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Gene Expression of Ternary Complexes through the Compaction of Nanofiber-Polyplexes by Mixing with Lipofectamine

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For the development of an effective nonviral gene vector, ternary complexes were prepared through the compaction of nanofiber-polyplexes. These were formed using pDNA and a head-tail type polycation bearing a multi-arm poly(ethylene glycol) head and a poly(L-lysine) tail, and this strategy was based on the crowding effect of poly(ethylene glycol) in the polyplex. Mixing was carried out using a cationic lipid (lipofectamine), which is a commercially available transfection reagent. Through ternary complex formation, the elongated morphology of nanofiber-polyplexes was found to compact into a spherical shape with an average diameter of ca. 100 nm. Accompanying ternary complex formation, the compaction of the nanofiber-polyplexes can improve cellular uptake and helps the ternary complex to retain its smooth transcription / translation process, which is characteristic of nanofiber-polyplexes. As a result, ternary complexes prepared at an optimal mixing ratio exhibit a high transfection efficiency compared with lipofectamine lipoplexes.

Introduction

Gene therapy has received considerable attention as a treatment for various diseases such as cancer and native genetic diseases. Various types of cationic compounds including polymers and lipid molecules have been designed and evaluated for use in effective gene therapy,[1-4] Cationic polymers can electrostatically form complexes with DNA and this complex is called polyplex. Polyplex formation induces DNA condensation through the charge neutralization of DNA molecules. This condensation has both advantages and disadvantages for the use of polyplexes as a nonviral gene vector. Although condensed DNA molecules offer effective protection from degradation by DNase, overstabilization is observed as a general tendency during polyplex formation.[5] The overstabilization of polyplexes inhibits the smooth release of DNA through an exchange reaction with counter polyanions within the cells.[6,7] The complete inhibition of enzymatic activity such as transcription has been reported when condensation is complete and the DNA molecules are fully packed.[8] This behavior reinforces the importance of DNA packing control in the polyplex for successful gene delivery.

Additionally, the instability of polyplexes towards incubation with serum proteins because of the positive zeta-potentials of polyplexes is a common problem for most cationic vector systems in clinical applications. After intravenous injection, the polyplex vectors tend to form aggregates and are subsequently dismantled because of the electrostatic interactions between cationic vectors and serum components. To overcome this issue, the conjugation of cationic polymers with biocompatible polymers such as poly(ethylene glycol) (PEG), poly[N-(2-hydroxypropyl)methacrylamide] and dextran has also been reported, and such approaches have been found to be effective at reducing the overall surface charge of polycationic vectors.[9-18]

We have investigated head-tail type polycations composed of a polyamidoamine (PAMAM) dendron head and a poly(L-lysine) (PLL) tail as a non-viral gene vector.[19,20] The construction of a multi-arm PEG (maPEG) structure by the introduction of PEG chains to the periphery of the PAMAM dendron head results in an effective improvement in the stability of the polyplexes against incubation with serum proteins.[21] Additionally, an improvement in the inhibition ability of DNA condensation occurs despite the cationic property of maPEG-PLL.[22] We recently found that polyplex morphology can be elongated by increasing the size of maPEG head, in which the exclusion volume of the maPEG head can be controlled by changing the molecular weight of the PEG chains and by the generation of a PAMAM dendron.[23] Importantly, this elongation of polyplex morphology to a nanorod or a nanofiber results in an increase in cell-free gene expr ession reflecting the efficiency of the transcription / translation process. However, the most-elongated polyplex morphology, i.e. nanofiber-polyplexes, is a disadvantage in terms of cellular uptake for gene delivery. In this study, we prepared ternary complexes through the compaction of nanofiber-polyplexes by mixing with lipofectamine for the development of a cationic vector that exhibits both the valuable features of nanofiber-polyplexes and lipofectamine. These are a relatively smooth transcription / translation process and the endosomal escape ability of lipofectamine. Ternary complex formation was confirmed by atomic force microscopy and by dynamic light scattering measurements, and the gene expression behavior of the ternary complexes is discussed considering an evaluation of

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the transfection efficiency, cellular uptake, the intracellular distribution and cell-free gene expression.

Experimental

Materials

Plasmid DNA (pCMV-Luc and pEGF-C1) were amplified in E. coli, isolated and purified using a QIAGEN plasmid Maxi Kit. The head-tail type polycation bearing a multi-arm PEG head (number of PEG arms = 16 and the Mn of PEG = 2000) and the poly(L-lysine) tail (number-averaged polymerization degree = 71) were synthesized according to our previous report.[21] Lipofectamine reagent was purchased from Invitrogen. Fetal bovine serum (FBS) was purchased from JRH Biosciences. Dulbecco's modified Eagle's medium (DMEM) was purchased from Nissui Pharmaceutical. Luc-PGC-50 detergent was purchased from Toyo Ink. Coomassie Protein Assay Reagent was purchased from Pierce. A label IT Fluorescein Labeling Kit was purchased from Mirus.

Preparation of ternary complexes

The ternary complexes were prepared by mixing lipofectamine with the nanofiber-polyplexes formed by maPEG-PLL and pDNA. maPEG-PLL and pDNA were separately dissolved in 20 mM phosphate buffer (pH 7.4), and the maPEG-PLL solutions were added to the pDNA solution at N/P = 1 and 3, and the N/P ratio was defined as the number of Lys residues to the number of phosphates. After storing at least overnight, the ternary complexes were prepared by adding lipofectamine to nanofiber-polyplex solutions at various mixing ratios (N/P = 0.13, 0.25, 0.52, 1.03, 2.06, 4.12 and 8.24). The N/P ratio was defined as the number of amines to the number of phosphates. Therefore, the prepared ternary complexes were stored overnight at room temperature before further evaluation. The ternary complexes prepared are abbreviated FxLy, which indicates the N/P ratios of the nanofiber-polyplex (x = 1 and 3) and lipofectamine (y = 0.13, 0.25, 0.5, 1, 2, 4 and 8).

Transfection to HeLa Cells

The transfection of the ternary complexes to HeLa cells was evaluated by luciferase and GFP assay. In the case of luciferase assay, the cells were seeded in 0.5 mL of DMEM supplemented with 10% FBS in 24-well culture plates at 5×10^4 cells per well the day before the transfection. The cells were washed with phosphate buffer saline containing 0.36 mM $CaCl_2$ and 0.42 mM $MgCl_2$ [PBS(+)] and then incubated with DMEM in 10% FBS (0.5 mL). Ternary complex solutions containing 1 μg of pDNA were gently added to the cells and then incubated at 37 °C for 24 hours. The cells were rinsed with PBS(+) and incubated with DMEM in 10% FBS at 37 °C. After 24 hours of incubation the cells were lysed by adding 50 μL of the Luc-PGC-50 detergent. A 20 μL aliquot from each well was used for the luciferase assay using a kit (Toyo Ink) and a Lumat LB9507 luminometer (Berthold). Also, in the case of GFP assay, the cells were treated by same procedures with luciferase assay. The transfection time were changed to varying time (24, 48, 72 and 96 hours). After the transfection time, total fluorescence intensity of the cells were evaluated using flow cytometry (EPICS XL, Beckman Coulter, Inc.). For both assays, the protein content of the lysate was measured by Coomassie Protein Assay Reagent using bovine serum albumin as the standard. Further, the cytotoxicity of ternary complexes were evaluated by MTT assay, in which the cells were treated by same condition with luciferase assay. The cells were incubated with the ternary complexes for 24 hours with 10 % FCS. The culture medium was replaced with 0.2 mL of DMEM with 10 % FCS containing 40 μL of MTT dissolved in PBS(-) (10 mg/mL) was added to each well. After 3 hours incubation, the medium was removed, and the cells were solubilized in 500 μL of 2-propanol containing 0.1 M HCl. The viable cells were counted from the absorbance at 490 nm using $ARVO_{SX}$ multilabel counter (Perkin Elmer).

Physicochemical characterization

Atomic force microscopy (AFM) was performed using a SPA-400 atomic force microscope (Seiko Instruments Inc.) containing a cantilever made of silicon (SI-DF40; Seiko Instruments Inc., Japan) and its spring constant was 16 N/m. The ternary complexes (F1L1, F1L2, F3L1 and F3L2) and nanofiber-polyplexes (F1L0 and F3L0) were applied to freshly cleaved mica and incubated overnight. AFM images were collected from the prepared mica. Dynamic light scattering (DLS) and laser-Doppler electrophoresis measurements were performed using an ELS-8000 (Otsuka Electronics Co., Ltd., Osaka, Japan) equipped with a He/Ne ion laser (λ = 633 nm) at 25 °C. For the DLS measurements the detection angle was fixed at 90°. The hydrodynamic diameter (D_h) was calculated by the Stokes–Einstein equation and the size distribution was obtained by CONTIN analysis. For the laser-Doppler electrophoresis measurements, the particle velocity was measured using a laser light scattering technique. Because of the Doppler effect the frequency of the scattered laser light was different from that of the original beam. From the electrophoretic mobility determined by the frequency shift from the Doppler effect the zeta-potential was calculated using the Smoluchouski equation.

Cellular uptake of the ternary complexes by HeLa cells

Cellular uptake was evaluated using FITC-labeled pDNA, which was labeled using a label IT Fluorescein Labeling Kit according to the protocol provided by the manufacturer. HeLa cells were seeded in 0.5 mL of DMEM supplemented with 10% FBS in 24-well culture plates at 5×10^4 cells per a well the day before the uptake experiments. The cells were washed with PBS(+) and then incubated with DMEM in 10% FBS (1 mL). The ternary complexes prepared using the FITC-labeled pDNA were gently added to the cells and incubated at 37 °C for varying incubation times (1, 2, 4, 8, and 24 h). The cells were washed with PBS(+) and detached using trypsin. Cellular fluorescence was then evaluated using flow cytometry (EPICS XL, Beckman Coulter, Inc.).

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Intracellular distribution of the ternary complexes

HeLa cells cultured in 2 mL DMEM were supplemented with 10% FBS using 2×10^5 cells the day before washing with PBS(+) and they were then incubated with DMEM containing RITC-dextran (0.28 mg/mL). The ternary complexes prepared using FITC-labeled pDNA were gently added to the cells and incubated at 37 °C for 24 h. After washing three times with PBS(+), confocal laser scanning microscopic (CLSM) analysis of the cells was performed using an LSM 5 EXCITER (Carl Zeiss Co. Ltd.).

Cell-free gene expression

Cell-free gene expression of pDNA was evaluated using the Quick Coupled Transcription/Translation system (Promega). The experiments were performed according to the protocol provided by Promega as follows: 9 μL of ternary complex solutions including 1 μg of pDNA were mixed with 40 μL of TNT Quick Master Mix (Promega) and 1 μL of 1 mM methionine solution, and the mixed solutions were then incubated for 60 min at 30 °C. 5 μL of the mixtures were treated with 50 μL Luciferase Assay Reagent (Promega) and the luminescence intensities were then monitored using an ARVOSX multilabel counter (Perkin-Elmer).

Stability of the ternary complexes

The stability of the ternary complexes, the nanofiber-polyplexes and the lipoplexes were evaluated by polyanion competition assay using agarose gel electrophoresis. Varying amounts of heparin were added to the ternary complex, the nanofiber-polyplex and the lipoplex solutions, and the mixtures had a constant pDNA concentration. After 30 min of incubation, the sample solutions were electrophoresed at 100 V/cm for 30 min with 0.6 w/w% agarose gel in the buffer solution (40 mM Tris, 20 mM sodium acetate, and 2 mM EDTA buffer containing 1 μg/mL EtBr). The migrated EtBr-stained bands were visualized and the magnitude of the band intensity was analyzed using a LAS-1000UVmini (FUJIFILM, Japan).

Results and discussion

Fig. 1a shows the lipofectamine-mixing effect on the transfection efficiency of the nanofiber-polyplexes. The transfection efficiency of the nanofiber-polyplexes dramatically increased upon the addition of lipofectamine, suggesting the formation of ternary complexes. The low transfection efficiency of the nanofiber-polyplexes comes from low cellular uptake because of their elongated morphology. All the complexes, including the F1 and F3 ternary complexes and the lipoplexes prepared using pDNA and lipofectamine showed maximal transfection efficiency upon a change in the N/P ratio of lipofectamine to pDNA. The F3 ternary complexes gave a lower transfection efficiency compared with the lipoplexes at all N/P ratios. On the other hand, the transfection efficiency of the F1 ternary complexes is higher than that of the lipoplexes at N/P ≥ 2. The transfection efficiency of the F1L2 ternary polyplexes is 600 times that of the nanofiber-polyplexes (F1L0), and 1.6 times that of lipoplexes. High transfection efficiency of F1L2 ternary complexes compared with lipofectamine was also observed for GFP gene expression (Fig. 1b). At optimal transfection time (48 hours), GFP gene expression of F1L2 ternary complexes is 13 times that of lipofectamine. A high transfection efficiency might be expected where F1 ternary complexes retain the advantages of the nanofiber-polyplex and of lipofectamine, which are a relatively high efficiency for the transcription / translation process of a nanofiber-polyplex[23] and the membrane fusion ability of the dioleoyl phosphatidylethanolamine (DOPE) in lipofectamine for endosomal escape.[24,25] The morphology, cellular uptake, intracellular distribution and cell-free gene expression data were obtained for the F1L2 and F3L2 ternary complexes to determine the effect of ternary complex formation on transfection efficiency. The F1L2 and F3L2 ternary polyplexes showed an opposite effect to that of lipoplexes in the transfection experiment, as shown in Fig. 1a.

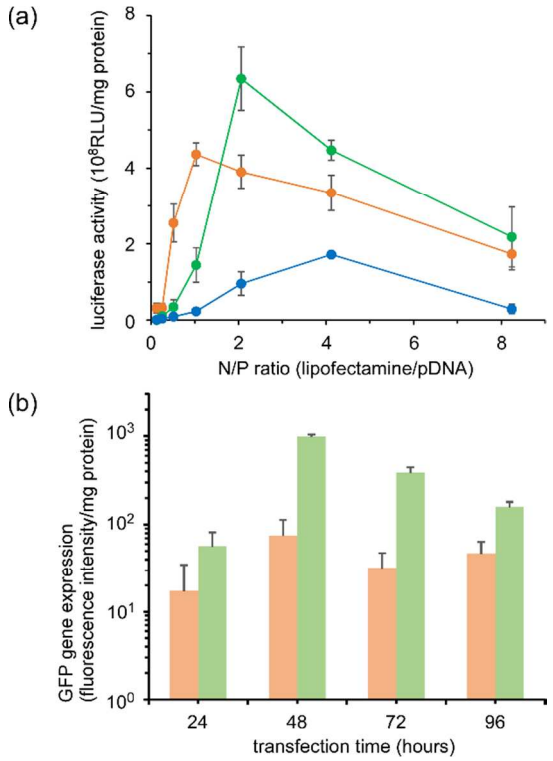

Fig. 1 Luciferase activity of HeLa cells treated with the mixture of nanofiber-polyplexes and lipofectamine (F1, green symbols; F3, blue symbols) and lipofectamine lipoplexes (orange symbols) (a). Data presented as the average of three experiments ± SD. GFP expression of HeLa cells treated with F1L2 ternary complexes (green) and lipofectamine (orange) for varying transfection time (b).

To confirm the morphology and size distribution of ternary complexes, AFM observation and DLS measurements were performed. Fig. 2 show AFM images of nanofiber-polyplexes, lipofectamine lipoplexes and ternary complexes, and size

distributions of the F1L2 and F3L2 ternary complexes. The size distributions were obtained by a CONTIN analysis of the DLS measurements. The elongated nanofiber-like morphology as shown in Fig. 2a was not observed in the AFM images and only spherical complexes of ca. 100 nm in diameter were present for the ternary complexes (Fig. 2d and 2f). Also, lipofectamine lipoplexes shown in Fig. 2c had quite large size compared with ternary complexes. These observations suggest that ternary complex formation induces a change in morphology from an elongated to a spherical shape. The exchange reaction between maPEG-PLL and cationic self-assembly of lipofectamine occur in the mixture of nanofiber-polyplexes and lipofectamine. Presumably hydrophobic domain coming from lipofectamine self-assembly are formed in nanofiber-polyplex, and then, the compaction of nanofiber-polyplex occurs so that nanofiber-polyplex surrounds hydrophobic domain. This change to a compact morphology might be effective in improving cellular uptake. Indeed, this compaction is important to improve the transfection efficiency of nanofiber-polyplexes, and both of the elongated polyplexes and spherical complexes were observed in AFM images (Fig. 2b) for F1L1 ternary complexes which ternary complexes showed quite low luciferase activity (Fig. 1a). Additionally, the diameters obtained from the AFM images agree with the hydrodynamic diameters (D_h) determined by DLS. The D_h of the F1L2 and F3L2 ternary complexes were found to be 106 nm and 117 nm, respectively. Importantly, the ternary complexes have quite a low polydispersity index (PDI) and the PDI of the F1L2 and F3L2 ternary complexes are 0.07 and 0.08, respectively. These low PDI values indicate that the ternary complexes have a narrow size distribution. Furthermore, the zeta-potentials of the F1L2 and F3L2 ternary complexes are 0.7 mV and 0.4 mV, respectively. These neutral zeta-potentials of the ternary complexes suggest that electrically neutral PEG chains surround the surface of the ternary complexes although the exact structure of the ternary complexes is still unclear. Being surrounding by PEG chains is consistent with the low cytotoxicity of the F1L2 and F3L2 ternary complexes, as evaluated by an MTT assay. The F1L2 and F3L2 ternary complexes retain high cell viability (more than 80%) indicating their negligible cytotoxicity toward HeLa cells.

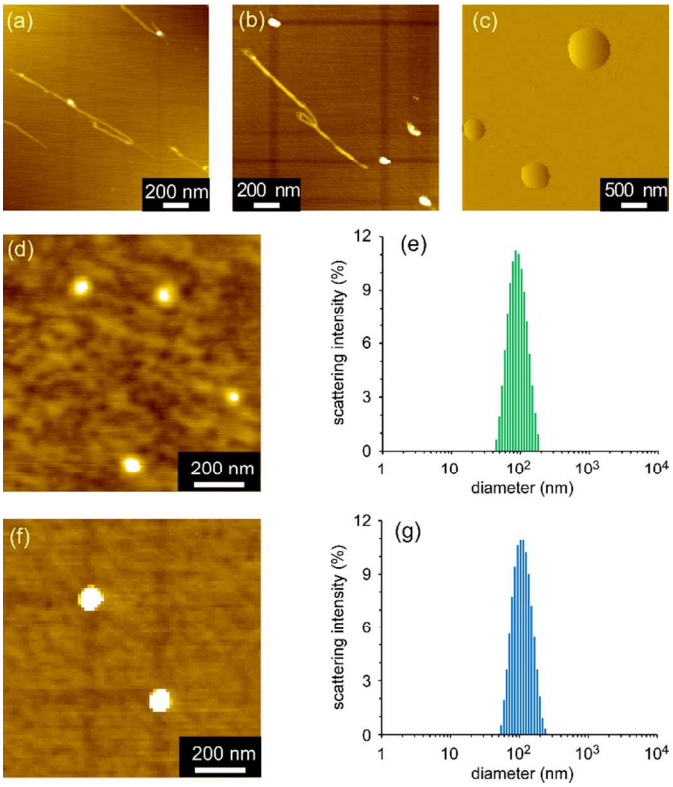

Fig. 2 AFM images of F1L0 nanofiber-polyplexes (a), F0L2 lipoplexes (c) and F1L1 (b), F1L2 (d) and F3L2 (f) ternary complexes, and DLS size distribution of the F1L2 (e) and F3L2 (g) ternary complexes.

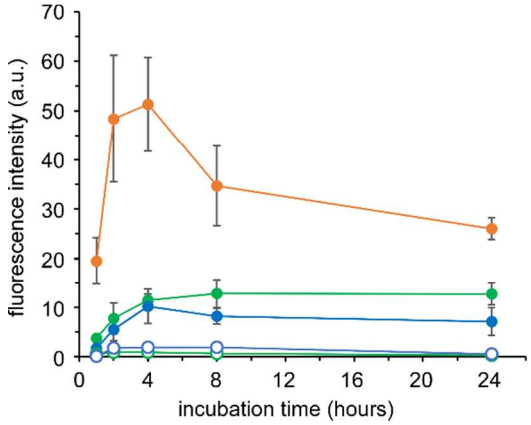

Fig. 3 Cellular uptake of ternary complexes (F1L2, closed green symbols; F3L2, closed blue symbols), nanofiber-polyplexes (F1L0, open green symbols; F3L0, open blue symbols) and lipofectamine lipoplexes (F0L2, orange symbols) into HeLa cells. Data presented as the average of three experiments ± SD.

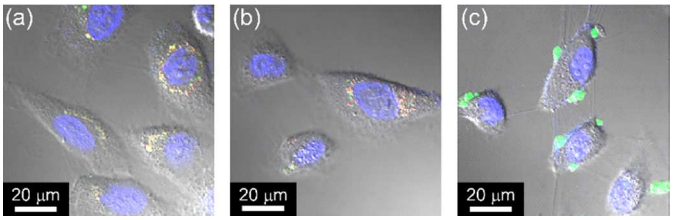

Fig. 4 Confocal laser scanning microscopy images of HeLa cells treated by the F1L2 (a), F3L2 (b) ternary complexes and lipofectamine lipoplexes (F0L2) (c).

The cellular uptake of ternary complexes was evaluated by flow cytometry using FITC-labeled pDNA. Fig. 3 shows the incubation time dependency of the fluorescence intensity of the HeLa cells treated with the ternary complexes, the nanofiber-polyplexes and the lipoplexes. The fluorescence intensity of the HeLa cells treated with lipofectamine immediately increased because of the strong electrostatic interaction between the anionic cellular surface and the cationic lipoplexes. The FITC-labeled pDNA was mainly located at the cellular surface as

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determined by confocal microscopy observations (Fig. 4c). As described above, nanofiber-polyplexes do not enter the cells even after 24 h of incubation. In our previous study, nanorod-polyplexes with major and minor axes at 428 ± 261 nm and 142 ± 43 nm showed good tolerance against incubation with serum proteins and were gradually taken up by the cells.[21,23] As a result of this incubation time dependent cellular uptake the transfection efficiency gradually increased with an increase in incubation time. The nanofiber-polyplexes have a more elongated morphology and their major axis is at 965 ± 247 nm.[23] This elongated morphology is a disadvantage in the cellular uptake process and the fluorescence intensity of the HeLa cells did not increase, even after 24 h of incubation with the nanofiber-polyplexes. However, the fluorescence intensity of the HeLa cells treated by the ternary complexes gradually increase with an increase in incubation time without a steep increase during the initial incubation period. There was no meaningful difference between the F1L2 and F3L2 ternary complexes in the cellular uptake experiment, and it is difficult to explain the difference in transfection efficiency shown in Fig. 1 from a cellular uptake point of view. As a follow up experiment, we compared the intracellular distribution of the ternary complexes by confocal laser scanning microscopy to clarify differences in the effect of ternary complex formation by the F1L2 and F3L2 ternary complexes on transfection efficiency. Figure 4a and b show confocal microscopy images of the HeLa cells treated with the F1L2 and F3L2 ternary complexes. Green, red and blue fluorescence images for FITC-labeled pDNA, RITC-labeled dextran and Hoechst were obtained separately and merged using a differential interference contrast image. For both ternary complexes, the yellow dots indicate an identical distribution of green and red fluorescence from pDNA and dextran as an indicator of endosomes. These dots are distributed near a nucleus. In addition to the cellular uptake experiments shown in Figure 4, no obvious difference was found for the intracellular distribution of the F1L2 and F3L2 ternary complexes.

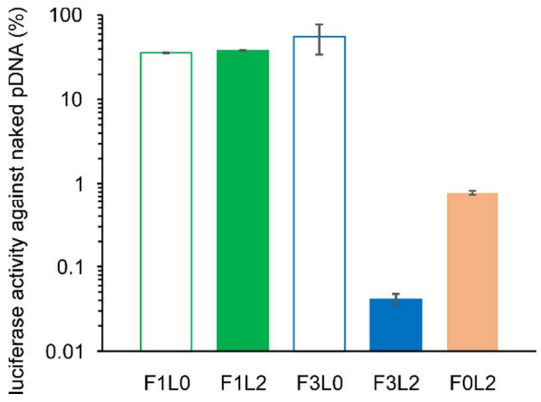

Fig. 5. Relative gene expression of ternary complexes, nanofiber-polyplexes and lipofectamine lipoplexes. Luciferase activity against naked pDNA is calculated as relative gene expression. Data presented as the average of three experiments ± SD.

The cell-free gene expression of ternary complexes, nanofiber-polyplexes and lipofectamine lipoplexes was evaluated to compare the efficiency of the transcription / translation process. Fig. 5 shows the relative gene expression of the ternary complexes and the nanofiber-polyplexes in which the relative gene expression is a percentage of luciferase activity in the ternary complexes, the nanofiber-polyplexes and lipofectamine lipoplexes against that of naked pDNA. The relative gene expression reflects the efficiency of the transcription / translation process. Obviously, only the F3L2 ternary complexes exhibit extremely low gene expression. The F1L2 ternary complexes maintained a similar level of gene expression to that of the nanofiber-polyplexes. The difference in relative gene expression between the F1L2 and F3L2 ternary complexes is quite large and the gene expression of the F1L2 ternary complexes is more than 10^3 times that of the F3L2 ternary complexes. This significant difference can influence the HeLa cell transfection experiments, as shown in Fig. 1.

Finally, the stability of the ternary complexes and the lipoplexes were compared by polyanion competition assay using gel electrophoresis. Varying amounts of heparin, which is a negatively charged linear polysaccharide bearing sulfonates and carboxylates were added to the ternary complexes and lipoplexes, and the amounts of released pDNA were determined from the band intensity in the agarose gel electrophoretic images after 30 min of incubation. Fig. 6 shows these electrophoretic images and changes in the amounts of released pDNA upon the addition of heparin. The ternary complexes and the lipoplexes have different release profiles upon a change in the amount of heparin added. In the case of lipoplexes, the release of pDNA depends on the amount of added heparin, and the pDNA molecules were completely released when 2.5 μg of heparin was added. The ternary complexes did not release pDNA, even upon the addition of 2.5 μg of heparin. The ternary complexes released different amounts of pDNA upon an increase in the amount of heparin and a critical amount of heparin was required to initiate pDNA release. At less than this critical amount, pDNA molecules are not released from the ternary complexes and the addition of more heparin than the critical amount promotes the release of pDNA molecules by an exchange reaction between pDNA and heparin. The ternary complexes are highly stable upon co-incubation with the polyanion compared with the lipoplexes. The critical heparin amounts are different for the F1L2 and F3L2 ternary complexes and were found to be 2.5 and 3.5 μg of heparin for F1L2 and F3L2, respectively. These values are not different when considering the charge balance between the positive (Lys residue) and the negative (phosphates of pDNA, sulfates and carboxylates of heparin) groups in each mixture. Also, this difference in critical heparin amounts between F1L2 and F3L2 ternary complexes suggests that there are a difference in the composition of ternary complexes. Although it is difficult to determine exact composition of ternary complexes, F3L2 ternary complexes might have higher content of cationic molecules including both of maPEG-PLL and lipofectamine. Excess cationic molecules induce the overstabilization of the

ternary complexes, and the overstabilization might inhibit the transcription process as suggested from a decrease in cell-free gene expression (Fig. 5). On the contrary, highly condensation was not induced for F1L2 ternary complexes and F1L2 ternary complexes exhibit high transfection efficiency compared with lipofectamine lipoplexes.

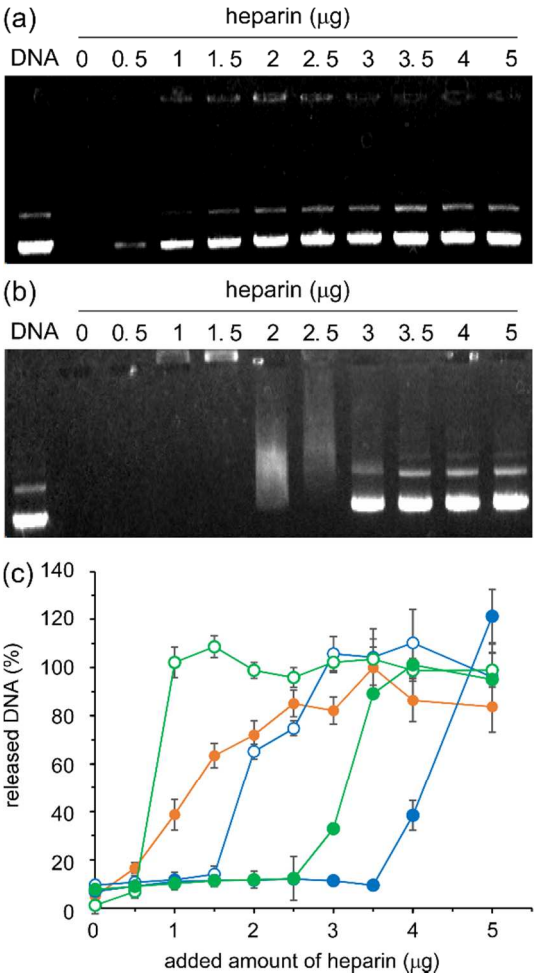

Fig. 6. Stability of the ternary complexes and the lipoplexes determined by polyanion competition assay using gel electrophoresis. Gel electrophoretic image of lipoplexes (a) and F1L2 ternary complexes (b) mixed with varying amounts of heparin. (c) Change in the amounts of released pDNA with an increase in the amount of heparin added (F1L2, green closed symbols; F1L0, green open symbols; F3L2, blue closed symbols; F3L0, blue open symbols; lipoplexes, orange symbols). Data presented as the average of three experiments ± SD.

Conclusions

In this study, ternary complexes were prepared by mixing nanofiber-polyplexes, which are formed by pDNA and maPEG-PLL bearing large maPEG, in addition to lipofectamine for effective gene delivery. Ternary complex formation induces the compaction of nanofiber-polyplexes to a spherical morphology with a narrow size distribution and with a ca. 100 nm hydrodynamic diameter. These compact ternary complexes entered HeLa cells but the nanofiber-polyplexes showed almost no cellular uptake because of their elongated morphology. Additionally, the ternary complexes were surrounded by electrically neutral PEG chains. Non-specific interactions between the ternary complexes and the cellular surface were effectively inhibited by the effect of the surrounding PEG chains. Furthermore, the ternary complexes prepared using an optimal mixing ratio maintained a relatively smooth transcription / translation process leading to nanofiber-polyplexes with good properties. The ternary complexes prepared by the compaction of the nanofiber-polyplexes and upon mixing with lipofectamine exhibited high transfection efficiency toward HeLa cells compared with lipofectamine lipoplexes.

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Notes and references

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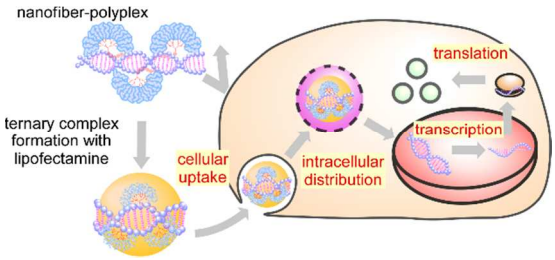

The compaction of the nanofiber-polyplexes by mixing with cationic lipofectamine can improve cellular uptake and helps the ternary complex to retain its smooth transcription / translation process, and ternary complexes exhibit a high transfection efficiency.

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