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Stability of DNA/Td3717 complexes for gene transfection, defined by the size and polydispersity of the complex

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ABSTRACT

The amphiphilic α-helical peptide, Td3717, is a bi-functional synthetic peptide that acts as both a polycation for DNA binding and a ligand for targeted delivery to tumor cells. Td3717 forms a stable complex with plasmid DNA, and the complex maintained high transfection efficiency after storage at 4 °C for six months and after four freeze/thaw cycles. During the storage and freeze/thaw cycling, the particle size of the DNA/Td3717 complex remained less than 100 nm. The size of the complex is an important factor for its internalization into cells via the endocytosis pathway; therefore, the stability of the particles will strongly contribute to high transfection efficiencies after storage and repeated freezing/thawing.

1. Introduction

Non-viral vectors for gene delivery, such as chemically synthesized cationic lipids and cationic polymers, are being sought to overcome the disadvantages of viral vectors.[1–5] In addition to an unexpected immune response, viral vectors are difficult to handle, for example, strict storage conditions and short expiration date. Of course, repeated freezing/thawing is usually not permitted. Non-viral vectors should be rather easier to handle than the viral vectors. The storage stability of DNA/non-viral vector complex in aqueous solution, however, is still low in many cases because the formulation of the complex can change during storage, resulting in decreased transfection efficiency. Therefore, a fresh preparation of the complex is usually required. It would be more convenient if the complex in aqueous solution could be stored for a long time in a refrigerator in the same way as common reagents.

Previous studies have shown that complex formation between cationic molecules and DNA is a key parameter of its transfection efficiency.[6,7] That is, non-viral transfection agents make complexes with DNA mainly by electrostatic interaction, and in some cases, by additional hydrophobic interaction. The resulting complex, with an appropriate particle size, is well adapted to enter into the cells via the endocytosis-mediated pathway. Therefore, elucidation of the structural features of the DNA/cationic molecules complex is important for discussing its transfection efficiency.

We have developed Td3717, a bi-functional synthetic peptide that acts as both a polycation for DNA binding and a ligand for targeted delivery to tumor cells expressing phosphatidylserine (PS) on their cell surfaces (Fig. 1).[8,9] Td3717 was designed on the basis of the amino acid sequence of the carboxy-terminal C2 domain of human coagulation factor VIII (hFVIII), which is known to contribute to efficient anchoring of hFVIII on the surface of PS-exposed cells, such as activated platelets.[10–13] Td3717, therefore, would be a promising vector for the specific delivery of DNA into tumor cells. In this paper, to demonstrate the advantages of Td3717, we evaluated the long-term storage stability of DNA/Td3717 complexes in aqueous solution and its stability after freeze/thaw cycling. We discuss the stability of the complexes with regard to the size of the complexes and their polydispersity.

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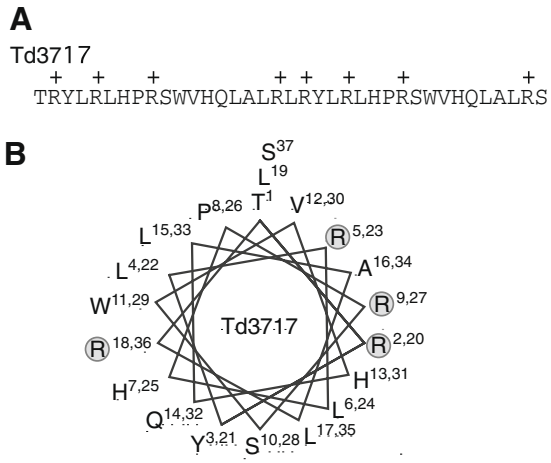

Figure 1. Amino acid sequence (A) and predicted schematic α-helical structure (B) of Td3717.

2. Results and discussion

2.1. Storage stability of DNA complex with Td3717

Figure 2 shows the storage stability of DNA complexes with Td3717 and with the commercially available liposome, Lipofectin™, in aqueous solution at 4 °C. A plasmid DNA encoding luciferase was employed as a reporter gene. The stability of the complex was evaluated by measuring luciferase expression in Vero cells (a cell line of kidney epithelial cells from African green monkey) after transfection with the complex. The transfection efficiency of the Td3717 remained at a high level for six months without significant cytotoxicity, whereas that of Lipofectin™ was lower than Td3717 and decreased to half of the original efficiency after 14 days. This result indicated that the DNA/Td3717 complex was stable, and remained transfection competent for at least for six months in aqueous solution at 4 °C.

The stability of the DNA complex after repeated cycles of freezing/thawing was then evaluated (Fig. 3). The transfection efficiency of the DNA/Td3717 complexes increased significantly after freeze/thaw cycling, whereas that of the DNA/Lipofectin™ complexes decreased after each cycle, and reached to 40% of its original activity

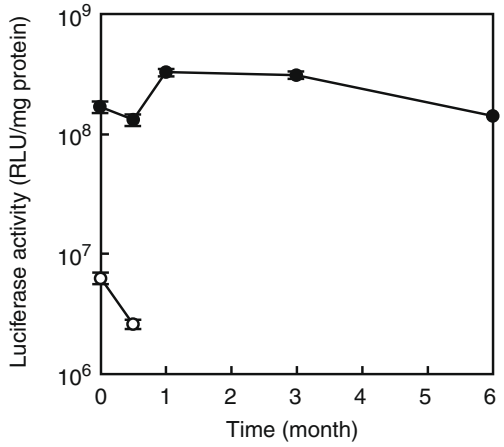

Figure 2. Transfection efficiencies after storage in aqueous solution at 4 °C. *Closed circles* and *open circles* indicate transfection efficiencies of Td3717 and Lipofectin™. Plasmid DNA and Td3717 or Lipofectin™ were mixed to concentrations of 8 μg/mL and 20 nmol/mL or 128 μg/mL, respectively. After storage at 4 °C, transfection efficiency into Vero cells was evaluated.

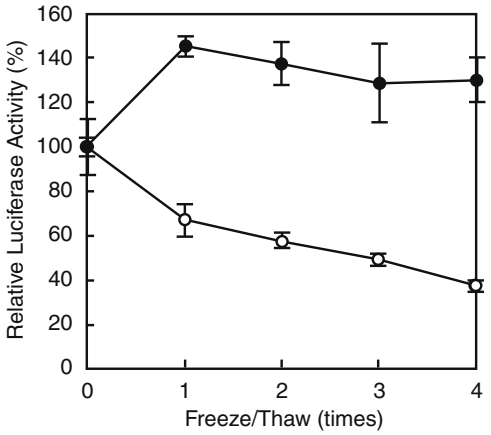

Figure 3. Transfection efficiencies after freeze/thaw cycling. *Closed circles* and *open circles* indicate transfection efficiencies of Td3717 and Lipofectin™. Plasmid DNA and Td3717 or Lipofectin™ were mixed to concentrations of 8 μg/mL and 20 nmol/mL or 128 μg/mL, respectively. After repeated cycles of freezing and thawing, transfection efficiency into Vero cells was evaluated. Relative activities to those of freshly prepared complexes are shown.

after four cycles. We cannot explain why the transfection efficiency of the DNA/Td3717 complexes increased after the first freeze/thaw procedure, though it might be due to maturation of the complex. This feature will be an advantage in transfection into cells.

In the presence of 10% serum during the transfection for 5 h, the efficiencies decreased to less than 10% of the original efficiency without serum. However, when the amount of DNA was increased to fourfold (from 1 μg to 4 μg), the efficiency was fully restored (data not shown), suggesting that the stability of the complex will reflect application to in vivo use.

2.2. Physicochemical properties of Td3717/DNA complexes

It has already reported that the time-dependent increase in the size of cationic lipid/DNA complex causes a decrease in its transfection efficiency.[14,15] To confirm the relationship between the structure of the complex and the activity of the DNA/Td3717 complexes, we monitored the particle sizes of the complexes during storage and freeze/thaw cycling and compared them with those of Lipofectin™ complexes.

Table 1 shows the changes of the particle sizes of the DNA/Td3717 complexes and DNA/Lipofectin™ complexes during storage in aqueous solution at 4 °C. The initial particle size of the complex with Td3717 was about 50 nm. The polydispersity of the particle size, which is indicated as a standard deviation of the evaluation, was less than 1 nm, and both the particle size and the polydispersity did not change after one month of storage. As a result of measuring zeta-potential of the complexes, the initial complex showed slightly positive charge (about +10 mV), and it did not also change after the storage (data not shown). On the other hand, the initial particle size of the DNA/Lipofectin™ complex was about 250 nm, which was consistent with previous observations,[16,17] and it increased to 450 nm after storage for 15 days. In addition, the poly-

Table 1
Change of particles sizes of DNA/Td3717 and DNA/Lipofectin™ complexes during storage

Storage (day)	Particle size of DNA complex (nm)	
	Td3717	Lipofectin™
0	51.3 ± 0.4	262.4 ± 97.5
15	48.3 ± 3.8	443.4 ± 110.1
30	49.2 ± 0.1	445.5 ± 213.3

dispersities of the DNA/Lipofectin™ complex were higher than those of the DNA/Td3717 complex, and they also increased during storage. These results indicated that once Td3717 made a complex with the plasmid DNA, the complex was stable, even in aqueous solution. In contrast, DNA/Lipofectin™ complexes gradually formed aggregates.

The particle size of a DNA/cationic molecules complex is an important factor for the transfection mediated by endocytosis pathway;[18] thus we evaluated the effect of inhibition of the endocytosis pathway on the transfection efficiencies of T3717. Endocytosis is an energy-dependent pathway, and it can be inhibited by lowering the temperature or by the use of metabolic inhibitors to deplete the ATP pool.[19] Furthermore, it can be classified into two broad categories, phagocytosis and pinocytosis.[20] Cellular uptake triggered by the cell-penetrating peptide 'TAT' was demonstrated to be mediated by macropinocytosis.[21,22] Based on these data, we evaluated the effect of lowering the temperature during transfection and of the addition of Wortmannin,[23] a specific inhibitor of macropinocytosis, on the transfection efficiency. Transfection efficiencies were dramatically decreased in both cases (Fig. 4), indicating that cellular uptake of the DNA/Td3717 complex is mediated by the endocytosis pathway, which generally mediates non-viral gene delivery.[24,25] The difference in storage stability between the DNA/Td3717 complexes and the DNA/Lipofectin™ complexes could be explained by differences in the stability of the complex formulation, that is, the size of the particles and their polydispersity. The optimum particle size of the complex would depend on the type of carrier molecule and the cell lines used for transfection. The small observed particle size (~50 nm) of the DNA/Td3717 complex clearly indicated that the DNA was well compacted and that this particle size would be favorable to achieve highly efficient transfection.

Table 2 shows the changes in particle size of DNA complexed with Td3717 and with Lipofectin™ during freeze/thaw cycling. No dramatic changes in size or polydispersity, indicated as the standard deviation, were observed in the case of Td3717 after two freeze/thaw cycles. However, although they slightly increased after three to four cycles, the size and standard deviation were kept to less than 100 nm and 10 nm, respectively. Thus, it can be said that the DNA/Td3717 complex is resistant against freeze/thaw cycling. As shown in Figure 3, a significant increase in the transfection efficiency was detected after the first freeze/thaw cycle. We hypothesize that this is due to the maturation to the optimum complex for transfection. However, no evidence supporting this hypothesis was observed in the particle size analysis. We tried to find a clue to the hypothesis in the zeta-potential analysis. However, no significant change of the zeta-potential was observed after the freeze/thaw cycles. At this stage, we could not explain clear relationship between the physicochemical properties of the complex and its expression efficiency. On the other hand, DNA complexed with Lipofectin™ showed a dramatic increase in particle size, reaching 1360 nm. The standard deviation of the size also increased, indicating that heterogeneous aggregates were formed during freeze/thaw cycling. This would explain why the transfection efficiency of Lipofectin™ decreased during freeze/thaw cycling.

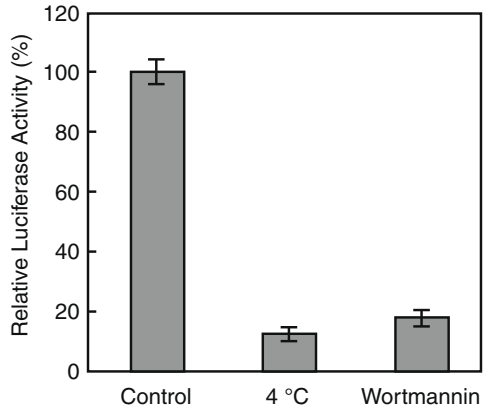

Figure 4. Effects on transfection efficiency of lowering temperature and addition of Wortmannin. Vero cells were transfected using an ice-cooled solution containing DNA complexed with Td3717 and incubated for 5 h at 4 °C. After washing with growth medium, the cells were cultured at 37 °C for 24 h. Wortmannin (1 μM) was added to the transfection medium and the cells were incubated for 5 h at 37 °C. After washing with growth medium, the cells were cultured at 37 °C for 24 h. Transfection efficiencies were evaluated as described in Section 4.

Table 2
Change of particles sizes of DNA/Td3717 and DNA/Lipofectin™ complexes after freeze/thaw cycling

Repetition (time)	Particle size of DNA complex (nm)	
	Td3717	Lipofectin™
0	56.4 ± 0.9	157.3 ± 74.6
1	55.3 ± 0.5	182.4 ± 100.7
2	56.4 ± 2.4	384.7 ± 200.2
3	71.6 ± 6.8	407.4 ± 154.1
4	99.0 ± 3.4	1359.1 ± 1416.0

3. Conclusion

DNA/Td3717 complex showed storage stability in aqueous solution at 4 °C for six months. It also showed resistance against at least four freeze/thaw cycles. The initial particle size of the complex was about 50 nm and its polydispersity was low, and these parameters were not significantly altered during long-term storage and freeze/thaw cycling. The size of the complex is an important factor in the endocytotic uptake by cells; therefore, the stability of the nano-sized particle of the DNA complex would strongly contribute to high transfection efficiencies after storage in such severe conditions.

4. Experimental

4.1. Reagents

Lipofectin™ and opti-MEM were purchased from Invitrogen. Wortmannin was purchased from Calbiochem. The other reagents used for analysis were of reagent grade.

4.2. Preparation of peptides

Peptide Td3717 (TRYL-RIHP-RSWV-HQIA-LRLR-YLRI-HPRS-WV HQ-IALR-S) was synthesized on an automated peptide synthesizer (model 433A, Applied Biosystems Inc.) and purified by reversed phase high-performance liquid chromatography (HPLC) on a C18 column (CAPCELL PAK C18AG120, Shiseido Fine Chemicals Co.) with a linear gradient established between 30% and 70% acetonitrile containing 10 mM HCl, for 15 min. The final products were identified by amino acids analysis and matrix-assisted laser desorption ionization mass spectrometry (VoyagerDE-STR, PE Biosystems).

4.3. Cell culture

Vero cells (a cell line of kidney epithelial cells from African green monkey) were cultured with DMEM supplemented with 10% FBS, 100 U/mL penicillin G, and 0.1 mg/mL streptomycin. Cells were maintained at 37 °C with 5% CO_2 in a humidified incubator and passaged every three to four days.

4.4. Complex formation of Td3717 or Lipofectin™ with plasmid DNA

For the preparation of DNA complexed with Td3717, 16 μg/mL plasmid DNA solution was mixed with the equivalent volume of 40 nmol/mL Td3717 solution in 5% glucose and allowed to stand for 30 min at room temperature. For transfection, this solution was diluted with opti-MEM to obtain an appropriate plasmid DNA concentration.

For the preparation of DNA complexed with Lipofectin™, 16 μg/mL plasmid DNA solution was mixed with equivalent volume of 256 μg/mL Lipofectin™ in opti-MEM. For transfection, this solution was diluted with opti-MEM to obtain an appropriate plasmid DNA concentration.

4.5. Evaluation of transfection efficiency into cells

A luciferase expression plasmid was employed as reporter. Vero cells were inoculated into 24-well plastic plates at a density of 1.0×10^5 cells/well and cultured at 37 °C for 24 h. Cells were washed twice with saline and 0.25 mL of opti-MEM and 0.25 mL of the vector (Td3717 or Lipofectin™)/plasmid DNA complex solution containing 2 μg/mL the plasmid DNA in opti-MEM were added to each well. The cells were then incubated for 5 h at 37 °C in 5% CO_2/95% air, washed once with growth medium, and cultured at 37 °C for 24 h. Luciferase expression was measured using a luciferase assay kit (Promega). After transfection, the cells were washed twice with saline. Subsequently, 200 μL of passive lysis buffer (Promega) was added to each well and allowed to stand for 5 min at room temperature. The cells were then scraped from the culture plates and the resulting cell homogenates were vortexed vigorously for 5 min in a 1.5 mL plastic tube. After centrifugation for 30 s at 10,000*g*, luciferase activities in the supernatants were quantified by luminometer (Arvo™ SX 1420 multilabel counter, Perkin–Elmer). The amount of protein in the supernatants was determined using a protein assay reagent (Bio-Rad).

4.6. Stability of DNA complex with Td3717 and Lipofectin™

Storage stability of DNA complexed with Td3717 or Lipofectin™ in an aqueous solution at 4 °C was carried out for six months. For the evaluation of resistance against freeze/thaw cycling, the complex solution was frozen and thawed four times using liquid nitrogen and a water bath. Briefly, plasmid DNA and Td3717 or Lipofectin™ were mixed at concentrations of 8 μg/mL and 20 nmol/mL or 128 μg/mL, respectively. After storage or freeze/thaw cycling, transfection efficiency was evaluated as described above and the luciferase activity of the complex was compared with that of freshly prepared complex. The particle sizes of the complexes were measured by a Zetasizer Nano ZS (Malvern Instruments Ltd, Malvern,).

4.7. Evaluation of the mechanism of cellular uptake of Td3717/DNA complex

For evaluation of transfection efficiency at 4 °C, transfection was carried out using Vero cells as described above, except for the temperature of transfection. Briefly, the cells were washed twice with saline on the next day of inoculation, and an ice-cooled solution containing DNA complexed with Td3717 was added. After 5 h incubation at 4 °C in refrigerator, the cells were washed once with growth medium. More growth medium was then added, and cells were cultured at 37 °C for 24 h. For the evaluation of transfection efficiency in the presence of Wortmannin, 1 μM of Wortmannin was mixed into the medium during transfection.

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