. 8, preincubation of transferrin did not affect the β-galactosidase expression from DNA only, DDAB liposome, Lipofectin™/DNA, or DDAB/DNA formulations. However, about a 72% decrease in β-galactosidase expression was observed from T_f-liposome/DNA formulation, suggesting that T_f-liposome uptake into the cells is mainly by receptor-mediated endocytosis.

CONCLUSION

Development of safe and efficient nonviral gene delivery systems applicable for human use is one of

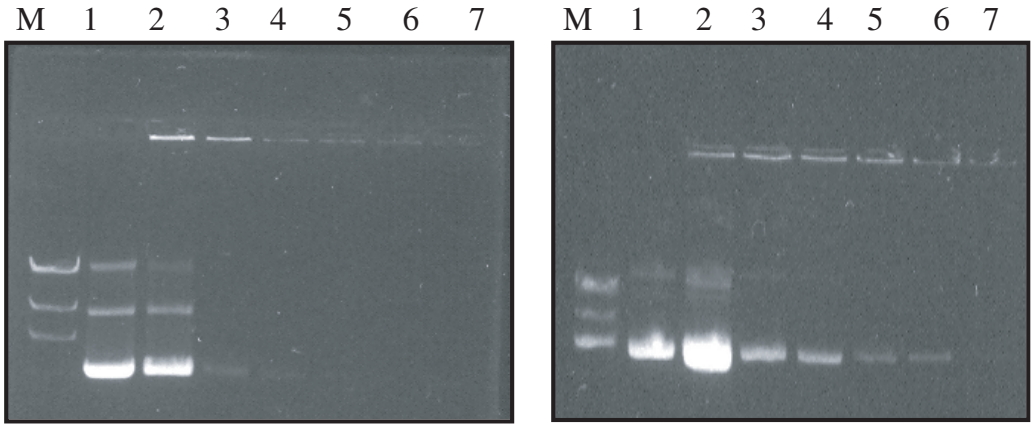


Figure 3. Electrophoretic mobility of liposomes on a 1% agarose gel electrophoresis. Fixed amount (1 μg) of plasmid DNA was mixed with increasing amounts of DDAB in liposomes. The DNA was visualized with ethidium bromide staining: (A) DDAB liposome and (B) T_f-liposome (M, marker; lane 1, DNA only; lane 2, 1:1; lane 3, 5:1; lane 4, 6:1; lane 5, 7:1; lane 6, 8.5:1; lane 7, 10:1 DDAB:DNA weight ratio).

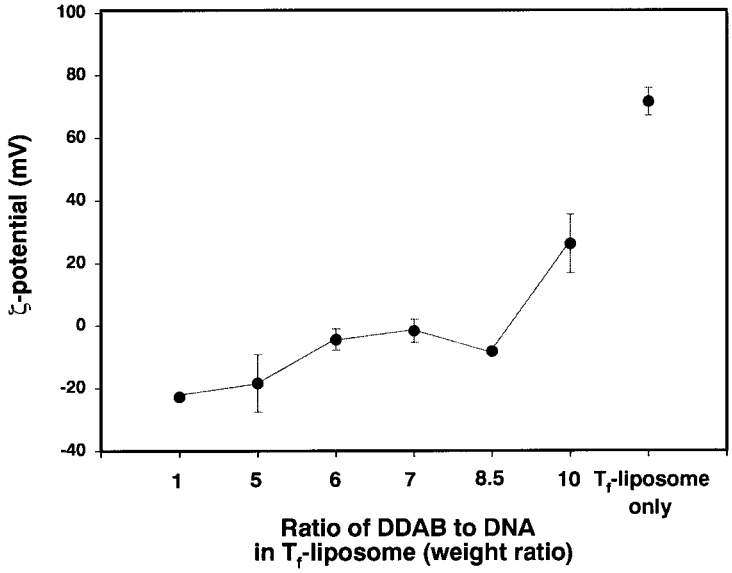


Figure 4. Zeta-potential of the T_f-liposome/DNA complex. Fixed amount (1 μg) of plasmid DNA was mixed with increasing amounts of DDAB in liposomes. Zeta-potential showed about −4.5 mV at a 6:1 ratio of DDAB:DNA. Data represent the mean ±SD obtained from triplicate experiments.

the major hurdles for the successful outcome of human gene therapy, especially when considering the unknown risks of viral gene delivery systems. We report here the use of transferrin as an efficient targeting ligand for gene delivery using cationic liposomes. As tumor cells replicate more rapidly compared with normal ones, they need to uptake more ions in the form of transferrin through transferrin receptors. For this reason, transferrin has been used in targeted drug delivery systems

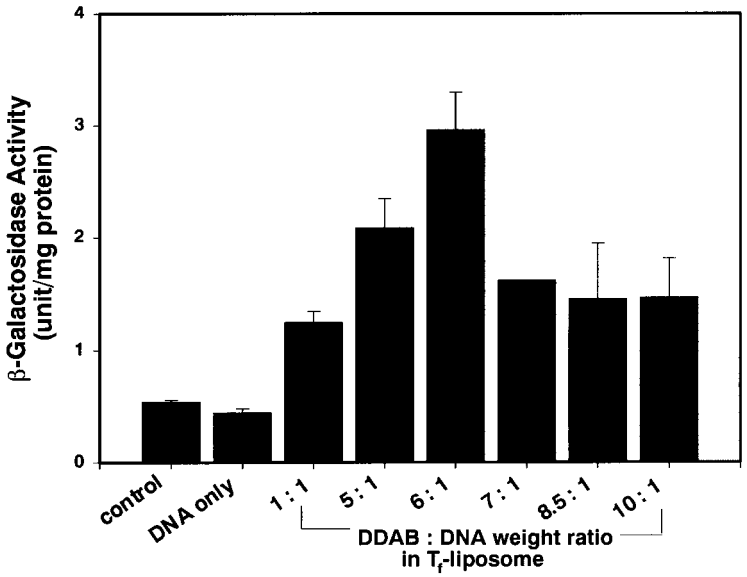


Figure 5. Charge effect on transfection efficiency in HeLa cells. Fixed amount (1 μg) of plasmid DNA was mixed with 1, 5, 6, 7, 8.5 and 10 μg of DDAB in liposomes. Cells were incubated with transfection materials for 48 hr in the presence of 10% fetal bovine serum before measurement of enzyme activity. Data represent the mean ±SD obtained from triplicate wells.

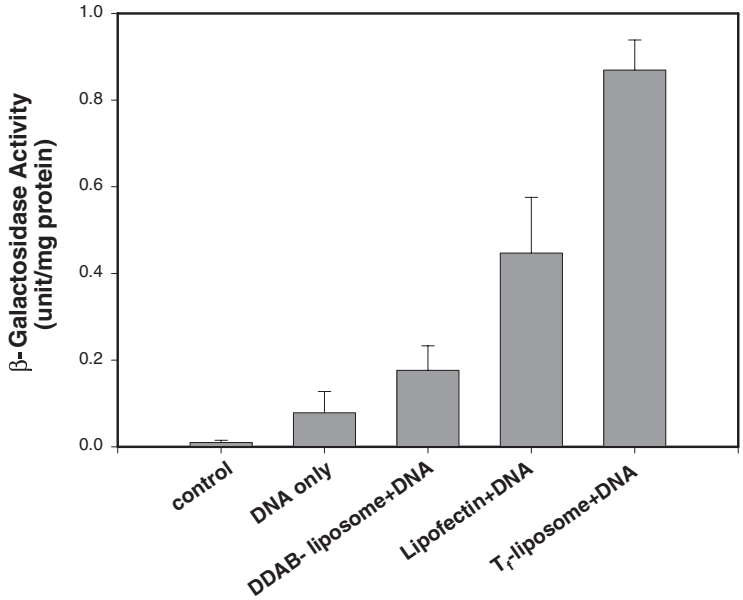


Figure 6. In vitro β-galactosidase activity (enzyme unit/mg total protein) after transfection on HeLa cells. The weight ratio of the Lipofectin™/DNA was 1:1. Cells were incubated with transfection materials for 48 hr in the presence of 10% fetal bovine serum before measurement of enzyme activity. Data represent the mean±SD obtained from triplicate wells.

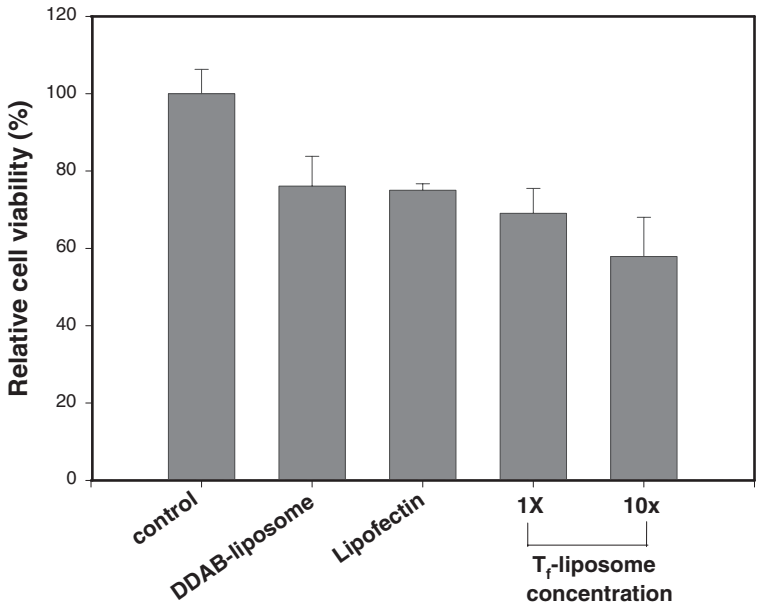


Figure 7. Effect of various transfection materials on the viability of HeLa cells in the presence of 10% FBS. Cell viability was expressed as percentage of untreated control cells. The concentration of gene delivery vector was 2 μg per well of the 96-well plate [20 μg/mL (1×) in cell culture medium]. The vectors were incubated for 4 hr with cells at 37°C, then cell viability was determined using MTT assay. Data represent the mean±SD obtained from triplicate wells.

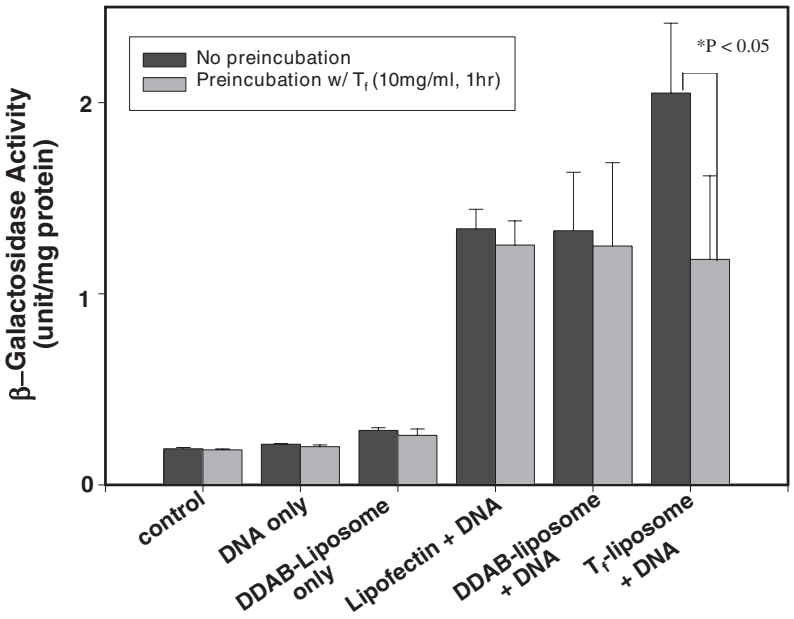


Figure 8. Effect of specific competition for transferrin receptors on gene transfer by T_f-liposomes. Excess amount of free transferrin (10 mg/mL) was added to the cells prior to the transfection. Data represent the mean±SD obtained from triplicate wells.

for a long time, and proved to be safe and efficient. We have successfully conjugated transferrin to MPB-PE using heterobifunctional cross-linking agent (SPDP) via thioether linkage. Among the physico-chemical properties of T_f-liposome and/or its DNA complex include the size, shape, zeta-potential, and gel retardation properties. The size of T_f-liposome, determined by dynamic laser-light scattering, was slightly bigger than that of DDAB liposome (584 nm vs. 466 nm in diameter). This is partially due to the increased hydrodynamic radius of T_f-liposome after transferrin attachment. Fusion between T_f-liposomes seems to be less likely to happen, as the resulting complex after fusion should be much larger than the observed value. A typical multi-lamellar liposomal structure was seen from the transmission electron micrographs. The zeta-potential of the T_f-liposome/DNA complex at 6:1 ratio (w/w) was -4.5 mV, and this formulation exhibited the highest transfection efficiency among those tested. This result is consistent with the literature, where the best transfection result usually comes with almost neutrally charged complexes, including both slightly positive and slightly negative ones. Electrophoresis was performed to examine the nature of interactions between the plasmid DNA and T_f-liposomes (or DDAB liposomes), where stronger interaction resulted in more retardation of gel due to the charge neutralization. Therefore, the T_f-liposome system might be useful in targeted gene delivery, and further studies using animal models are underway.

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