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Intranuclear DNA Release Is a Determinant of Transfection Activity for a Non-viral Vector: Biocleavable Polyrotaxane as a Supramolecularly Dissociative Condenser for Efficient Intranuclear DNA Release

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It has been believed that nuclear gene delivery is the most important process for gene expression, and various non-viral vectors are currently being developed with this assumption. However, some of our earlier studies revealed a surprising difference in transfection activity between viral and non-viral vectors: this difference is largely due to the result of the intranuclear disposition of DNA rather than its delivery to the nucleus (Hama S. *et al.* (2006), Quantitative comparison of intracellular trafficking and nuclear transcription between adenoviral and lipoplex systems. *Mol. Ther.*, 13, 786–794). Here, we report on some direct evidence that demonstrates the importance of the release of intranuclear DNA on transfection activity. The data show that transfection activity can be substantially enhanced by integrating a multifunctional envelope-type nano device (MEND) and a biocleavable polyrotaxane (DMAE-SS-PRX) as an artificial condenser. Our integration system showed significantly higher transfection activity compared to conventional gene delivery system. Moreover, this system provides a strong support for our hypothesis that intranuclear DNA disposition plays a critical role in gene expression for non-viral vectors.

Key words biocleavable polyrotaxane; multifunctional envelope-type nano device; liposome; intranuclear DNA release; nanoparticle; drug delivery system

It is generally thought that nuclear gene delivery is the most important process for gene expression. As a result, various non-viral vectors are currently being developed with this assumption in mind.^{2–5)} We have reported on the development of a non-viral gene delivery system, a multifunctional envelope-type nano device (MEND).^{6–10)} The device consists of plasmid DNA (pDNA) particles condensed with a polycation and outer lipid envelopes that coat the particles, which mimic envelope-type viruses, as shown in Fig. 1A, which aid in regulating various phases of intracellular trafficking, including cellular-uptake, endosomal escape and nuclear transfer. An advanced MEND system modified with a cell-penetrating peptide, octaarginine (R8), (R8-MEND) showed a high transfection activity comparable to that of potent viral vectors.⁷⁾ The R8-MEND stimulates macropinocytosis (clathrin-independent endocytosis) and is efficiently taken up by cells,⁸⁾ then introducing condensed pDNA particles into the intranuclear compartment. Finally, pDNA is released from the polycation in the nucleus, leading to gene expression (Fig. 1A). On the other hand, some of our earlier studies that involve the quantitative analysis of the intracellular trafficking of pDNA revealed a surprising difference in transfection activity between the viral and non-viral vectors. This difference is largely the result of the intranuclear disposition of pDNA rather than its delivery to the nucleus.^{11,12)} The intranuclear disposition assumes the following processes, DNA release from a vector, transcription to mRNA and translation to proteins (Fig. 1B). This led us to the hypothesis that DNA release from a vector in the nucleus represents a critical and dominant event in transfection activity. In this context, we employed biocleavable polyrotaxanes (DMAE-SS-PRX) as an artificial DNA condenser,^{13–15)} which can condense pDNA and then release it under intracel-

lular reductive condition. It is anticipated that cationic cyclodextrin (dimethylaminoethyl-modified α -CD, DMAE- α -CD) molecules threaded onto a poly(ethylene glycol) (PEG) chain capped *via* disulfide linkages (Fig. 1C) can be de-threaded through a supramolecular dissociation triggered by cleavage of the disulfide linkage(s), thus leading to the release of pDNA. In this report, we propose an innovative gene delivery system, prepared by integrating R8-MEND^{6–10)} and DMAE-SS-PRX^{13–15)} for improved intranuclear DNA disposition (Fig. 2), and provide some evidence to verify this hypothesis that the release of intranuclear DNA from condensed pDNA particles using a polycation is a determinant process for efficient transfection using a non-viral vector.

MATERIALS AND METHODS

Materials pDNA encoding enhanced green fluorescent protein and luciferase protein (pEGFP-Luc) was obtained from BD Bioscience Clontech (Palo Alto, CA, U.S.A.). pDNA was purified using a Qiagen EndoFree Plasmid Mega Kit (Qiagen GmbH, Hilden, Germany). Phosphatidic acid (PA) was purchased from Sigma (St. Louis, MO, U.S.A.). 1,2-Dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) was purchased from AVANTI Polar Lipids Inc. (Alabaster, AL, U.S.A.). Protamine was purchased from CALBIO CHEM (Darmstadt, Germany). 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, U.S.A.). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Invitrogen Corp. (Carlsbad, CA, U.S.A.). All other chemicals were commercially available reagent-grade products.

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Fig. 1. Schematic Image of Gene Delivery by R8-MEND (A), Intracellular Fate of pDNA with Gene Vectors (B) and Structure of DMAE-SS-PRX and Image of DNA Release from Condensed pDNA Particle (C)

Fig. 2. Schematic Representation of Integrated System of R8-MEND with DMAE-SS-PRX

Fig. 3. Relationship between N/P Ratio and Diameter or ζ Potential of pDNA Particles Condensed with Polyrotaxanes

A pDNA solution was vortexed with 14DMAE (open circles) or 29DMAE (closed circles) at room temperature and the diameter (A) and ζ potential (B) were then measured. Data are means ± S.D. ($n=3-6$).

Fig. 4. Agarose Gel Electrophoresis Data of DNA Release from the Condensed pDNA Particles with Protamine, 14DMAE or 29DMAE (N/P Ratio=0.5 (A) or 5 (B)) and Efficiency of DNA Release of Condensed pDNA Particles Formed at an N/P Ratio of 0.5 (C) or 5 (D)

Efficiency of DNA release was calculated based on the intensity of released pDNA band, as described in Materials and Methods.

Fig. 5. Transfection Activity of R8-MEND Containing Condensed pDNA Particles Formed at an N/P Ratio of 0.5 (A) or 5 (B), Was Evaluated by Luciferase Assay

Data are means ± S.D. ($n=3$). * Significant difference ($p < 0.05$ by one-way ANOVA, followed by Bonferroni correction *post hoc* test). N.S., no significant difference. Relationship between efficiency of DNA release (x-axis) and transfection activity (y-axis) (C).

Synthesis of DMAE-SS-PRX We prepared a biocleavable polyrotaxane DMAE-SS-PRX, in which 18 mol of DMAE-α-CD are threaded onto a PEG chain capped with *N*-benzyloxycarbonyl L-tyrosine (Z-L-Tyr) via disulfide linkages, as previously reported.¹⁴ The number of DMAE groups in a polyrotaxane molecule was controlled to be 14 or 29, and are denoted as 14DMAE or 29DMAE, respectively.

Preparation of Condensed pDNA Particles Condensed pDNA particles were prepared using polycations *i.e.*, protamine, 14DMAE, or 29DMAE, in 10 mM *N*-(2-hydroxyethyl)-piperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer (pH 7.4). To condense the pDNA with polycations, solutions of pDNA (0.1 mg/ml) and a polycation solution were vortexed at room temperature. Condensed pDNA particles were prepared using a nitrogen/phosphate (N/P) ratio of 0.5 or 5. When the condensed pDNA particles were prepared at an N/P ratio of 5, the pDNA solution was added to the polycation solution. In contrast, condensed pDNA particles prepared using an N/P ratio of 0.5 were prepared by adding the polycation solution to the pDNA solution.

Validation of pDNA Condensation and Decondensation

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 and polyanion. Prior to electrophoresis, the samples were incubated for 1 h at 37 °C with the reducing agent, 10 mM dithiothreitol (DTT), to cleave the disulfide linkages in the DMAE-SS-PRX. After incubation, the resulting solutions were treated with a polyanion, *i.e.*, 1 mg/ml poly(L-aspartic acid) (pAsp), for 20 min at 25 °C to release the pDNA. A 0.1- μ g sample of pDNA was subjected to electrophoresis. Electrophoresis was performed on a 1% agarose gel in TAE (40 mM Tris-HCl, 40 mM acetic acid, 1 mM ethylenediaminetetraacetic acid (EDTA), pH 8.0) at 100 V for 20 min. The gel was stained with ethidium bromide (EtBr). The intensity of the supercoiled pDNA band was determined using the Image J v.1.40 software (National Institutes of Health, U.S.A.). Efficiency of DNA release was calculated as follows:

$$\text{efficiency of DNA release (\%)} = \left(\frac{\text{intensity of the pDNA band in samples treated with DTT and pAsp}}{\text{intensity of the pDNA band in naked pDNA}} \right) \times 100$$

Preparation of R8-MEND The R8-MEND was prepared using the lipid film hydration method, as previously reported.¹⁰ In a typical experiment, the pDNA solution was vortexed with either protamine, 14DMAE, or 29DMAE solution at an N/P ratio of 0.5 or 5 at room temperature to form pDNA condensed with protamine, 14DMAE or 29DMAE, respectively. In the case of condensed pDNA particles formed at an N/P ratio of 5, 0.25 ml of the condensed pDNA particle solution was added to the lipid film, which was formed by 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 30 min at room temperature to produce R8-MEND (Prot), R8-MEND (14DMAE) and R8-MEND (29DMAE). In the case of condensed pDNA particles formed at an N/P ratio of 0.5, solutions of condensed pDNA particles were added to the lipid film (DOPE/PA/STR-R8=7:2:2 (molar ratio)) to prepare R8-MEND using a procedure similar to that described above.

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

DNA Transfection Assay Transfection activity was assessed by measurement of luciferase activity described below. NIH3T3 cells (4×10^4 cells/well) were incubated in DMEM containing 10% FBS under 5% CO₂/air at 37 °C for 24 h. Samples containing 0.5 μ g of pDNA suspended in 0.25 ml of serum-free DMEM were added to the cells, followed by incubation under 5% CO₂ at 37 °C for 3 h. Then, 1 ml of fresh DMEM containing 10% serum was added to the cells, followed by incubation for 21 h. The cells were then washed with phosphate-buffer saline, and luciferase activity (relative light unit (RLU)) was measured using a Luciferase Assay System with a Reporter Lysis Buffer kit (Promega;

Madison, WI, U.S.A.) by means of a luminometer (Luminescence-PSN; ATTO, Japan). Cellular protein content was determined using a BCA protein assay kit (PIERCE; Rockford, IL, U.S.A.). Transfection activity was calculated as follows:

$$\text{transfection activity (RLU/mg protein)} = \frac{\text{luciferase activity}}{\text{cellular protein content}}$$

The experiment was repeated thrice. All the data are presented as means $\pm$ S.D. Statistical differences within multiple data sets were assessed by one-way ANOVA, followed by Bonferroni correction *post hoc* test. The level of statistical significance was fixed at $p < 0.05$.

To assess correlation between efficiency of DNA release and transfection activity, we created the graph in Fig. 5C. This graph shows the relationship between efficiency of DNA release (x-axis) and transfection activity, as assessed by measurement of luciferase activity (y-axis), based on results of a quantitative analysis for efficiency of DNA release (Figs. 4C, D) and the DNA transfection assay (Figs. 5A, B). We calculated Pearson's correlation coefficient, and determined if the correlation coefficient was significantly different from zero using a *t*-test. The level of statistical significance was fixed at $p < 0.05$.

RESULTS AND DISCUSSION

Preparation of Condensed pDNA Particles and R8-MEND Two polyrotaxanes, 14DMAE and 29DMAE, with different numbers of cationic DMAE groups were employed as an artificial condenser (Fig. 1C), and protamine, a natural DNA condenser, was selected as a control, since it shows a high transfection activity.^{16–18} It is generally recognized that the number of amino groups in a cationic polymer are closely related to polyplex formation, polyplex stability against a counter polyanion, transfection activity, *etc.*^{19,20} The size and charge of condensed pDNA particles, critical factors in the construction of a MEND,¹⁰ were investigated under various conditions (Fig. 3). Small positively charged particles were formed at nitrogen/phosphate (N/P) ratio of 5. At an N/P ratio of 0.5, small negatively charged particles were also formed. Aggregation occurred at an N/P ratios of 1 and 2, suggesting that aggregation resulted from the electrostatic neutralization of pDNA by the polycation. For R8-MEND preparation, negatively and positively charged nano-particles were formed at N/P ratios of 0.5 and 5, respectively. The observed diameter and ζ potential of condensed pDNA particles and their R8-MENDs are summarized in Table 1. The condensed pDNA particles were then covered with a lipid bilayer by hydration method,¹⁰ providing the desired R8-MEND, which were positively charged nano-particles (around 200–350 nm).

Here, we discuss about the influence that the sizes of R8-MENDs exert their intracellular trafficking, because the diameters of carriers had relatively large deviations. Previously, we reported that high-density R8-modified liposomes are taken up mainly through macropinocytosis, and delivered to the cytosol retaining the aqueous phase marker.⁸ Macropinocytosis refers to the formation of large endocytic vesicles of irregular size and shape, generated by actin-driven invagination of the plasma membrane.^{21,22} Therefore, we considered that each condensed pDNA particle encapsulated in

Table 1. Characteristics of Condensed pDNA Particle and R8-MEND

	N/P ratio		Diameter (nm)	ζ Potential (mV)
Condensed pDNA particle	0.5	Protamine	112±18	-35.1±12
		14DMAE	175±20	-29.0±6.3
		29DMAE	184±31	-27.1±5.6
	5	Protamine	72.3±14	22.4±6.8
		14DMAE	155±34	15.7±4.4
		29DMAE	167±130	20.6±5.3
R8-MEND	0.5	Protamine	281±69	58.4±1.9
		14DMAE	221±11	51.8±11
		29DMAE	206±75	61.8±5.5
	5	Protamine	356±180	63.1±4.7
		14DMAE	311±71	31.7±7.8
		29DMAE	295±93	53.0±8.0

Data are represented by the mean±S.D. (n=3).

R8-MEND would be delivered into nucleus at the same level, although diameters of these carriers had relatively large deviations.

Evaluation for Efficiency of DNA Release from Condensed pDNA Particles *In vitro* DNA release experiments using dithiothreitol (DTT) as a reducing agent were performed to determine the efficiency of DNA release from the condensed pDNA particles prepared at N/P ratios of 0.5 or 5 in the presence or absence of a counter polyanion, poly(L-aspartic acid) (pAsp) (Figs. 4A, B). In these experiments, efficiency of DNA release was defined, based on the intensity of released pDNA band, as described in Materials and Methods. In the presence of pAsp, the efficiency for the release of DNA for all particles that were condensed at an N/P ratio of 0.5 was around 30%, and appeared to be independent of the polycation type (Fig. 4C). In contrast, a significant difference in efficiency of DNA release was observed for particles that were condensed at an N/P ratio of 5 varying from a low of 10% to a high of 70% (Fig. 4D).

DNA release from condensed pDNA particles prepared using DMAE-SS-PRX at an N/P ratio of 0.5 was observed in both the presence and absence of pAsp (Fig. 4A, lanes 5, 6, 8, 9). In contrast, in the case of condensed pDNA particles prepared using protamine, which contains no disulfide linkages, DNA release occurred only in the presence of pAsp (Fig. 4A, lanes 4, 7). These results indicate that, at a lower N/P ratio (0.5), disulfide cleavage under reductive conditions can dominate the release of DNA from the condensed pDNA particles using DMAE-SS-PRXs, although condensed pDNA particles prepared using protamine require an exchange with counter polyanions for the release of pDNA. For the condensed pDNA particles formed at an N/P ratio of 5 (Fig. 4B, lanes 4–9), DNA release was clearly observed only in the presence of pAsp. It should be especially noted that efficiency of DNA release for 29DMAE was superior to that for protamine (Fig. 4D).

Evaluation for Transfection Activity of R8-MEND

We also evaluated the transfection activity of an R8-MEND containing condensed pDNA particles with DMAE-SS-PRX and protamine (Figs. 5A, B). In the case of an N/P ratio of 0.5, the transfection activity was found to be similar for all of

Table 2. Transfection Activity of Condensed pDNA Particle and R8-MEND

N/P ratio	Condenser	Transfection activity (RLU/mg protein) ^{a)}		Ratio ^{b)}
		Condensed pDNA particle	R8-MEND	
0.5	Protamine	1.8×10 ⁶	9.9×10 ⁸	5.5×10 ²
	14DMAE	2.1×10 ³	3.6×10 ⁸	1.7×10 ⁵
	29DMAE	4.2×10 ³	4.4×10 ⁸	1.0×10 ⁵
5	Protamine	2.8×10 ⁵	3.3×10 ⁸	1.2×10 ³
	14DMAE	1.3×10 ⁵	5.2×10 ⁷	3.9×10 ²
	29DMAE	1.7×10 ⁶	1.9×10 ⁹	1.1×10 ³

a) Transfection activities are determined from Luciferase activities (RLU/mg protein). b) Ratio (R8-MEND/condensed pDNA particle) is calculated the following equation. Ratio=luciferase activity of R8-MEND containing condensed pDNA particle/luciferase activity of condensed pDNA particle itself.

the R8-MENDs (Fig. 5A). In contrast, at an N/P ratio of 5, transfection activity was strongly dependent upon the polycation type. Of special interest in Fig. 5B is the fact that the transfection activity of the R8-MEND containing 29DMAE was approximately 5-fold greater than that of an R8-MEND containing protamine. The transfection activity obtained when DMAE-SS-PRX or protamine itself without coating by R8-MEND was used was 2–5 orders of magnitude lower at all of the N/P ratios tested (Table 2), indicating that the integration of R8-MEND and polycations are essential for achieving a promising gene delivery system, as expected.

According to our previous studies,^{6–10)} the efficiency of delivery of condensed pDNA particles to the nucleus is similar independent of the polycation condenser used. Therefore, the differences in transfection activity of the R8-MEND, as shown in Figs. 5A and B, can be explained in terms of intranuclear DNA disposition. The relationship between the efficiency of DNA release and transfection activity is summarized in Fig. 5C. This graph shows the relationship between efficiency of DNA release and transfection activity, as assessed by measurement of luciferase activity (see Materials and Methods for the detail). For this figure, we calculated Pearson's correlation coefficient ($r=0.84$), and determined if the correlation coefficient was significantly different from zero using a *t*-test ($p<0.05$). We found a significant correlation between the efficiency of DNA release and transfection activity, based on following assumption. After the delivery of DNA to the nucleus, 29DMAE, with a high efficiency of DNA release, is able to efficiently release pDNA, leading to a high gene expression, although 14DMAE showed poor gene expression because of its low efficiency of DNA release.

In this study, we demonstrated a close relationship between the efficiency of DNA release and transfection activity. It is particularly noteworthy that, from the standpoint of transfection, our integration system, comprised of R8-MEND with DMAE-SS-PRX was 5-fold greater than an R8-MEND with protamine. This finding provides strong support for our hypothesis that intranuclear DNA disposition plays a critical role in gene transfection, when non-viral vectors are used.

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