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Post-nuclear gene delivery events for transgene expression by biocleavable polyrotaxanes

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ABSTRACT

A quantitative comparison between nuclear DNA release from carriers and their transfection activity would be highly useful for improving the effectiveness of non-viral gene vectors. We previously reported that, for condensed DNA particles, a close relationship exists between the efficiency of DNA release and transfection activity, when biocleavable polyrotaxanes (DMAE-SS-PRX), in which the cationic density can be easily controlled. In this study, we first investigated the efficiencies of DNA release from condensed DNA particles with various types of DMAE-SS-PRX. The findings indicate that an optimal cationic density in DMAE-SS-PRX exists for DNA release. We then packaged condensed DNA particles in a multifunctional envelope-type nano device (MEND), and evaluated their transfection activities. The results showed that the transfection activity was increased and this increase was, to some extent, dependent on the efficiency of the DNA release. However, transfection activity decreased, when the value for the efficiency of DNA release was higher than a certain value. An investigation of the fate of intranuclear DNA indicated that a very high efficiency of DNA release has a positive influence on transcription, however, it would inhibit the post-transcription process; nuclear mRNA export, translation and related processes. Such information provides a new viewpoint for the development of cationic polymer-based vectors.

1. Introduction

Transgene expression has long been proposed as an innovative therapeutic strategy for directly treating diseases at the level of genes, and considerable efforts have been made to improve the efficiency of protein production [1-7]. To successfully achieve this, an efficient and targeted gene delivery system is necessary. Non-viral vectors would be useful gene delivery systems, especially from the viewpoint of safety, although they have a very lower transfection activity compared to viral vectors. A previous comparison revealed a surprising difference in transfection activity between viral and non-viral vectors: this difference is largely due to the intranuclear disposition of DNA rather than its delivery to the nucleus [8, 9]. The concept of intranuclear disposition assumes the following processes: DNA release from a vector, transcription to mRNA and translation to proteins (Fig. 1A). This led us to the hypothesis that DNA release from a vector in the nucleus represents a critical and dominant event in transfection activity.

To date, we reported on the development of an innovative gene delivery system [10, 11], prepared by integrating a multifunctional envelope-type nano device (MEND) [5, 12-14] and a biocleavable polyrotaxane (DMAE-SS-PRX) [15-17] (Fig. 1B). The MEND consists of plasmid DNA (pDNA) particles condensed with a polycation, with the outer envelopes coated with a lipid, which mimics envelope-type viruses [5, 12-14]. An advanced MEND system modified with a cell-penetrating peptide, octaarginine (R8), (R8-MEND) showed a high transfection activity comparable to that of reported potent viral vectors [13, 14]. On the other hand, DMAE-SS-PRX is an artificial DNA condenser [15-17], which can condense pDNA and then release it under intracellular reductive conditions. We concluded that DMAE-SS-PRX would be an appropriate model polycation for investigating the relationship between the DNA release in nucleus and transfection activity, because the cationic density in a DMAE-SS-PRX-molecule can be easily controlled, thus permitting the efficiency of DNA release to be adjusted [16].

We previously demonstrated the existence of a close relationship between the efficiency of DNA release and the transfection activity, using an integrated system of R8-MEND and DMAE-SS-PRX [10, 11]. It is particularly noteworthy that, from the standpoint of transfection, our integration system, which is comprised of the R8-MEND with DMAE-SS-PRX, was 5-fold greater than that for a conventional R8-MEND with protamine, which is a natural DNA condenser that shows a high transfection activity [18-20]. In vitro DNA release experiments showed that pDNA was efficiently released from the condensed pDNA particles, when DMAE-SS-PRX with a high cationic density

was used [11]. Moreover, we provide evidence to show that cationic density in DMAE-SS-PRX clearly has a positive influence on the efficiency of DNA release in the nucleus in living cells, resulting in high gene expression, as evidenced by imaging the nuclear condensation/decondensation status of pDNA using a new technology [10]

Based on the previous results, we hypothesized that a high cationic density in DMAE-SS-PRX would aid nuclear DNA release, resulting in strong transgene expression. In this study, we first screened the optimal DMAE-SS-PRX for nuclear DNA release. We next investigated the relationship between the efficiency of DNA release and transfection activity, and the results showed that the transfection activity was proportional to DNA release to some extent. Unexpectedly, transfection activity was decreased when the value for efficiency of DNA release was higher than a certain value. To clearly understand the intranuclear fate of condensed pDNA particles until gene expression could be achieved, we carried out a detailed investigation of the transgene process before and after the nuclear delivery of pDNA.

2. Materials and methods

2.1. Materials

pDNA encoding enhanced green fluorescent protein and luciferase protein (pEGFPLuc) was obtained from BD Bioscience Clontech (Palo Alto, CA, USA). pDNA was purified using a Qiagen EndoFree Plasmid Mega Kit (Qiagen GmbH, Hilden, Germany). Phosphatidic acid (PA) was purchased from Sigma (St. Louis, MO, USA). 1,2-Dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) was purchased from AVANTI Polar Lipids Inc. (Alabaster, AL, USA). Stearyl octaarginine (STR-R8) was obtained from KURABO INDUSTRIES LTD (Osaka, Japan). NIH3T3 mouse embryonic fibroblasts were obtained from the American Type Culture Collection (Manassas, VA, USA). Dulbecco's modified Eagle medium (DMEM) and fetal bovine serum (FBS) were purchased from Invitrogen Corp. (Carlsbad, CA, USA). All other chemicals were commercially available reagent-grade products.

2.2. Synthesis of DMAE-SS-PRX

We prepared various biocleavable polyrotaxane (DMAE-SS-PRX) preparations with different numbers of cations (Table 1), in which 18 or 29 molecules of cationic cyclodextrin (dimethylaminoethyl-modified α-CD, DMAE-α-CD) are threaded onto a poly(ethylene glycol) (PEG) chain capped with N-benzyloxycarbonyl L-tyrosine (Z-L-Tyr) via disulfide linkages, as previously reported [16]. The number of DMAE groups in a DMAE-SS-PRX molecule was controlled to be 17, 48, 52, 76, 99, 110 and

180, and are denoted as 17DMAE, 48DMAE, 52DMAE, 76DMAE, 99DMAE, 110DMAE and 180DMAE respectively. These DMAE-SS-PRXs have unique cationic densities.

2.3. Preparation of condensed pDNA particles

Condensed pDNA particles were prepared using polycations i.e., 17DMAE, 48DMAE, 52DMAE, 76DMAE, 99DMAE, 110DMAE or 180DMAE, in 10 mM HEPES buffer (pH 7.4). To condense the pDNA with polycations, a solution of pDNA (0.1 mg/mL) was added to a polycation solution were mixed under vortex at room temperature. Condensed pDNA particles were prepared using a nitrogen/phosphate (N/P) ratio of 5.

2.4. Evaluation of DNA-release efficiency from condensed pDNA particles

To evaluate the efficiency of DNA release, condensed pDNA particles were subjected to agarose gel electrophoresis before and after treatment with both a reducing agent, dithiothreitol (DTT), and polyanion, poly(L-aspartic acid) (pAsp). Prior to electrophoresis, the samples were incubated with 10 mM DTT for 1 hr at 37ºC to cleave the disulfide linkages in the DMAE-SS-PRX. After incubation, the resulting solutions were treated with a solution containing 1 mg/mL of pAsp for 20 min at room temperature to release the pDNA from the condensed pDNA particles. A 0.1-µg sample of pDNA was subjected to electrophoresis on a 1% agarose gel in TAE (40 mM Tris-HCl, 40 mM acetic acid, 1 mM EDTA, pH 8.0) at 100 V. The gel was stained with ethidium bromide. We observed the open circular form of pDNA (O.C.) and the supercoiled form of pDNA (S.C.), which is the major contributor to gene expression, as shown in Figure 2A. The intensity of the supercoiled pDNA band was determined using the Image J v.1.40 software (National Institutes of Health, USA). Efficiency of DNA release was calculated as follows:

Efficiency of DNA release (%) = (intensity of the supercoiled form of the pDNA band in samples treated with DTT and pAsp / intensity of the supercoiled form of the pDNA band in naked pDNA) ×100.

2.5. Preparation of R8-MEND

The R8-MEND was prepared by the lipid film hydration method, as previously reported [12]. First, condensed pDNA particles were prepared with DMAE-SS-PRX at an N/P ratio of 5. Then, 0.25 mL of the condensed pDNA particle solution was added to the lipid film, which was formed by the evaporation of a chloroform solution of 137.5

nmol lipid (DOPE/PA = 7 : 2 (molar ratio)), on the bottom of a glass tube, followed by a 15-min incubation to hydrate the lipids. The glass tube was sonicated to produce the MEND in a bath-type sonicator (85 W; Aiwa Co., Tokyo, Japan). To attach R8 to the surface of the carrier, a solution of STR-R8 (20 mol% lipids) was incubated with the MEND for 10 min at room temperature to produce R8-MEND.

2.6. Measurement of diameter and ζ potential

Particle diameter was measured using a quasi-elastic light scattering method and the ζ potential was determined electrophoretically using laser doppler velocimetry (Zetasizer Nano ZS; Malvern Instruments, Herrenberg, Germany).

2.7. Evaluation of transfection activity by measuring luciferase activity.

Transfection activity was assessed by measurement of luciferase activity described below. NIH3T3 cells (4 X 10^4 cells/well) were incubated in 24 well plate (Corning, NY) with DMEM containing 10% FBS, under 5% CO_2/air at 37ºC for 24 hr. Samples containing 0.5 µg of pDNA suspended in 0.25 mL of serum-free DMEM were added to the cells, followed by incubation under 5% CO_2 at 37ºC for 3 hr. Then, 1 mL of fresh DMEM containing 10% serum was added to the cells, followed by incubation for 21 hr. The cells were washed with phosphate-buffer saline (PBS (-)), and luciferase activity (relative light unit (RLU)) was measured using a Luciferase Assay System with a Reporter Lysis Buffer kit (Promega; Madison, WI, USA) by means of a luminometer (Luminescencer-PSN; ATTO, Japan). Cellular protein content was determined using a BCA protein assay kit (PIERCE; Rockford, IL, USA). Transfection activity was calculated as follows:

Transfection activity (RLU/mg protein) = Luciferase activity / Cellular protein content.

2.8. Quantification of nuclear pDNA and mRNA production after transfection using R8-MEND

One day before the transfection, NIH3T3 cells (2 X 10^5 cells/well) were seeded on a 6-well plate (Corning, NY, USA) in DMEM containing 10% FBS under 5% CO_2/air at 37ºC. Samples containing 2.5 µg of pDNA suspended in 1 mL of serum-free DMEM were added to the cells, followed by incubation under 5% CO_2 at 37ºC for 3 hr. Then, 1 mL of fresh DMEM containing 10% serum was added to the cells, followed by incubation for 21 hr. The cell surface-bound carriers were removed by washing twice with 1 mL PBS (-) supplemented with heparin (20 units/mL), the cells were then

collected by trypsinization.

To quantify the nuclear pDNA, the fraction was further purified as previously described [21, 22]. The collected cells were further washed twice with PBS (-) supplemented with heparin (20 units/mL), and incubated with 250 μL of CellScrubBuffer (Gene Therapy Systems Inc., San Diego, CA, USA) for 30 min on ice. The suspension was centrifuged at 9,200g for 2 min at 4°C, and the supernatant removed. The collected cells were suspended in 375 μL of CellScrubBuffer, and 125 μL of cell lysis solution (2% IGEPAL CA630, 40 mM NaCl, 12 mM MgCl$_2$, and 40 mM Tris–HCl, pH 7.4) was then added. The suspension was centrifuged at 9,200g for 2 min at 4°C, and the supernatant removed. This operation was repeated 3 times, and the obtained pellet was used as the nuclear fraction. pDNA in the nuclear fraction was extracted using a GenElute Mammalian Genomic DNA Miniprep Kit (Sigma) according to the manufacture's protocol. During this operation, it was treated with both a reducing agent (DTT) and a polyanion (pAsp) to sufficiently dissociate pDNA from DMAE-SS-PRX, as described above. The collected pDNA was subjected to the SYBR Green real-time PCR with ABI Prism 7700 sequence detection system (Life Technologies Corporation; Carlsbad, CA USA). The reaction mixture consisted of 5 μL of diluted sample DNA solution, 5 pmol of two types of primers (Luc (+): GGTCCTATGATTATGTCCGGTTATG and Luc (-): ATGTAGCCATCCATCCTTGTCAAT, or b-actin (+): AGAGGGAAATCGTGCGTGAC and b-actin (-): CAATAGTGATGACCTGGCCGT) and 12.5 μL of SYBR Green Real time PCR Master Mix (Toyobo, Osaka, Japan). The DNA was denaturized at 95°C for 15 sec, and annealing/extension was performed at 60°C for 1 min. The denaturation/annealing cycle was repeated 40 times. The amount of pDNA was normalized by the number of nuclei quantified by the copy number of b-actin DNA.

For the quantification of luciferase-encoding mRNA, total RNA was extracted from the collected cells using a GenElute Mammalian Total RNA Kit (Sigma) according to the manufacture's protocol, combined with DNase I digestion for the degradation of genomic DNA in total RNA samples. First-strand cDNA was synthesized from 500 ng of total RNA by using a PrimeScript RT reagent Kit and an oligo dT primer (Takara, Kusatsu, Japan), as described manufacture's protocol. Each of the mRNA transcripts was subjected to real-time PCR with an ABI 7500 real-time system using Luc (+), Luc (-), b-actin (+) and b-actin (-) primers, as described above. The amount of luciferase-encoding mRNA was normalized by the number of nuclei quantified by the copy number of b-actin DNA. In this experiment, the relative amounts of mRNA were

calculated from the amount of mRNA after transfection with R8-MEND containing condensed pDNA particles with 48DMAE as a standard.

3. Results

3.1. Evaluation for efficiency of DNA release from condensed pDNA particles

  The efficiency of DNA release from condensed pDNA particles was investigated using various types of DMAE-SS-PRXs with different numbers of cationic DMAE groups (Table 1). It is generally accepted that the number of amino groups in a cationic polymer are closely related to polyplex formation, polyplex stability against a counter polyanion, transfection activity and etc. [23, 24]. We previously confirmed that pDNA particles condensed with DMAE-SS-PRX efficiently released DNA and showed a high transfection activity, when the positive particles were formed at a nitrogen/phosphate (N/P) ratio of 5 [11]. In this study, we prepared condensed pDNA nanoparticles at an N/P ratio of 5, and their diameters and ζ potentials were measured (Table 2). In each experiment, positively charged nanoparticles were allowed to form.

  To determine the efficiency of DNA release, condensed pDNA particles were subjected to gel electrophoresis before and after treatment with both DTT as a reducing agent and a counter polyanion (pAsp) (Fig. 2A). We hypothesized that cationic DMAE-α-CD molecules threaded onto a poly(ethylene glycol) (PEG) chain that had been capped via disulfide linkages could be dethreaded through a supermolecular dissociation triggered by the cleavage of the disulfide linkages, thus leading to the release of pDNA. In this experiment, we determined that pDNA was completely released from nanoparticle, when the fluorescent band was detected at the same location where naked pDNA is detected (Fig. 2A, lanes 1). In the absence of pAsp and DTT, we were unable to observe DNA release in any types of DMAE-SS-PRXs (Fig. 2A (a)), and the results suggest that condensed pDNA nanoparticles can be formed. On the other hand, we detected the DNA released from condensed pDNA particles in the presence of pAsp and DTT, although the efficiencies of DNA release were different in each types of DMAE-SS-PRX (Fig. 2A (b)). We also observed different population of open circular form (O.C.) and supercoiled form (S.C.), when pDNA was released from condensed pDNA particles.

  In these experiments, DNA-release efficiency was defined based on the intensity of the band corresponding to the released supercoiled pDNA, as described in the Materials and methods section (Fig. 2B). This graph shows the relationship between the number of DMAE per α-CD in DMAE-SS-PRX as a cationic density parameter

(x-axis) and the efficiency of DNA release (y-axis). The results show that the increase in the efficiency of DNA release was dependent on the cationic density in DMAE-SS-PRX to some extent. We also observed that the value was decreased when the cationic number in DMAE-SS-PRX was higher than a certain value (number of DMAE/α-CD = 4).

3.2. Evaluation for transfection activity of R8-MEND

We evaluated the transfection activity of an R8-MEND containing condensed pDNA particles with various DMAE-SS-PRXs. The condensed pDNA particles were covered with a lipid bilayer using the hydration method [12], providing the desired R8-MENDs, which were positively charged nanoparticles without aggregation (around 150-300 nm). The observed diameters and ζ potentials of R8-MENDs are summarized in Table 3. Transfection activity was assessed by measuring luciferase activity at 24-hr after transfection in NIH3T3 cells. The values of the transfection activities are summarized in Figure S1. Figure 3 shows the relationship between efficiency of DNA release (x-axis) and transfection activity (y-axis). Based on previous report that there would be a positive correlation between the efficiency of DNA release and transfection activity [11], it was expected that DMAE-SS-PRX with a high efficiency for DNA release would result in the production of a strong gene expression. The results in Figure 3 showed that the transfection activity was proportional to the efficiency of the DNA release to some extent. However, the transfection activity was decreased when the value for efficiency of DNA release was higher than a certain value. The result indicated that an adequate efficiency of nuclear DNA release is required in order to achieve an effective level of transgene expression.

3.3. Quantification of the amount of nuclear pDNA

In this study, we encapsulated condensed pDNA particles with various DMAE-SS-PRXs in an R8-MEND, a vehicle for nuclear targeting, using a similar packaging method. Therefore, it would be expected that the amounts of nuclear pDNA delivered were similar to the values for any of the DMAE-SS-PRXs. To validate this assumption, we quantified the amounts of pDNA in the nuclear fraction after transfection (Fig. 4). After the transfection, nuclear pDNA was collected and subjected to real time PCR. No significant differences were found in the amounts of nuclear pDNA among the condensed pDNA particles with any of the DMAE-SS-PRXs, suggesting that their transfection activities are not largely influenced by the nuclear delivery of pDNA.

3.4. Evaluation for transcription efficiency of condensed pDNA particles and comparison with their efficiencies of DNA release

To investigate how gene expression after nuclear pDNA delivery was affected, we evaluated the relationship between the DNA release from condensed pDNA particles and their transcription efficiencies. The transcription efficiency of condensed pDNA particles was estimated as described in the Materials and methods section. Cellular mRNA was collected and subjected to reverse transcription real-time PCR to quantitatively determine the mRNA (Fig. S2). The relationship between the efficiency of DNA release and their transcription efficiencies is summarized in Figure 5. The results show that the transcription efficiency is proportional to the efficiency of DNA release. Collectively, these findings indicate that the strong gene expression involved with the increase in DNA release is influenced by the transcription process. Whereas, a higher DNA release above a certain value might inhibit gene expression (Figure 3), although the transcription efficiency was high in this case.

4. Discussion

Maintaining the supercoiled form of pDNA when condensed pDNA nanoparticles are designed is a major issue in this field of study. Since it is known that free pDNA possesses some unique features; its supercoiled form is more efficient in gene expression than the linear and open circular forms, which can be damaged. Several studies showed that the supercoiled form of pDNA is susceptible to being converted into the linear or open circular form during microencapsulation, resulting in a significant reduction in gene expression [25, 26]. An evaluation of the efficiency of DNA-release showed that there were different amounts of open circular and supercoiled forms, when pDNA was released from condensed pDNA particles (Fig. 2A). Fortunately, the supercoiled form of pDNA was easily released from pDNA particles that had been condensed with DMAE-SS-PRX compared with open circular form of pDNA, although the mechanism for this is not clear. In this experiment, the efficiency of DNA release, as shown in Fig. 2B was defined based on the intensity of the band corresponding to the released supercoiled form of pDNA, which is the major contributor to gene expression.

Moreover, we indicated that an optimal cationic density of DMAE-SS-PRX should exist for DNA release (Fig. 2). The efficiency in DNA release was proportional, to some extent, to the cationic density in DMAE-SS-PRX. This tendency was also observed in a previous study, in which 14DMAE and 29DMAE were employed as an artificial condenser [11]. Additionally, we showed that the cationic density in

DMAE-SS-PRX has a positive influence on the efficiency of DNA release in the nucleus in living cells [10], suggesting that the conditions used in the in vitro experiment would reflect the condition of the nucleus. In this study, we used condensed pDNA particles at an N/P ratio of 5 in all of the DMAE-SS-PRXs. In this situation, condensed pDNA particles with high numbers of DMAE could be formed by small molar amounts of DMAE-SS-PRX. For example, in the case of 17DMAE, the condensed pDNA particle consists of 3765 DMAE-SS-PRX molecules per pDNA molecule; whereas, in the case of 48 DMAE, the particle consists of 1333 DMAE-SS-PRX molecules per pDNA molecule. Therefore, pDNA might be easily released from the nanoparticle with a high number of DMAE molecules, because the amounts of DMAE-SS-PRX bound to pDNA were low in the condensed pDNA nanoparticle. On the other hand, we observed that the value decreased, when the cationic density in DMAE-SS-PRX was higher than a certain value. When the condensed pDNA particles were formed by DMAE-SS-PRX with too high a number of DMAE molecules, a high cationic density in DMAE- SS-PRX might result in pDNA being strongly bound in the nanoparticle, thus decreasing the efficiency of DNA release. Based on these results, we concluded that the optimal cationic density in DMAE-SS-PRX for DNA release is dominated by a delicate balance between the molar amounts of DMAE-SS-PRX in the nanoparticle and the cationic density of the DMAE-SS-PRX.

We expected that DMAE-SS-PRX with a high efficiency of DNA release would show strong gene expression; however, the DNA-release efficiency was not completely correlated with the transfection activity (Fig. 3). This result was supported by a previous report that the correlation was poor in a scattered plot of mRNA expression versus protein production in individual cells [27]. The finding that a too high efficiency in nuclear DNA release inhibited the transfection activity (Fig. 3) led us to conclude that some factor might disturb the post transcription process; transcription, nuclear mRNA export, translation and related processes. Such an assumption was verified in cases where the amount of nuclear pDNA was similar in all types of DMAE-SS-PRX used. Therefore, we quantitatively determined the amounts of nuclear pDNA when R8-MENDs containing various DMAE-SS-PRXs were used (Fig. 4). The result showed that the condensed pDNA particles were delivered to the nucleus with a similar efficiency in any of the types of DMAE-SS-PRXs used. We next investigated the transcription efficiency after transfection, an important process in post nuclear delivery. We compared the efficiency of DNA release and transcription efficiency (Fig. 5). As a result, we found a positive correlation between the efficiency of DNA release

and transcription efficiency. These collective data indicate that a highly efficient release of nuclear DNA would negatively influence the post transcription process.

We focused the free cations released from the condensed pDNA particles as one of the reason for the inhibition of transgene expression. One possibility is that the free cations might bind to the mRNA transcribed from the pDNA, and this would inhibit nuclear mRNA export and the translation process. The cations might also depress the expression of protein related post transcription process, resulting in low transgene expression. Another possibility is that the cations might influence the quality of mRNA, when pDNA bound to cations was transcribed. In such situations, the levels of free cations were increased depending on the increase in DNA release efficiency, resulting in the translational depression of mRNA. Therefore, a too high efficiency in DNA release might negatively contribute to gene expression. On the other hand, a moderate efficiency in DNA release would decrease the amounts of free cations, resulting in low levels of interactions between free cations and mRNA, nuclear protein, pDNA and etc. In the case of a low efficiency of DNA release, small amounts of mRNA would be transcribed because the levels of released pDNA are low, although the amounts of free cations might be low. Collectively, strong gene expression using cationic polymer-based gene vector would require a balance between DNA release efficiency and the amounts of free cations.

5. Conclusion

In this study, we demonstrated a close relationship between the efficiency of DNA release and transcription efficiency. We also found that when the efficiency of DNA release from condensed pDNA particles is too high, this constitutes a negative contribution to transfection activity. Based on these results and our conclusions, it was presumed that a large amount of free cations released from the condensed pDNA particles might inhibit the post-transcription process. This finding provides a new viewpoint that cationic polymer-based gene vectors require efficient DNA release and a depression in free cation levels for strong transgene expression to be achieved.

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Figure legends

Figure 1. Conceptual diagram of intracellular fate of pDNA with gene vectors (A) and schematic representation of integrated system of R8-MEND with DMAE-SS-PRX (B).

Figure 2. Data of DNA release from the condensed pDNA particles with various types of DMAE-SS-PRXs (A). The samples, before (a) and after (b) treatment by DTT (reducing agent) and pAsp (polyanion) to release pDNA, were subjected to agarose gel electrophoresis. Lane 1, naked pDNA; lanes 2—8, pDNA particles condensed with 17DMAE, 48DMAE, 52DMAE, 76DMAE, 99DMAE, 110DMAE and 180DMAE. OC, open circular form of pDNA; SC, supercoiled form of pDNA. Based on the band intensity corresponding to the released pDNA as shown in Fig.2A (b), efficiency of DNA release was calculated (B). Data are means ± S.E. (n = 7-10).

Figure 3. Relationship between efficiency of DNA release (x-axis) and transfection activity (y-axis). For x-axis, we used data regarding efficiency of DNA release shown in Fig.2B. For the y-axis, we evaluated transfection activity of R8-MEND containing each

condensed pDNA particles (Fig. S1). Data are represented by the mean ± S.E. (n = 7-11).

Figure 4. Quantification of nuclear pDNA delivery. R8-MEND containing pDNA condensed with various DMAE-SS-PRX was transfected to HeLa cells. After transfection, pDNA was purified from an isolated nucleus and subjected to the real-time PCR, as described in Materials and methods. Data are means ± S.E. (n = 3-4). No significant difference (N.S.) indicates the lack of a statistically significant difference (p > 0.05 by one-way ANOVA, followed by Bonferroni correction post hoc test).

Figure 5. Relationship between efficiency of DNA release (x-axis) and transcription efficiency (y-axis). For x-axis, we used data regarding efficiency of DNA release shown in Fig.2B. The data are represented by the mean ± S.E. (n = 3-10). For the y-axis, we calculated transcription efficiency as described in Materials and methods.
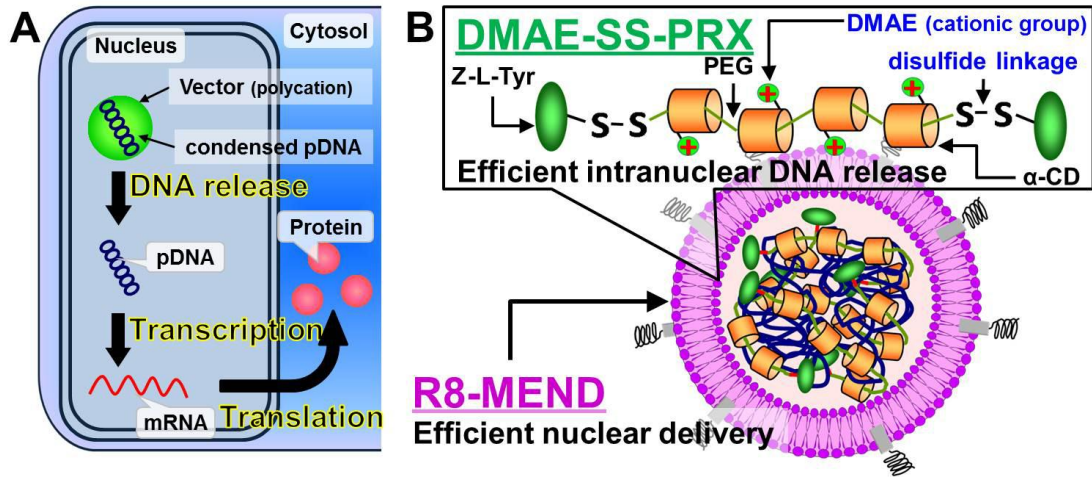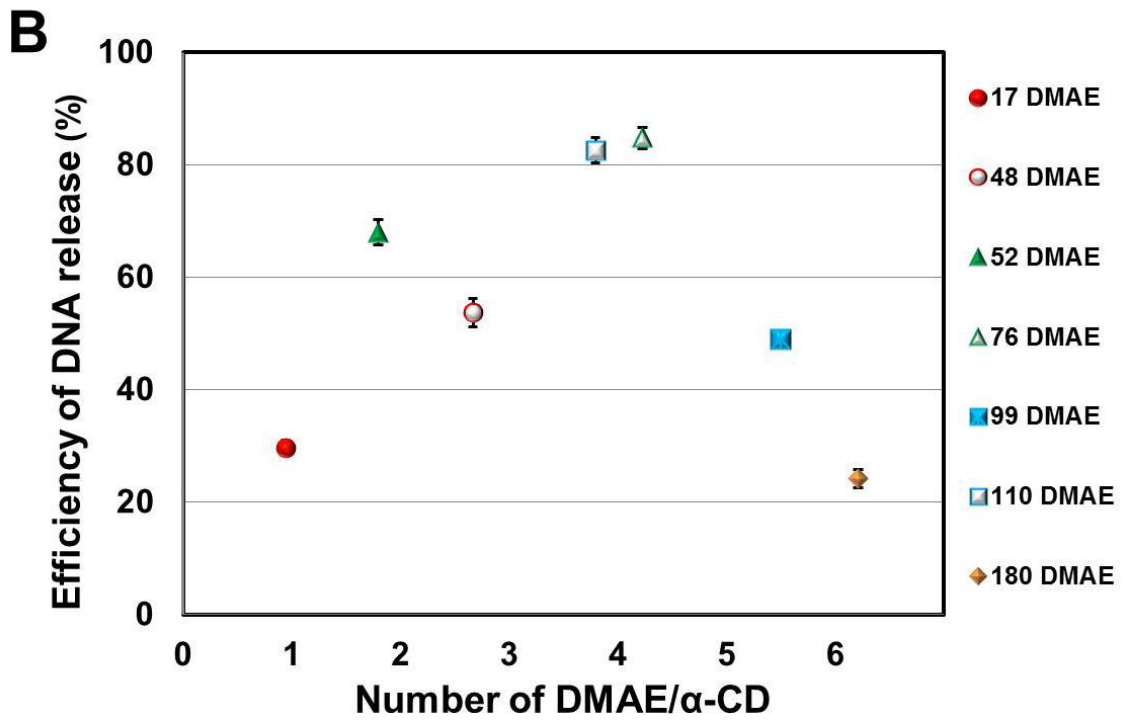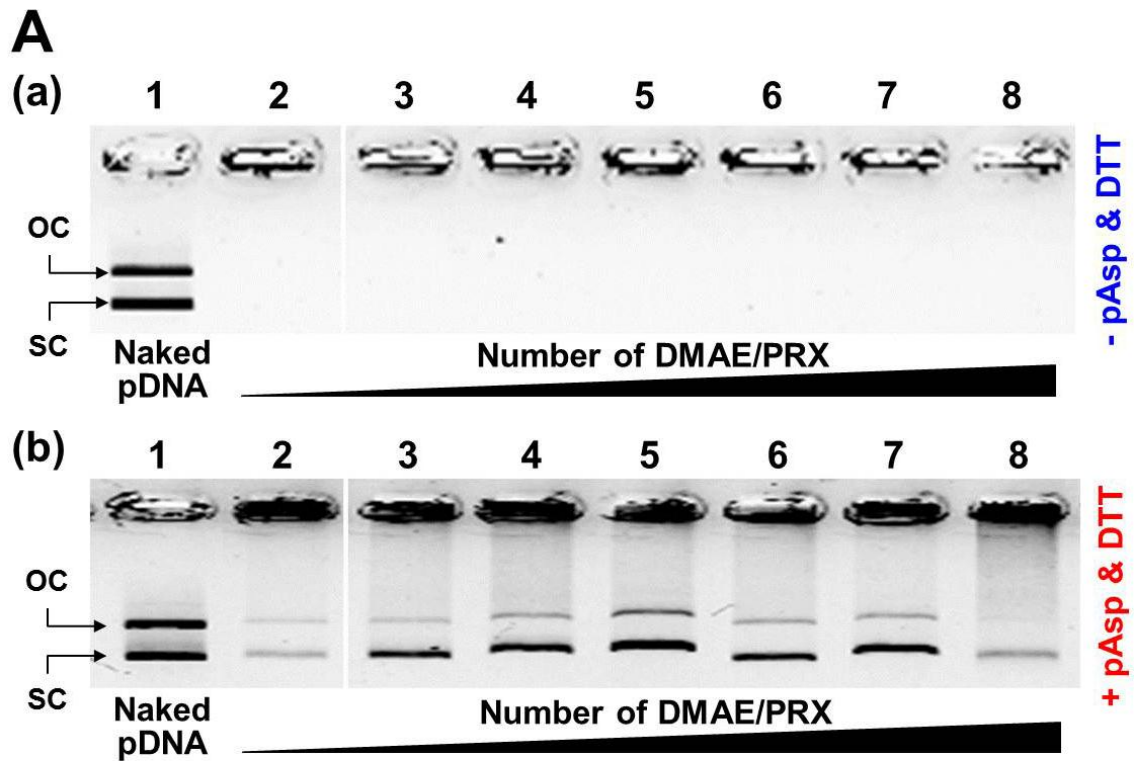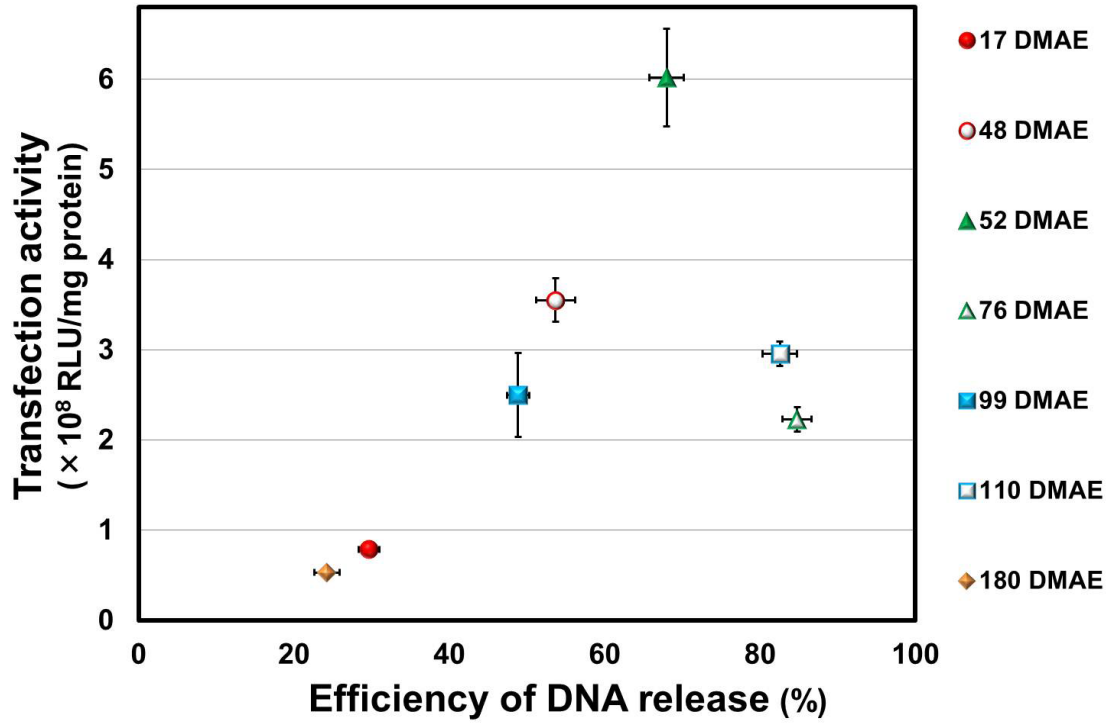
Fig. 1

A

Cytosol

Nucleus

Vector (polycation)

condensed pDNA

DNA release

pDNA

Protein

Transcription

mRNA   Translation

B **DMAE-SS-PRX**   DMAE (cationic group)

disulfide linkage

Z-L-Tyr   PEG

S-S   S-S

α-CD

Efficient intranuclear DNA release

R8-MEND
Efficient nuclear delivery

Fig. 2

A

(a) — pAsp & DTT

Lanes: 1 2 3 4 5 6 7 8

OC
SC

Naked pDNA

Number of DMAE/PRX

(b) + pAsp & DTT

Lanes: 1 2 3 4 5 6 7 8

OC
SC

Naked pDNA

Number of DMAE/PRX

B

Efficiency of DNA release (%) vs Number of DMAE/α-CD

- 17 DMAE
- 48 DMAE
- 52 DMAE
- 76 DMAE
- 99 DMAE
- 110 DMAE
- 180 DMAE

Fig. 3

Fig. 4

Fig. 5

- 17 DMAE
- 48 DMAE
- 52 DMAE
- 76 DMAE
- 99 DMAE
- 110 DMAE
- 180 DMAE

Table 1

Table 1   Characteristics of DMAE-SS-PRX

DMAE-SS-PRX	No. of CD[a]	No. of DMAE /CD[b]	No. of DMAE /PRX[c]
17DMAE	18	0.94	17
48DMAE	18	2.67	48
52DMAE	29	1.79	52
76DMAE	18	4.22	76
99DMAE	18	5.50	99
110DMAE	29	3.79	110
180DMAE	29	6.21	180

Mw of PEG = 3500-4500. [a], [b] and [c] represent the averaged threading number of an α-CDs in a DMAE-SS-PRX, the averaged number of DMAE groups in an α-CD and the number of DMAE groups in a DMAE-SS-PRX, respectively.

Table 2

Table 2   Characteristics of pDNA particles condensed with various DMAE-SS-PRX

DMAE-SS-PRX	Diameter (nm)	ζ Potential (mV)
17DMAE	182 ± 1.0	18 ± 0.3
48DMAE	122 ± 1.7	21 ± 0.3
52DMAE	107 ± 0.8	26 ± 0.3
76DMAE	95 ± 1.0	23 ± 0.9
99DMAE	90 ± 0.6	24 ± 0.3
110DMAE	83 ± 0.3	22 ± 0.4
180DMAE	78 ± 0.4	22 ± 0.5

Data are represented by the mean ± S.E. (n = 9-37)

Table 3

Table 3   Characteristics of R8-MEND containing pDNA particles condensed with various DMAE-SS-PRX

| DMAE-SS-PRX	Diameter (nm)	ζ Potential (mV)
17DMAE	280 ± 3.8	33 ± 11
48DMAE	248 ± 3.3	45 ± 21
52DMAE	321 ± 4.3	52 ± 14
76DMAE	165 ± 1.2	51 ± 19
99DMAE	143 ± 17	56 ± 15
110DMAE	220 ± 43	50 ± 20
180DMAE	168 ± 40	45 ± 26

Data are represented by the mean ± S.E. (n = 13-23)

Evaluation for transfection activity of R8-MEND (Figure S1)

R8-MEND containing pDNA particles condensed with various DMAE-SS-PRXs was prepared, and their transfection activities were evaluated by a luciferase assay. The transfection activities are summarized in Figure S1.

**Figure S1.** Transfection activities of R8-MEND by luciferase assay.
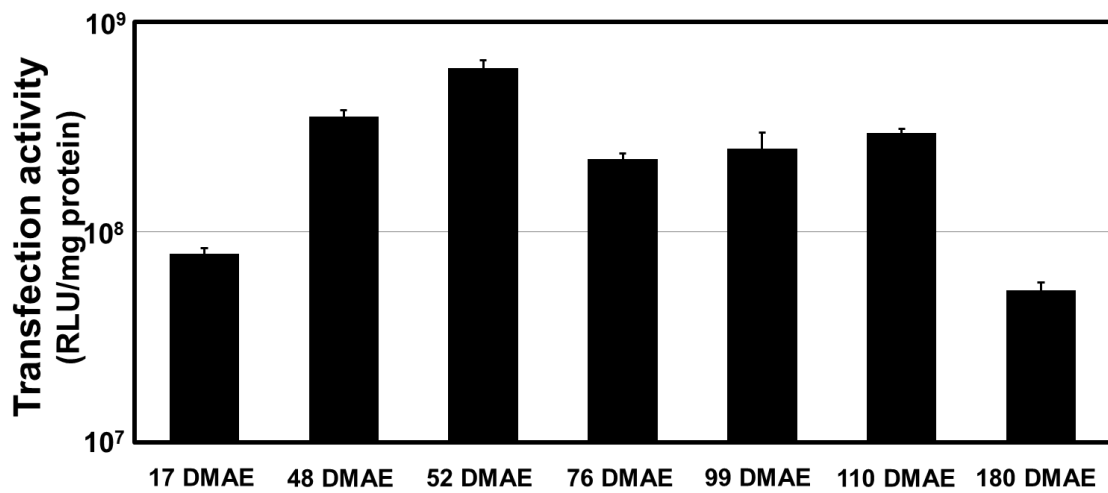
Transfection activities of R8-MEND were evaluated by means of a luciferase assay.

Data are means ± S.E. (n = 11-17).

Quantification of cellular mRNA after transfection by R8-MEND (Figure S2)

After transfection by an R8-MEND containing pDNA particles condensed with various DMAE-SS-PRX, we quantified the cellular mRNA transcribed from the pDNA using reverse transcription real-time PCR. Cellular mRNA was measured in several independent experiments and these data were normalized by the amount of cellular mRNA in R8-MEND with 48 DMAE (relative value of cellular mRNA). The transcription efficiency was then calculated as follows: Transcription efficiency = relative value of cellular mRNA / amount of nuclear pDNA. The values shown in Figure S1 were used as the amount of nuclear pDNA.

Figure S2. Quantification of cellular mRNA by reverse transcription real-time PCR.
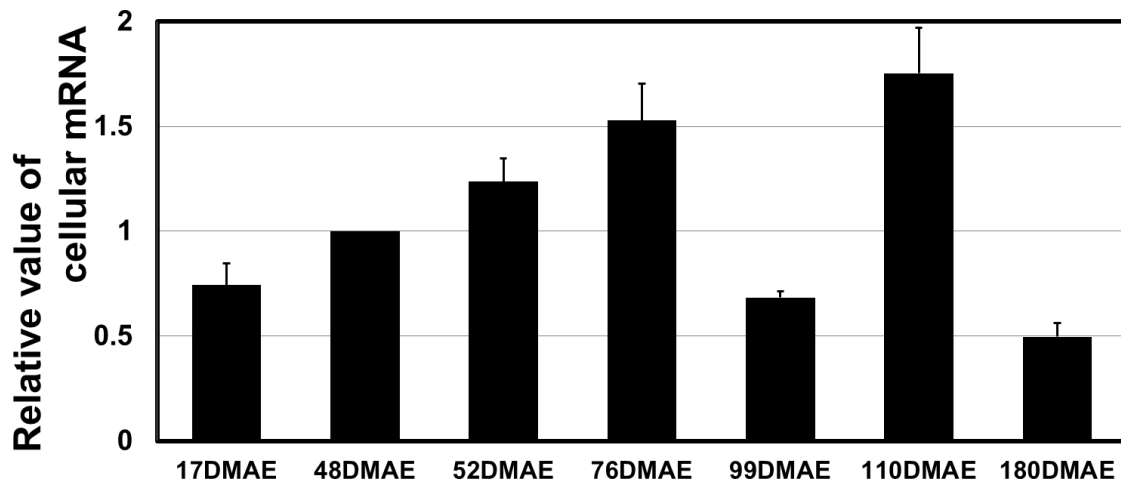
After transfection, cellular mRNA was quantified by reverse transcription real-time PCR. The relative values of cellular mRNA were estimated. Data are means ± S.E. (n = 3-4).