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Characterization of Transferrin-Modified Procationic-Liposome Protamine-DNA Complexes

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We developed a novel transferrin modified non-viral gene delivery system, transferrin-modified procationic-liposome-protamine-DNA complexes (Tf-PLPD) and investigated its characteristics. Blank procationic liposomes were prepared using the film dispersion filter method. Protamine was used to condense plasmid DNA to form protamine-DNA complexes and the complexes were further incubated with blank procationic liposomes to form PLPD. Transferrin was adsorbed onto the surface of PLPD *via* an electrostatic interaction, and thus Tf-PLPD was produced. Characteristics such as stability in rat serum, morphology, average particle size, zeta potential, and transfection efficiency in HepG2 cells were further investigated. The results indicated that the procationic liposomes remained stable in rat serum for 24 h. Tf-PLPD protected plasmid DNA from enzymatic degradation even after lyophilization. The size distribution of Tf-PLPD was in the range of 240±12 nm and the zeta potential was −24.10±2.5 mV (n=3), respectively. The transfection efficiencies of Tf-PLPD were 24.26±2.6 mU β-galactosidase/mg protein. Lyophilization and the presence of serum did not affect the transfectivity of Tf-PLPD and the procationic liposomes also had low cytotoxicity to cells.

Key words——procationic liposomes; transferrin; transfection efficiency; stability

INTRODUCTION

Gene transfer systems with high transfection efficiency and improved safety are of paramount importance for successful *in vivo* tumor gene therapy. Although viral vectors can facilitate the uptake of nucleic acids by target cells and also promote transport of the genetic material into the nucleus,[1] the immunogenicity problem and inability to penetrate target tissue are obstacles for the successful application of this strategy. As a nonviral delivery system, cationic liposomes may be a promising alternative to viral vectors, for which the feasibility for gene delivery has been verified by numerous studies both *in vivo* and *in vitro*.[2] However, the transfectivity of cationic liposomes *in vivo* is not as efficient as *in vitro*, which may be due to the fact that their surface is net positively charged, which might lead to aggregation with plasma protein and accumulation in lung tissue. In addition, anionic liposomes have been extensively studied as vehicles for the delivery of pharmaceutical cargo.[3] They may be used as a drug reservoir for controlled release, to reduce toxicities associated with drugs exhibiting narrow therapeutic indices, and to deliver drugs directly to target cells.[4]

Cholest-5-en-3-ol-(3β)-[2-[[4-[(carboxymethyl) dithio]-1-iminobutyl] amino] ethyl] carbamate (CHETA, $C_{36}H_{61}N_3O_4S_2$, Fig. 1), a cholesterol derivative, was used to prepare procationic liposomes. Procationic liposomes are neutral or negatively charged in the physiologic environment. However, when in contact with cellular membranes or internalized by cells, their surface charge becomes positive.[5] Therefore procationic liposomes have the characteristics of both cationic liposomes and anionic liposomes.

Moreover, protamine not only condenses plasmid DNA but also provides complete protection of DNA from nuclease degradation. Some researchers showed that protamine could markedly enhance the transfection efficiency of several types of cationic liposome by

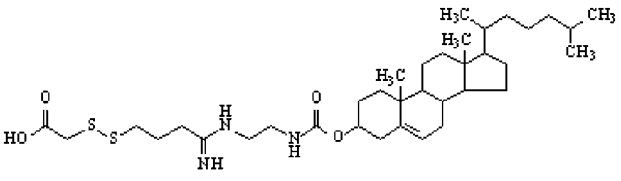

Fig. 1. Chemical Structure of CHETA

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2-28-fold in a number of cell lines *in vitro* and *in vivo*.[6] Therefore protamine was used in this study to condense plasmid DNA. Moreover, some studies indicated that transferring (Tf) could be efficiently uptaken by cells through receptor-mediated endocytosis. It was reported that the expression of Tf receptors was relatively higher in hepatoma cells than in normal tissues, and hence it was expected that the uptake of the procationic liposomes should be improved by incorporating Tf.[7,8] Lactoferrin, a member of the Tf family,[9] was tested as a ligand to target procationic liposomes to human hepatoma cells. The isoelectric point of lactoferrin is 8.70 and as a result, lactoferrin was net positively charged at physiologic pH, which made it feasible to be adsorbed onto the surface of procationic liposomes *via* an electrostatic interaction.

In the present study, novel procationic-piposome-protamine-DNA complexes (PLPD) were constructed, which could associate with the targeting ligand of Tf to form Tf-PLPD and their characteristics *in vitro* were further investigated.

MATERIALS AND METHODS

Materials pORF-LacZ plasmid (3.54 kb), purchased from InvivoGen (USA), was transformed into DH5-α *E. coli* and purified following amplification using the Qiagen Giga Endo-free plasmid purification kit (CA, USA). Protamine sulfate (derived from salmon) and Tf (lactoferrin) from a bovine source was purchased from Sigma (USA). The β-Gal assay kit was from Invitrogen (USA), and the BCA Protein Assay Kit was purchased from Pierce (USA). Cell culture medium DMEM was obtained from Gibco (USA). Chang liver cells and hepatoma cells (HepG2, SMMC7721) were obtained from Shanghai Cell Institute, China Academy of Sciences. CHETA was synthesized according to the patent.[5]

Preparation of PLPD and Tf-PLPD The lipid mixture of CHETA/PC/Chol with a molar ratio of 9 : 10 : 1 (according to the optimized formulation) was dissolved in chloroform 10 ml and dried under a vacuum until a thin film formed on the flask wall. The dried lipid film was hydrated with phosphate-buffered saline (NaCl 6.85 mM, KCl 0.13 mM, Na_2HPO_4 0.32 mM and KH_2PO_4 0.07 mM, pH 7.4). Then the hydrated solution was sonicated in a bath sonicator for 2 min and in a probe sonicator for 100 s intermittently to form plain procationic liposomes.

DNA and protamine sulfate were dissolved in 5% glucose solution, respectively. Then equal volumes were mixed together, and the mixture was incubated at room temperature for 10 min to produce DNA/protamine complexes. Plain procationic liposomes were subsequently added to the DNA/protamine complex solutions to form PLPD.

Tf was dissolved in HEPES-buffered saline solution (HEPES 20 mM, NaCl 100 mM, pH 7.4, Sigma) to reach 420 μg/ml. Tf-PLPD was obtained by gently mixing Tf solution with a predetermined volume of PLPD according to different ratios and further incubation for 15 min at room temperature.

Lyophilization of PLPD and Tf-PLPD Lactose 150 mg (5%, w/v) was added into PLPD 3 ml and Tf-PLPD solution 3 ml, respectively. Then the samples were frozen at -40°C for 8 h and lyophilized for 24 h on a ThermoSavant ModulyoD freeze-dryer (Thermo, USA). After lyophilization, vials were sealed with rubber stoppers and aluminum seals. Prior to determining the particle size and transfection efficiency, the lyophilized samples were resuspended with distilled water to the original volume.

Negative-staining Microscopy The morphology of the procationic liposomes was observed under transmission electron microscopy (TEM) on a JEM-100SX electron microscope (Japan). One drop of PLPD suspension was placed on collodion-carbon-coated copper grid and left for 2 min for attachment of PLPD to the grid. The excess of the sample was removed using filter paper before it was stained with 1% (w/v) phosphotungstic acid. After complete air-drying, the grids were screened in a transmission electron microscope in bright field mode at an accelerating voltage of 60 kV.

Particle Size and Zeta Potential The size distribution and surface charge (known as zeta potential) of the liposomes were determined using a Malvern Zetasizer Nano ZS90 (Malvern instruments Ltd., UK) instrument with a 50-mV laser at a scattering angle of 90°. All measurements were carried out at 25°C in triplicate immediately after preparation of the complexes.

Assay of the Zeta Potential of Liposomes Treated with Dithiothreitol To verify whether the reductive agent could change the zeta potential of procationic liposomes from negative to positive, the preformulated procationic liposomes were diluted with distilled water to a final concentration of 1 mmol/ml total lipid. One milliliter of dilution was placed in

each of four tubes. While the test tubes were being vortexed, 0 μl, 10 μl, 20 μl, and 30 μl of the disulfide reducing agent dithiothreitol (DTT 0.5 mM, NaCl 6.85 mM, KCl 0.13 mM, Na_2HPO_4 0.32 mM, and KH_2PO_4 0.07 mM, pH 7.4) was added. The mixtures were then incubated for 30 min at room temperature and their zeta potentials were measured as described above.

Protection Assay against Nuclease Degradation To investigate the ability of procationic liposomes to protect plasmid DNA from enzymatic degradation, DNaseI-mediated digestion of the liposomes was evaluated using agarose gel electrophoresis. For the DNase digestion, different samples containing DNA 1 μg were exposed to DNaseI solution (DNA 3 U/μg) with $MnCl_2$ 10 mM in phosphate buffered saline (PBS, pH 8.0). After incubation for different time periods at 37℃, EDTA 0.5 M, NaCl 1 M, and 1% Triton X-100 solution were added. Then the samples were shaken gently for 2 min and 0.9% (w/v) heparin solution was added to help to release DNA from PLPD or from Tf-PLPD. These samples were carefully loaded on a 1% (w/v) agarose gel column containing ethidium bromide for electrophoresis.

Transfection *In vitro* HepG2 cells were seeded on 12-well plates at a density of 100000 cells/well in DMEM, supplemented with 10% fetal calf serum. Before transfection, the semi confluent monolayer was washed twice with PBS, and then DMEM 0.3 ml (with or without serum and antibiotics) together with 0.2 ml of test complexes (total lipid 0.1 mM) was added to each well. After incubation for 5 h at 37℃ in 5 % CO_2, the medium was replaced with DMEM containing 10% fetal bovine serum (FBS) and antibiotics, and then the cells were further incubated for 48 h. Each well was washed twice with PBS and the cells were lysed with 100 μl mammalian cell lysis buffer (Tris 0.25 M, pH 8.0) at room temperature for 10 min, followed by alternating freeze-thaw cycles. The cell lysate was centrifuged for 5 min at 10000×g to pellet debris. Average β-galactosidase activities per well were determined using the β-galactosidase enzyme assay system. Supernatant 50 μl was assayed for total β-galactosidase activity as described by Invitrogen using a model 550 plate reader (Biorad, USA).

The total protein content of the lysates was measured using the BCA assay (Pierce, USA) using bovine serum albumin as the standard. Lysate 25 μl was placed into 96-well plate and mixed with 200 μl of freshly prepared reaction solution. The plate was incubated for 30 min at room temperature to reach the plateau of light emission and then placed in the model 550 plate reader for absorbance determination.

Cell Viability Assay Cells were seeded in 96-well plates at a density of 20000 cells per well and grown to 60-80% confluence. After 5 h of incubation with different compositions of transfection complexes (total lipid 0.1 mM), the cells were further incubated for 48 h with DMEM-FBS and the cell viability was measured using the 3-(4,5-Dimethylthialzol)-2,5-diphenyl tetrazolium bromide (MTT) assay. Briefly, MTT was dissolved in PBS at 5 mg/ml, followed by adding 20 μl to each well and incubating for 3.5 h. Each well was washed with PBS 100 μl, followed by the addition of DMSO to dissolve the MTT formazan crystals. The absorbance was read at 570 nm using a model 550 microplate reader. Data were calculated according to the formula: Viability$=(A_{treated}-A_{background})/(A_{control}-A_{background})\times 100$, in which the control cells were not exposed to liposome and the background well contained no cells.

Stability Assay of Liposomes in the Presence of Serum The protein adsorption on the surface of liposomes in serum was estimated by measuring the change of size and turbidity. The prepared liposome solution (total lipid 0.5 mg/ml) was added to rat serum (0%—15%, v/v) and the mixed solution was incubated at 37℃ with gentle stirring before determining size distribution and turbidity. The turbidity change in the mixed solution was measured using a UV spectrophotometer (UV GBC Cintra 10e, GBC, Inc, Australia) at a wavelength of 450 nm.[10]

RESULTS

Size, Zeta Potential, and Morphology of PLPD and Tf-PLPD The average size of the resultant PLPD was 228.9±8 nm with polydispersity index of 0.122±0.02 (n=3) and the Zeta potential was −25.08±2.5 mV (n=3). When PLPD was mixed with Tf at the Tf/CHETA (w/w) ratio of 1∶1, Tf-PLPD had a mean diameter of 240±12 nm with a polydispersity index of 0.150±0.03 (n=3), and zeta potential of −24.10±2.5 mV (n=3). The zeta potential of plain procationic liposomes (without DNA) was −33.83±1.23 mV, but when adding DTT 20 μl, it was +22.74±1.08 mV. However, no significant difference was observed between DTT 20 μl and 30 μl, which indicated that the disulfide bonds

had almost been completely cleaved by DTT. In addition, both the lyophilized PLPD and, the lyophilized Tf-PLPD maintained almost the same average size and zeta potential with fresh preparations. As shown in Fig. 2, the morphology of PLPD remained unchanged after lyophilization and most liposomes were relatively regular and spherical with a condensed core inside.

Cell Viability and Transfection Efficiency The cell viabilities of HepG2, SMMC7721, and Chang normal liver cells showed a similar trend (Fig. 3). Here, we show only the viability of Chang liver cells under different experimental conditions. Compared with Lipofectamine 2000, the procationic liposomes had less cytotoxicity to cells. Transfectivity was quantified as milliunits of β-galactosidase per milligram of total cell protein (mU β-galactosidase/mg protein).

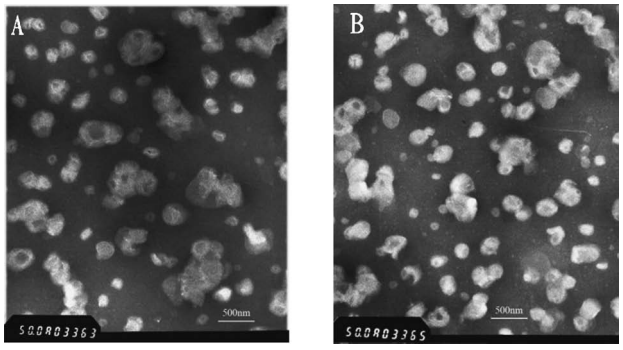

Fig. 2. Transmission Electron Microscopy of PLPD (× 50,000)

(A) Fresh PLPD and (B) lyophilized PLPD. The morphology of the samples was observed on a JEM-100SX electron microscope (Japan).

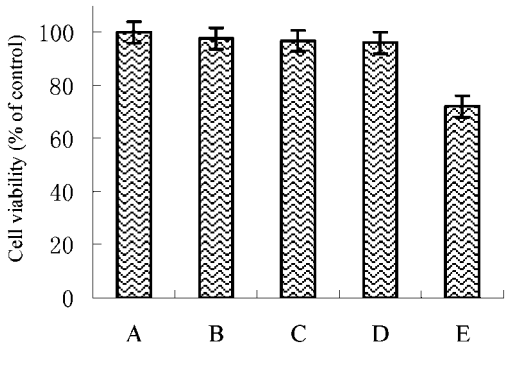

Fig. 3. Viability of Chang Liver Cells Determined in the MTT Assay under Different Experimental Conditions (Total Lipid 0.1 mM): (A) Fresh PLPD, (B) Lyophilized PLPD, (C) Fresh Tf-PLPD (Tf/CHETA=1 : 1, w/w), (D) Lyophilized Tf-PLPD (Tf/CHETA=1 : 1, w/w) and (E) Lipofectamine 2000

Results are presented as mean±standard deviation (S.D.) (n=5).

The results indicate that the transfection efficiency of Tf-PLPD and PLPD showed no significant difference in the presence or absence of serum (10%, v/v), while that of Lipofectamine 2000 was markedly inhibited by serum (Fig. 4). Figure 4 also shows that Tf-PLPD enhanced the transfetion efficiency by nearly 2-fold compared with PLPD. Their transfection efficiencies were about 24.26±2.6 and 12.18±3.8 mU β-galactosidase/mg protein, respectively. Both Figs. 3 and 4 also indicate that the lyophilization did not affect the cell viability induced by the procationic liposomes and it also had no effects on their transfectivity.

Protection against Nuclease Degradation As shown in Fig. 5, lane D, the plasmid DNA incorporated into liposomes remained in a supercoiled form after incubation, while naked DNA was completely digested under identical conditions (lane B). The results demonstrated that plasmid DNA inside PLPD or Tf-PLPD is well protected from the external environment even after lyophilization (lane F and lane G). The results also suggest that the procedure of procationic liposome preparation was gentle and resulted in little, if any, plasmid degradation.

Stability of Liposomes in the Presence of Serum Different concentrations of serum (v/v) were tested in order to determine the appropriate volume of serum to be added into liposomes while fixing the incubation time at 8 h (Fig. 6A). At the concentrations examined (0.5 mg lipid/ml), the sizes of procationic

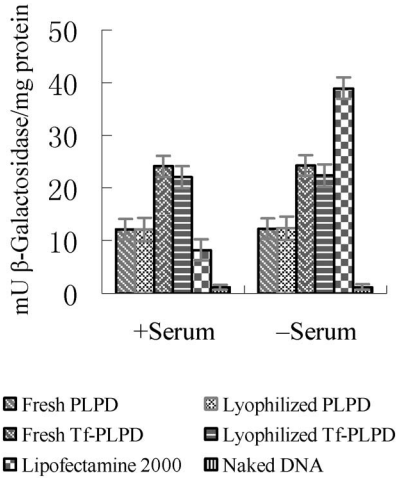

Fig. 4. Transfectivities of PLPD, Tf-PLPD, Lipofectamine 2000 (Total Lipid 0.1 mM) and Naked DNA in HepG2 Cells in the Presence or Absence of Serum (10%, v/v)

The data represent the mean±S.D. of three wells and was representative of three independent experiments.

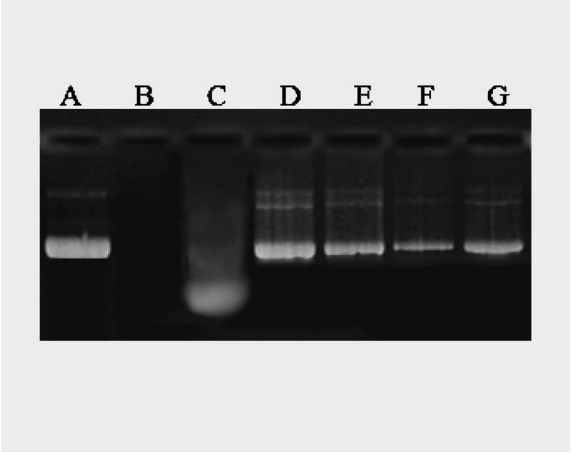

Fig. 5. Resistance of PLPD and Tf-PLPD to DNase

Lane A: free DNA, lane B: naked DNA incubated with DNase for 1 h, lane C: naked DNA incubated with DNase for 5 min, lane D: fresh PLPD incubated with DNase for 1 h, lane E: fresh Tf-PLPD incubated with DNase for 1 h, lane F: lyophilized PLPD incubated with DNase for 1 h, lane G: lyophilized Tf-PLPD incubated with DNase for 1 h.

liposomes and cationic liposomes[11] showed no obvious change when serum percent reached 10%. Therefore 10% serum was used in all subsequent studies.

Figure 6(B) shows that average particle size of cationic liposomes increased greatly from 85.0 to 638.2 nm after incubation for 24 h in rat serum at 37℃, while that of procationic liposomes (without protamine and DNA) and Tf-modified procationic liposomes changed slightly. On the other hand, to determine whether the size change of cationic liposomes was caused by the 37℃ temperature, the solution without serum was tested at the same temperature and the particle size showed no change (data not shown). This result indicates that the size change shown in Fig. 6 was not due to the temperature factor.

Similarly, Fig. 6(C) shows the turbidity change in liposome suspension in serum. The optical density of cationic liposomes rapidly increased but the optical density of both procationic liposomes and Tf-

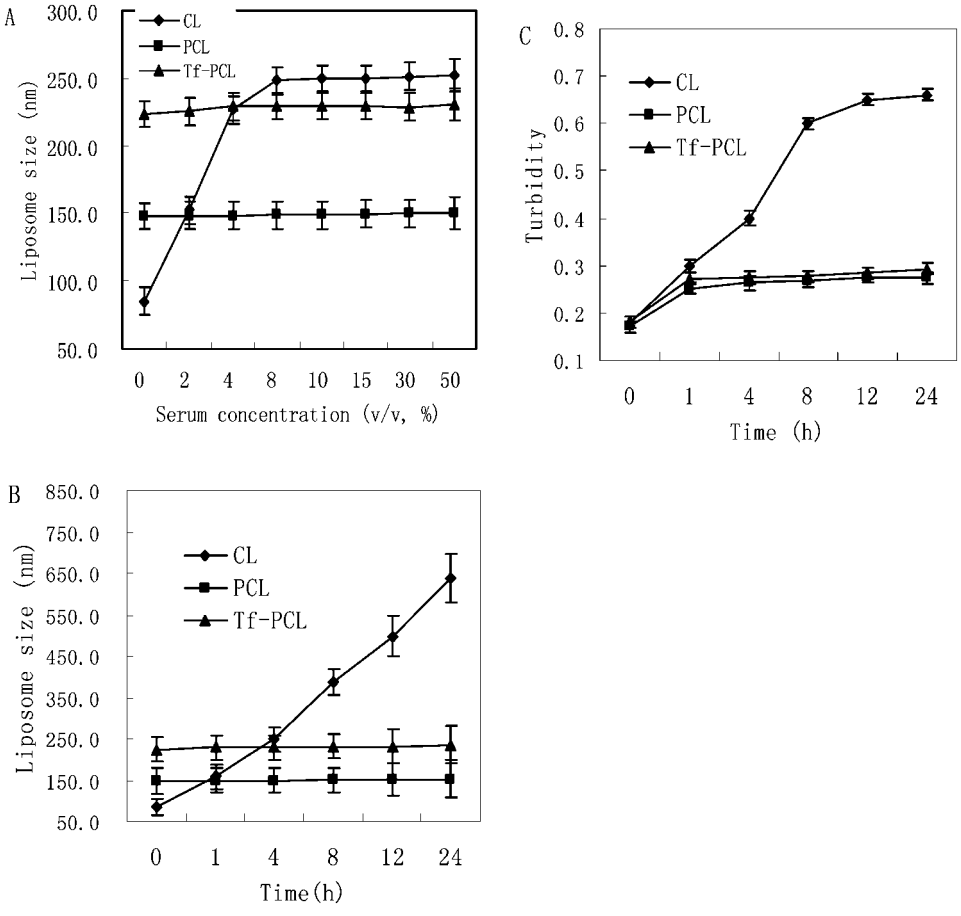

Fig. 6. (A) Particle Size of Liposomes after 8-h Incubation with Different Amounts of Rat Serum (v/v) at 37℃, (B) Turbidity Changes in Liposomes after Incubation at 37℃ in Rat Serum (10%, v/v) and (C) Particle Size of Liposomes after Incubation at 37℃ in Serum (10%, v/v)

Data are shown as mean±SD (n=3).

procationic liposomes suspensions at first slowly increased for 5-8 h and then showed a constant density for 24 h. There were no marked differences between them.

DISCUSSION

TEM provided visual confirmation of the formation of liposomes. The fact that the transfection efficiency of Tf-PLPD was nearly 2-fold higher than that of PLPD indicated that the positively charged Tf had been adsorbed on the surface of PLPD. The average size of PLPD was smaller than that of Tf-PLPD and the comparison of the zeta potential of PLPD and Tf-PLPD also gave similar results. We evaluated the binding amount of Tf to the complexes using gel filtration, the free Tf concentration was examined in the BCA assay, and the binding rate of Tf was calculated to be 70%.

Cytotoxicity is an important parameter for consideration in addition to gene transfection efficiency. Generally, cell toxicity is described in terms of LD_{50} or IC_{50}. Transfection efficiency is closely related to cytotoxicity, that is specific transfection efficiency may produce corresponding cytotoxicity. Therefore it would be reasonable to compare the cytotoxicity at the highest transfection efficiency. In this study, the cell survival rates of three cell lines were investigated when the total lipid concentration was equal to the transfection concentration. In another test, it was found that when the total lipid concentration was 1.5 mM, the cell viability of Chang liver cells was 70%, which indicated that the transfection concentration of total lipid 0.1 mM mentioned above was safe for use *in vivo*. Figure 3 demonstrated that PLPD and Tf-PLPD were less cytotoxic to cells than Lipofectamine 2000. When in contact with cells, the surface charges of PLPD and Tf-PLPD were negative, but the zeta potentials gradually became positive due to the existence of reductant factors. However, the surface charge of Lipofectamine 2000 was originally positive, and therefore, it had no such potential changing process as PLPD or Tf-PLPD have.

According to the stability assay, procationic liposomes and Tf-procationic liposomes showed low protein adsorption and retained their initial particle sizes during incubation in serum, because the negative charge might prevent protein adsorption between the serum protein and liposome surface. In other words, the procationic liposomes were more stable than cationic liposomes in rat serum.

One important factor for simulating *in vivo* conditions is using a high concentration of serum for transfection experiments *in vitro*, rather than serum-free medium. So far, many studies have been carried out on the impact of serum on transfection.[12] Figure 4 also illustrated that Tf-PLPD enhanced the transfection efficiency by nearly 2-fold compared with PLPD and PLPD maintained similar transfection efficiency after lyophilization. Although compared with freshly prepared Tf-PLPD the transfection efficiency of lyophilized Tf-PLPD decreased slightly, it still maintained a relatively high level of transfection efficiency. The decrease may be explained by the fact that a small part of the Tf underwent irreversible denaturation upon lyophilization and lost its activity.

CHETA consists of a hydrophobic cholesterol tail group (X) and a hydrophilic head group (Y^+-S-S-Z^-), of which the head group incorporates both positively and negatively charged regions connected by a disulfide bond. The disulfide bond is susceptible to cleavage ($X\text{-}Y^+\text{-}S\text{-}S\text{-}Z^- \rightarrow X\text{-}Y^+\text{-}SH + HS\text{-}Z^-$) by components of the intracellular membrane such as protein disulfide isomerase or thioredoxin reductase (TrxR). Since the content of TrxR in tumor cells is 10 times higher than in normal tissues,[13,14] when the procationic liposomes reach the surface of tumor cells, the disulfide bond will be cleaved, which results in the removal of the negatively charged region from the head group and the formation of cationic liposomes.

In our study, the dispersion of PLPD and Tf-PLPD was relatively stable when stored at 4°C: over a period of 6 weeks, no aggregates were observed and the transfection efficiency remained unchanged, but considering long-term storage and convenience of use, lactose (5%, w/v) was found to be the best lyoprotectant of PLPD and Tf-PLPD. The results indicated that the lyophilization procedure did not affect the transfectivity and physicochemical characteristics of PLPD or Tf-PLPD.

In sum, we prepared PLPD and Tf-PLPD, which could protect DNA from DNase degradation. Compared with cationic liposomes, both PLPD and Tf-PLPD had many advantages such as decreased cytotoxicity, increased stability in rat serum, relatively high transfectivity, etc. Moreover, Tf-PLPD mediated higher gene expression than PLPD in the presence or absence of serum. Therefore further

efforts should be made to investigate the ability of Tf-PLPD to target animal tumors *in vivo* and its application in tumor gene therapy.

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