

Preparation of a DNA Complex with Lipoglutamide Having Tetraethylene Glycol Tails, and Its Application to DNA Delivery into Tumor Cells¹⁾

Toshinori Sato, Hiroyuki Akino, and Yoshio Okahata*

Department of Biomolecular Engineering, Tokyo Institute of Technology, Nagatsuda, Midori-ku, Yokohama 226

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We newly prepared a polyion complex of DNA with lipoglutamide, *N,N'*-bis(18-hydroxy-7,10,13,16-tetraoxaoctadecyl)-L-glutamide hydrochloride (abbreviated to $2\text{EO}_4\text{C}_6\text{N}^+$). Formation and characterization of the DNA/ $2\text{EO}_4\text{C}_6\text{N}^+$ complex were investigated by elemental analysis, melting temperature, and light-scattering measurements. Compaction of DNA by binding with the cationic $2\text{EO}_4\text{C}_6\text{N}^+$ was seen by multi-angle light scattering. Aggregation of the DNA/ $2\text{EO}_4\text{C}_6\text{N}^+$ complex was significantly depressed compared with the DNA complex without tetraethylene glycol. The DNA/ $2\text{EO}_4\text{C}_6\text{N}^+$ complex had low cytotoxicity and efficient internalization into tumor cells compared with native DNA.

A number of new techniques have been developed to introduce oligonucleotides and foreign genes into mammalian cells. A basic requirement for the therapeutic use of nucleotides is efficient cell uptake. Several modifications have been proposed to increase the cell uptake of nucleic acids. Substitutions of nucleic acids with lipophilic groups^{2–5)} or cell receptor ligands^{6–8)} have been explored as a method of efficient transport into cells. An alternative approach would be to use molecular assemblies without any chemical modification of the nucleic acids themselves. Encapsulations of nucleic acid into liposomes^{9,10)} increased the activity. Bindings of nucleic acid to polycations such as cationic liposomes,¹¹⁾ lipopolyamines,¹²⁾ poly(L-lysine),¹³⁾ or DEAE-dextran¹⁴⁾ through ionic interaction have been developed to increase cell uptake. Though those chemically modified DNAs and the DNA complexes had high cell uptake, one of the problems is poor water-solubility. Especially DNA complexes with cationic lipids and cation polymers precipitate easily. Production of precipitates restricts the dose of the DNA complex. Exploration of a DNA complex that does not produce precipitate and shows strong interaction with cells is necessary to the progress in DNA delivery systems.

On the other hand, it is known that administered proteins are cleared from circulation by the reticuloendothelial system. Proteins are also metabolized by peptidases and thus lose their biological activity. Similar problems will happen in the administration of DNA. However, it was found that conjugation of proteins with ethylene glycol chains resulted in increased blood clearance time and decreased immunogenicity or antigenicity.^{15,16)} Based on this knowledge, we considered that modification of DNA with ethylene glycol chain might be important in the development of DNA delivery systems.

In our previous paper,¹⁷⁾ we developed DNA complexes with cationic lipoglutamates such as α,γ -dibutyl glutamate ($2\text{C}_4\text{N}^+$) and α,γ -dihexyl glutamate ($2\text{C}_6\text{N}^+$) through ionic

interaction, as shown in Fig. 1. We investigated the structure and cell uptake of the DNA/lipoglutamate complex. In this paper, we newly synthesized a cationic lipoglutamide having tetraethylene glycol tails, *N,N'*-bis(18-hydroxy-7,10,13,16-tetraoxaoctadecyl)-L-glutamide hydrochloride (abbreviated to $2\text{EO}_4\text{C}_6\text{N}^+$, Fig. 1). The DNA/ $2\text{EO}_4\text{C}_6\text{N}^+$ complex would be expected to show higher water-solubility than the previous DNA/lipoglutamate complexes. We investigated formation, water solubility, structure, and cellular interaction of the DNA/ $2\text{EO}_4\text{C}_6\text{N}^+$ complex.

Experimental

Chemicals. Syntheses of $2\text{C}_4\text{N}^+$, $2\text{C}_6\text{N}^+$, and FITC (fluorocein isothiocyanate)-labeled DNA were described in detail in our previous paper.¹⁷⁾

$2\text{EO}_4\text{C}_6\text{N}^+$ was synthesized as follows:¹⁾ 1,6-Dibromohexane and potassium phthalimide were reacted in DMF at 70–80 °C to obtain *N*-(6-bromohexyl)phthalimide in a yield of 41%. Tetraethylene glycol was conjugated with *N*-(6-bromohexyl)phthalimide in THF by refluxing for 15 h (yield, 31%). The *N*-(18-hydroxy-7,10,13,16-tetraoxaoctadecyl)phthalimide obtained was reacted with triphenylmethoxy chloride to protect the 18-hydroxy group in pyridine at r.t. for 15 h (yield, 55%). The phthalimide group was transformed into a primary amine by refluxing for 15 h in the presence of hydrazine monohydrate (yield, 90%). The 18-triphenylmethoxy-7,10,13,16-tetraoxaoctadecylamine was conjugated with *N*-benzyloxycarbonyl-L-glutamic acid in the presence of diethyl phosphorocyanidate at r.t. for 15 h. The product was purified with silica gel chromatography (ethyl acetate : methanol = 95 : 5). Removal of the benzyloxycarbonyl group was done by refluxing in the presence of ammonium formate and Pd-C for 15 h. The removal of the triphenylmethoxy group was done in the presence of formic acid overnight. The product was mixed with 20 vol% HCl in ethanol to give product $2\text{EO}_4\text{C}_6\text{N}^+$ (yield, 100%). ¹H NMR (300 MHz, CDCl_3) δ = 8.0 (3H, NH_3^+), 4.1 (1H, α -CH of glu), 3.5–3.8 (38H, 6, 8, 9, 11, 12, 14, 15, 17, 18-CH₂), 3.3 (4H, 1-CH₂), 2.1–2.5 (4H, β -, γ -CH₂ of glu), 1.5–1.7 (4H, 2-CH₂), 1.3–1.4 (8H, 3,

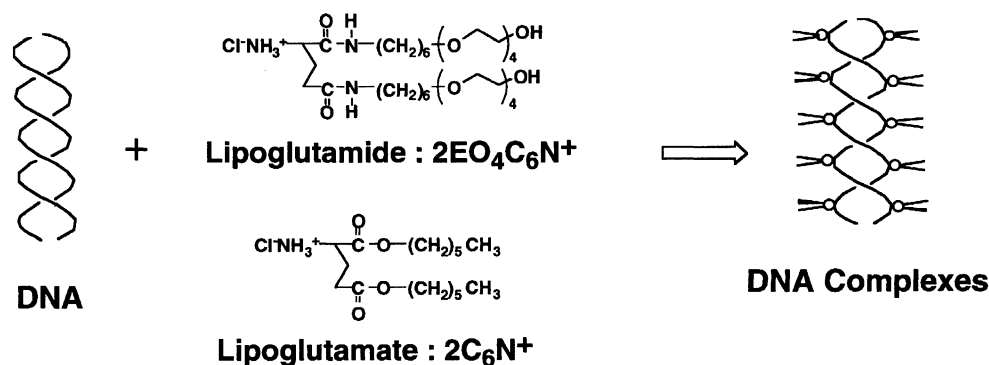


Fig. 1. A schematic illustration of DNA complexes.

4-CH₂). From the ¹H NMR, removal of Z- and triphenylmethoxy groups was confirmed by the disappearance of a peak at 7.2–7.5 ppm due to the phenyl group.

Preparation of DNA Complexes. Preparation of DNA/2EO₄C₆N⁺ complex was done by the same method as that of DNA/2C₆N⁺ complex previously reported.¹⁷⁾ Salmon sperm DNA (Na salt) was purchased from Sigma. The DNA was irradiated with a probe-type sonifier (UR-200P, Tomy Co., Tokyo) at 80 W and 4 °C for 30 min. The molecular weight of the sonicated DNA was 236000 (weight-average molecular weight, *M_w*) by the multi-angle light scattering method. The length of the sonicated DNA was estimated from the weight-averaged molecular weight to be about 357 base-pairs.

The aqueous solution of the sonicated DNA (10 mg DNA in 10 ml pure water) was mixed with 10 ml of pure water containing 22 mg of 2EO₄C₆N⁺ or 9.6 mg of 2C₆N⁺. The stoichiometry of phosphate anions of DNA to cationic glutamic acid derivatives was 1 : 1. The solution containing DNA and glutamic acid derivatives was stirred at room temperature for a couple of hours. Collection and purification of the DNA complexes were done by gel chromatography (Sephacryl S-1000 super fine, φ 16×330 mm).

Spectroscopic Observation. Absorbance and turbidity were recorded on a UV-240 spectrophotometer (Shimadzu Co., Tokyo). Circular dichroism (CD) spectra were recorded on a J-20A spectropolarimeter (JASCO Co., Tokyo) with a 10 mm path-length cuvette. Hyperchromicity changes of DNA and DNA complexes at elevated temperature (12 °C per hour) were monitored at 260 nm. Experiments were done in an aqueous solution containing 20 mM NaCl (1 M=1 mol dm⁻³). The concentration of DNA was 0.02 mg ml⁻¹.

The absolute molecular weight of DNA was measured by multi-angle light scattering photometers (Dawn Model F, Wyatt Technology, California) connected with a high-performance size exclusion chromatography (pump, JASCO Model PU-980; injector, Rheodyne; Model 7125; column, Shodex KW-804). The quantity of DNA injected was 0.1 mg in 20 μl. The elution solvent was 100 mM NaCl aqueous solution. The elution rate was 1 ml ml⁻¹. Elution was monitored by both a refractive index detector (Showa Denko K.K. Model SE-61) and a light-scattering detector (Dawn Model F). Analyses such as molecular weight and radius at an elution peak were done by the Wyatt Technology software program, ASTRA GPC. According to the software program, molecular weight were calculated from Debye plots.¹⁸⁾

MTT Assay. MTT assay is a convenient method to measure cell growth and cell viability.¹⁹⁾ Monkey kidney COS-7 cells (1×10⁴ cells) which are suspended in 0.1 ml of culture medium

(Eagle MEM, Nissui Pharmaceutical Co., Ltd., Japan) with 10% fetal bovine serum, were seeded into a 96-well multiplate, and were incubated overnight in 5% CO₂-95% air at 37 °C. The serum medium in each well was replaced with serum-free medium, and the solutions of DNA complex were added to each well. The final concentrations of DNA were 10–200 μg ml⁻¹.

The cells were incubated with DNA complexes at 37 °C for 6 h, and thereafter were washed with serum-free medium. MTT reagents (20 μg in 10 μl of phosphate-buffered saline, Sigma) were added to the cell-adherent wells, and the mixtures were incubated at 37 °C for 3 h. The supernatant was removed, and 100 μl dimethyl sulfoxide was added to each well. Absorbance at 550 nm due to formazane was measured by an ELISA reader.

Assay for Cellular Interaction of DNA Complexes. HeLa cells (2×10⁵ cells) in 1 ml of serum-free culture medium (ASF104, Ajinomoto Co., Inc., Japan) was seed into 24-well multiplates, and incubated in 5% CO₂-95% air at 37 °C. Then the cells were incubated with FITC-DNA and FITC-DNA complexes ([DNA]=10–150 μg ml⁻¹) for 3–24 h at 37 °C. Thereafter, cells were collected by treating with an aqueous solution of 0.05% trypsin–0.02% ethylenediaminetetraacetic acid. The cells loading with FITC-DNA was detected by a flow cytometer (EPICS-XL, Coulter, USA).

Results and Discussion

Formation of a DNA/2EO₄C₆N⁺ Complex. Mixing of DNA aqueous solution and 2EO₄C₆N⁺ aqueous solution gave transparent solutions. The DNA/2EO₄C₆N⁺ complex obtained was isolated by gel chromatography using pure water as the elution solvent. Elutions were monitored by a spectrophotometer (260 nm). The elution peak of the DNA complex appeared at the same volume (60 ml) as that of the native DNA. The stoichiometry of the anionic phosphate group of DNA to cationic 2EO₄C₆N⁺ was found to be 1 : 1 from the C/N ratio of elemental analysis.

Molecular weight was measured by multi-angle light-scattering method. The weight-averaged molecular weights (*M_w*) of the native DNA and the DNA/2EO₄C₆N⁺ complex were 236000 and 441000, respectively. The ratio of anionic phosphate groups of DNA to cationic 2EO₄C₆N⁺ was calculated to be 1 : 0.58, which was less than that found by elemental analysis (1 : 1). This may indicate that the DNA/2EO₄C₆N⁺ complex was partly dissociated in the presence of 100 mM NaCl.

Cofomational Changes of DNA Complexes. We

have already reported the CD spectrum of DNA/2C₆N⁺ complex.¹⁷⁾ The maximum wavelength of the DNA/2C₆N⁺ complex shifted from 277 nm to 284 nm. On the other hand, the CD spectrum of the DNA/2EO₄C₆N⁺ complex in aqueous solution showed no shift of maximum wavelength compared with the native DNA (Fig. 2). However, the ratio ($[\theta]_{280}/-[\theta]_{250}$) of the peak intensity in CD spectrum of the DNA/2EO₄C₆N⁺ complex was 0.5, which was smaller than 1.2 of the native DNA. This may indicate that the structure of the DNA changed in a C-type double helix form from a B-type form by the binding with 2EO₄C₆N⁺.²⁰⁾

Information about DNA structures in aqueous solution can be obtained by multi-angle light-scattering measurements. The slope of log-log plots of (root mean square radius, $\langle r^2 \rangle^{1/2}$) vs. (weight-averaged molecular weight, M_w) gives a conformation parameter.¹⁸⁾ This conformation parameter corresponds to the “ α ” value of the following equation.

$$\log \langle r^2 \rangle^{1/2} = ((\alpha + 1)/3) \log M_w + A \quad (1)$$

For example, α values for rod-like polymers, random coil polymers, and spherical polymers are 2, about 0.5, and 0, respectively.¹⁸⁾ The log-log plots for DNA/2EO₄C₆N⁺ complex gave a straight line as shown in Fig. 3. The α value was calculated from the slope of a solid line according to Eq. 1. The α value of the native DNA used in this study was found to be about 0.5, which meant that the conformation of the native DNA in water is nearly a random coil. In contrast, the α value of the DNA/2EO₄C₆N⁺ complex was about 0.12. From these values, it is expected that the DNA duplex was condensed to small particles by binding with cationic 2EO₄C₆N⁺.

The α value of DNA/2C₆N⁺ complex could not be measured by this method because of the turbidity of this complex. However, the α value of DNA/2C₄N⁺ complex, which has a

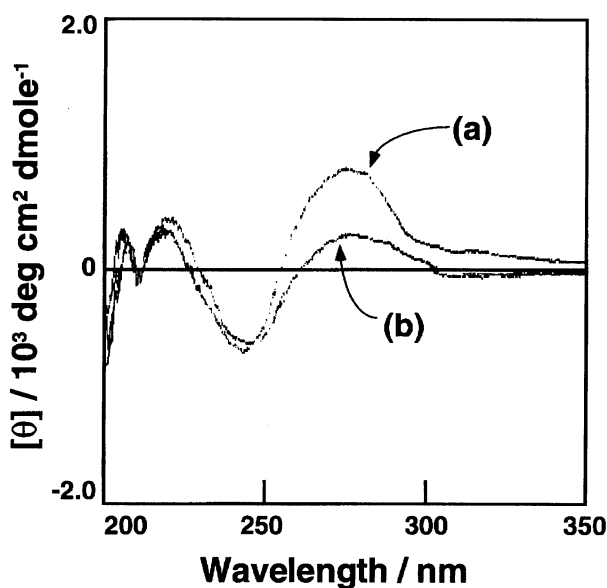


Fig. 2. CD spectra of (a) native DNA, (b) DNA/2EO₄C₆N⁺ complex in 20 mM NaCl containing aqueous solution. [DNA] = 0.02 mg ml⁻¹.

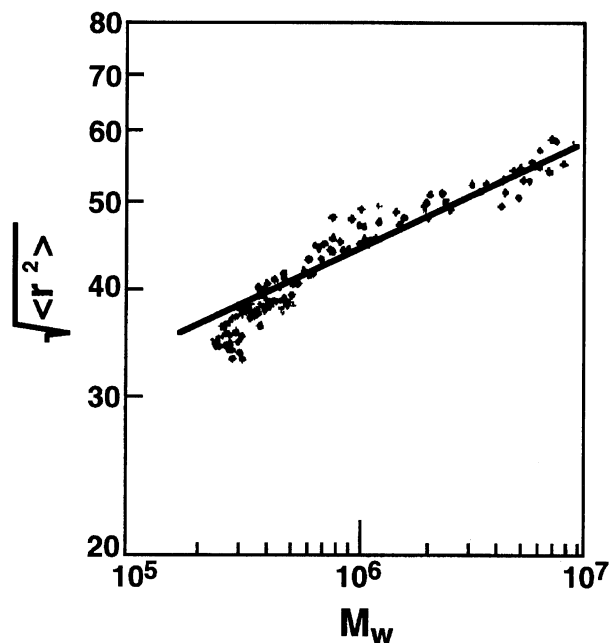


Fig. 3. log-log plots of (root mean square radius) vs. (weight averaged molecular weight) for DNA/2EO₄C₆N⁺ complex. According to Eq. 1 (see text), α value was calculated from the slope of a solid line.

shorter alkyl chain than 2C₆N⁺, was evaluated to be 0.05.¹⁷⁾ This indicated that the structure of DNA was condensed by the addition of 2C₄N⁺. It has been reported that similar condensation of DNA into small spheres was also induced by spermine,²¹⁾ lipospermine,²²⁾ and other counter cations.²³⁾ This DNA compaction is known to be induced by neutralization of the phosphate anions in the DNA.²¹⁾

Melting Temperature (T_m) of DNA Complexes. Melting temperatures of DNA and DNA/2EO₄C₆N⁺ complexes were obtained from hyperchromicity (increase in the absorbance at 260 nm) at elevated temperatures. The thermal profile of the DNA/2C₆N⁺ complex has been demonstrated in our previous paper, and found to be almost identical with the native DNA.¹⁷⁾ The temperature dependence of hyperchromicity of the native DNA and the DNA/2EO₄C₆N⁺ complexes was shown in Fig. 4. The thermal profile of the DNA/2EO₄C₆N⁺ complex was shifted to a lower temperature, and the phase transition occurred in two stages. The main T_m of the DNA/2EO₄C₆N⁺ (about 67 °C) was lower than those of the DNA/2C₆N⁺ complex and the native DNA (about 72 °C). The minor T_m was about 30 °C. The binding of 2EO₄C₆N⁺ to DNA seems to slightly lower the thermal stability of DNA. Though we could not understand why the DNA complex showed two stages in T_m profiles, the DNA complex may contain thermally unstable portions.

Turbidity of DNA Complexes. The mixture of salmon sperm DNA and 2C₆N⁺ became turbid in an aqueous solution.¹⁷⁾ Production of aggregation will be a disadvantage in the practical use as a gene delivery system. One of the methods for increasing water solubility of DNA complexes is the introduction of a hydrophilic ethylene glycol chain to

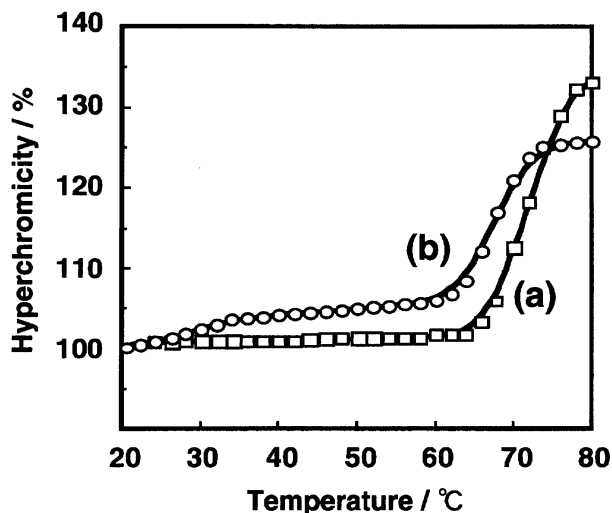


Fig. 4. Hyperchromicity changes of (a) native DNA and (b) DNA/2EO₄C₆N⁺ complex in 20 mM NaCl-containing aqueous solution at elevated temperature. Hyperchromicity was estimated from the absorbance at 260 nm. [DNA]=0.02 mg ml⁻¹.

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Figure 5A shows turbidity changes of DNA complexes as a function of DNA concentration. Figure 5B shows the courses of turbidity changes of DNA complexes. The two figures clearly indicate that aggregation of the DNA/2EO₄C₆N⁺ complex was depressed compared with the DNA/2C₆N⁺ complex without ethylene glycol tails. The solution of the DNA/2EO₄C₆N⁺ complex was transparent at least for a couple of days. DNA complex is considered to be in condensed particles in aqueous solution as suggested from the measurement of light scattering (Fig. 3). The water solubility of the DNA complex is dependent on the hydrophilicity/hydrophobicity balance of the particle surface. The hydrophilic

ethylene glycol tails of the DNA complex are probably exposed to the water phase. This may lead to the improvement of water-solubility of the DNA complex.

Cytotoxicity of DNA Complexes. Another major problem of the former DNA complexes with cationic lipids or cationic polymers is the strong cytotoxicity. Reduction of cytotoxicity will be indispensable to practical use of gene delivery systems. Cytotoxicity of DNA complexes was evaluated by an MTT assay. Percentages of cell viability are shown in Fig. 6. Cell viability in the presence of the DNA/2C₆N⁺ complex drastically decreased to about 20% when the DNA concentration was 200 μg ml⁻¹. The DNA/2EO₄C₆N⁺ complex, however, had high cell viability (about 90%) at the same DNA concentration. The difference in cell viability between the DNA/2EO₄C₆N⁺ and the DNA/2C₆N⁺ complexes was very significant. This indicates that the DNA/2EO₄C₆N⁺ complex can be used at high doses.

Interaction of DNA Complexes with Hela Cells. If we will aim at application to gene delivery systems, it is necessary to introduce foreign genes efficiently into the cells. Interaction of DNA complexes with the Hela cell, an established tumor cell, was investigated by flow cytofluorometry. Figure 7 shows fluorescence histograms of Hela cells incubated with native DNA or DNA/2EO₄C₆N⁺ complex at 37 °C for 6 h. The native DNA showed only a small shift to a higher fluorescence intensity, while the DNA/2EO₄C₆N⁺ complex showed a significant increase of fluorescence intensity. To calculate the uptake efficiencies of DNA complexes into cells, gates for the fluorescence intensities of histograms were created as shown in Fig. 7 (areas A and B in fluorescence histograms). The ratios of cell number in area B against area A were calculated as the uptake efficiency. The calculated percentages were 6, 22, and 62% for Hela cells (control), the native DNA, and the DNA/2EO₄C₆N⁺ complex, respectively.

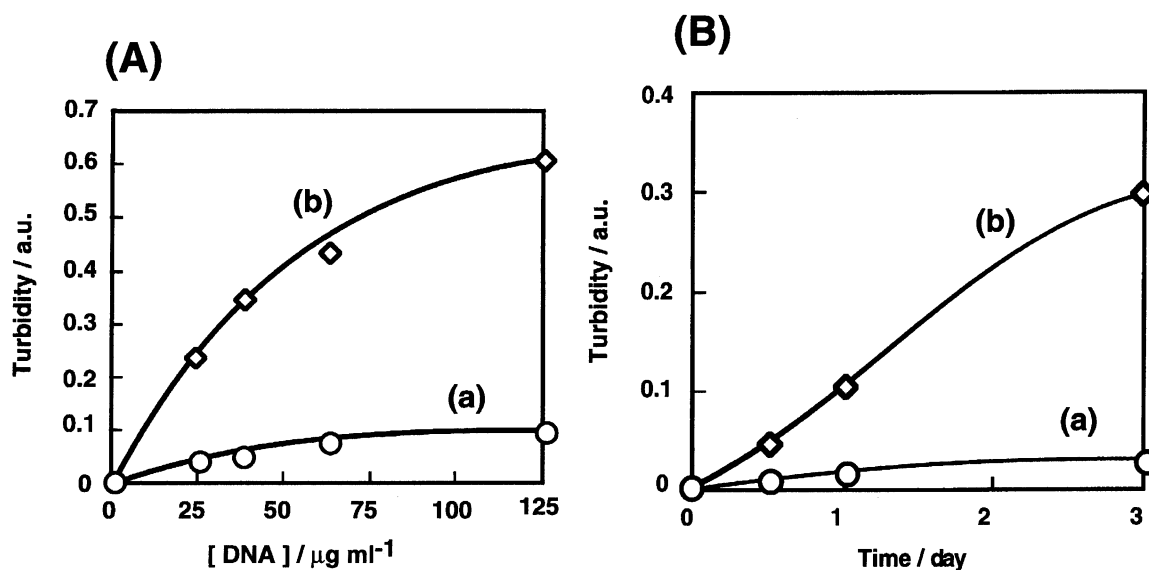


Fig. 5. (A) Turbidity changes as a function of DNA concentration at 1 day and (B) time courses of turbidity change at [DNA]=25 μg ml⁻¹ at 20 °C. (a) DNA/2EO₄C₆N⁺ complex and (b) DNA/2C₆N⁺ complex.

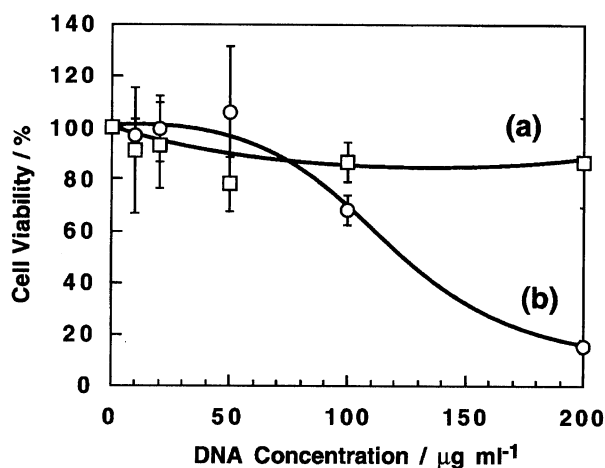


Fig. 6. Viability of COS-7 cells in the presence of (a) DNA/2EO₄C₆N⁺ and (b) DNA/2C₆N⁺ complex. COS-7 cells were incubated with the DNA complexes at 37 °C for 6 h.

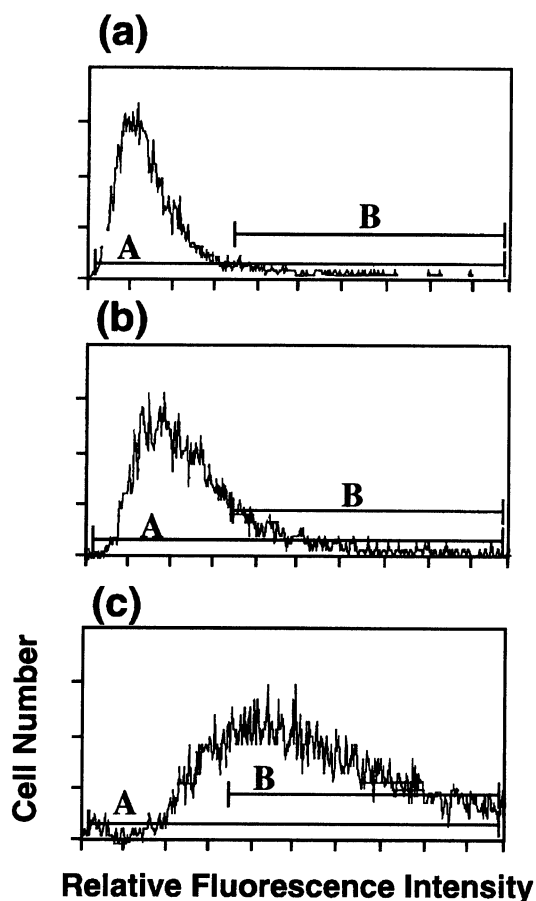


Fig. 7. Fluorescence histograms of HeLa cells in (a) control, (b) native DNA, and (c) DNA/2EO₄C₆N⁺ complex. The DNA was partly labeled with fluorescent probe (FITC). Incubation was carried out at 37 °C for 24 h. Cell number was 1×10^5 cells ml⁻¹, and the DNA concentration was 20 μg ml⁻¹. Area A and B in histograms mean all cells and the FITC-DNA bearing cells, respectively.

Figure 8 shows the courses of cell uptake of the native DNA, the DNA/2EO₄C₆N⁺, and the DNA/2C₆N⁺ complexes, when the DNA concentration was 50 μg ml⁻¹. Efficiency of cell uptake is represented as peak intensity of fluorescence shown in Fig. 7. Fluorescence intensities were leveled off by around 6 h. There are no significant differences in cell uptake between the DNA/2EO₄C₆N⁺ complex and the DNA/2C₆N⁺ complex. We worried that cell uptake of the hydrophilic DNA/2EO₄C₆N⁺ complex would be lower than that of the DNA/2C₆N⁺ complex, since Tabata et al. demonstrated the importance of appropriate hydrophobicity of the surface of polymer particle for cell uptake.²⁰ Fortunately, the hydrophilic DNA/2EO₄C₆N⁺ complex interacted with cells at the same efficiency as the DNA/2C₆N⁺ complex. Poly(ethylene glycol) is known as a fusogen,²⁴ and can increase membrane permeability.²⁵ Such a feature of the ethylene glycol chain may induce the strong cellular interaction of the DNA/2EO₄C₆N⁺ complex.

Figure 9 showed the cell uptake of the native DNA, the

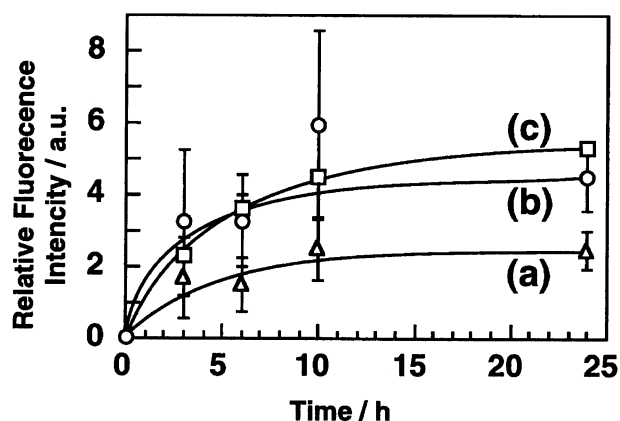


Fig. 8. Time courses on cell uptake of (a) native DNA, (b) DNA/2EO₄C₆N⁺, and (c) DNA/2C₆N⁺ complexes. Incubation was carried out at 37 °C. Cell number was 5×10^4 cells ml⁻¹, and the DNA concentration was 50 μg ml⁻¹.

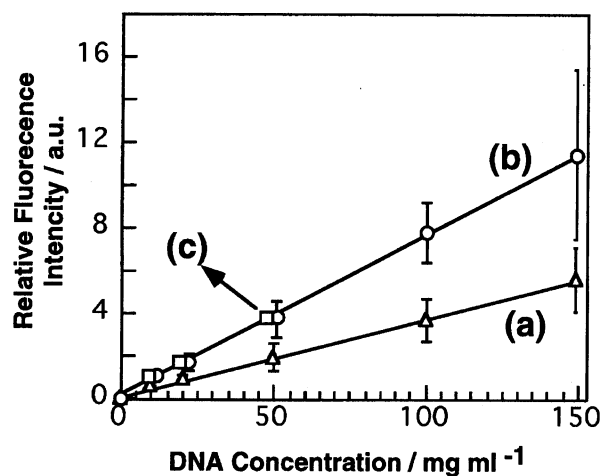


Fig. 9. Dependence of DNA concentration on cell uptake of (a) native DNA, (b) DNA/2EO₄C₆N⁺, and (c) DNA/2C₆N⁺ complexes. Incubation was carried out at 37 °C for 6 h. Cell number was 5×10^4 cells ml⁻¹.

DNA/ $2\text{EO}_4\text{C}_6\text{N}^+$, and the DNA/ $2\text{C}_6\text{N}^+$ complexes as a function of DNA concentration. Internalization efficiency increased linearly with increasing DNA concentration. It was found that the DNA/ $2\text{EO}_4\text{C}_6\text{N}^+$ complex could interact with cells at higher efficiency than the native DNA. In the case of the DNA/ $2\text{C}_6\text{N}^+$ complex, the doses have to be limited at DNA concentrations lower than $50\ \mu\text{g ml}^{-1}$ because of its cytotoxicity. On the other hand, the DNA/ $2\text{EO}_4\text{C}_6\text{N}^+$ complex was able to interact with cells at the higher concentration. These results indicate that the DNA/ $2\text{EO}_4\text{C}_6\text{N}^+$ complex is more useful than the DNA/ $2\text{C}_6\text{N}^+$ complex to introduce a larger amount of foreign DNAs into cells.

Cell uptake was also examined at $4\ ^\circ\text{C}$ to confirm that the DNA/ $2\text{EO}_4\text{C}_6\text{N}^+$ complex was internalized into the cells, but did not adhere only to the cell surface. It is known that low temperatures such as $4\ ^\circ\text{C}$ inhibit the endocytosis of cells. The internalization of the DNA/ $2\text{EO}_4\text{C}_6\text{N}^+$ complex was completely inhibited under such experimental conditions. This indicates that the DNA/ $2\text{EO}_4\text{C}_6\text{N}^+$ complex is certainly transported inside the cells.

Conclusion

We studied fundamental properties of DNA complexes with lipoglutamide having tetraethyleneglycol tails. The preparation method is simple, just mixing both aqueous solutions of native DNA and lipoglutamide. The DNA/ $2\text{EO}_4\text{C}_6\text{N}^+$ complex forms collapsed particles in the aqueous solution. Aggregation of the DNA/ $2\text{EO}_4\text{C}_6\text{N}^+$ complex was drastically depressed compared with the DNA/ $2\text{C}_6\text{N}^+$ complex without tetraethyleneglycol tails. Furthermore, the DNA/ $2\text{EO}_4\text{C}_6\text{N}^+$ complex showed no cytotoxicity and increased the uptake of DNA into tumor cells. The newly developed the DNA/ $2\text{EO}_4\text{C}_6\text{N}^+$ complex would be expected to be a more useful DNA carrier than the former DNA/ $2\text{C}_6\text{N}^+$ complex, since introduction of tetraethyleneglycol tails to DNA complexes brought several advantages desirable to DNA delivery systems.

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References

- 1) For preliminary report, see: T. Sato, H. Akino, and Y.

Okahata, *Chem. Lett.*, **1995**, 755.

- 2) A. V. Kabanov, S. V. Vinogradov, A. V. Ovcharenko, A. V. Krivonos, N. S. Melik-Nubarov, V. I. Kiselev, and E. S. Severin, *FEBS Lett.*, **259**, 327 (1990).

- 3) T. Saison-Behmoaras, B. Tocque, I. Rey, M. Chassignol, N. Thoung, and C. Helene, *EMBO J.*, **10**, 1111 (1991).

- 4) R. G. Shea, J. C. Marsters, and N. Bischofberger, *Nucleic Acids Res.*, **18**, 3777 (1990).

- 5) C. MacKellar, D. Graham, D. W. Will, S. Burgess, and T. Brown, *Nucleic Acids Res.*, **20**, 3411 (1992).

- 6) G. Y. Wu and C. H. Wu, *J. Biol. Chem.*, **263**, 14621 (1988).

- 7) D. Vestweber and G. Schatz, *Nature*, **338**, 170 (1989).

- 8) J.-S. Remy, A. Kichler, V. Mordvinov, F. Schuber, and J.-P. Behr, *Proc. Natl. Acad. Sci. U.S.A.*, **92**, 1744 (1995).

- 9) W. E. Magee, J. H. Cronenberger, D. E. Thor, and R. E. Paque, "Liposomes and Immunobiology," ed by B. H. Tom and H. R. Six, Elsevier, North-Holland (1980), p. 133.

- 10) M. Akashi, H. Iwasaki, N. Miyauchi, T. Sato, J. Sunamoto, and K. Takemoto, *J. Bioact. Compat. Polym.*, **4**, 124 (1989).

- 11) P. L. Felgner, T. R. Gadek, M. Holm, R. Roman, H. W. Chan, M. Wenz, J. P. Northrop, G. M. Ringold, and M. Danielsen, *Proc. Natl. Acad. Sci. U.S.A.*, **84**, 7413 (1987).

- 12) J.-P. Behr, B. Demeneix, J.-P. Loeffler, and J. Perez-Mutul, *Proc. Natl. Acad. Sci. U.S.A.*, **86**, 6982 (1989).

- 13) J. P. Lemaitre, B. Bayard, and B. Lebleu, *Proc. Natl. Acad. Sci. U.S.A.*, **84**, 648 (1990).

- 14) K. Ohtani, M. Nakamura, S. Saito, K. Nagata, K. Sugamura, and Y. Hinuma, *Nucleic Acids Res.*, **17**, 1589 (1989).

- 15) Y. Tsutsumi, S. Nakagawa, and T. Mayumi, *Drug Delivery Syst.*, **10**, 75 (1995).

- 16) F. Fuertges and A. Abuchowski, *J. Controlled Release*, **11**, 139 (1990).

- 17) T. Sato, T. Kawakami, N. Shirakawa, and Y. Okahata, *Bull. Chem. Soc. Jpn.*, **68**, 2709 (1995).

- 18) P. J. Wyatt, *Anal. Chim. Acta*, **272**, 1 (1993).

- 19) T. Mosmann, *J. Immunol. Methods*, **65**, 55 (1983).

- 20) S. Hanlon, S. Brudno, T. T. Wu, and B. Wolf, *Biochemistry*, **14**, 1648 (1975).

- 21) R. Wilson and V. Bloomfield, *Biochemistry*, **18**, 2192 (1979).

- 22) J.-P. Behr, *Tetrahedron Lett.*, **27**, 5861 (1986).

- 23) Y. Tabata and Y. Ikada, *J. Colloid Interface Sci.*, **127**, 132 (1989).

- 24) R. I. MacDonald, *Biochemistry*, **24**, 4058 (1985).

- 25) T. J. Aldwinckle, Q. F. Ahkong, A. D. Gangham, D. Fisher, and J. A. Lucy, *Biochim. Biophys. Acta*, **728**, 121 (1982).