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# A multifunctional envelope-type nano device for novel gene delivery of siRNA plasmids

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**Abstract**

A multifunctional envelope-type nano device (MEND) for use in the delivery of siRNA expression plasmids is described. The plasmid DNA encoding anti-luciferase short interfering RNA (siRNA) was condensed by poly-L-lysine (PLL) and packaged into the MEND. The silencing effect of the MEND(PLL) showed a 96% inhibition of luciferase activities in a co-transfection study. The silencing effect was maintained at more than 60%, even under the 100-fold diluted conditions. In the luciferase transformed cells, however, the MEND(PLL) showed no significant silencing effect (10%), indicating heterogeneity in transfection by the MEND(PLL). To solve this problem, the DNA condensing agents were optimized by comparing PLL, stearyl octaarginine (STR-R8) and protamine (Prot). No difference in silencing effect (95–97%) was found among these MENDs in a co-transfection study. However, the MEND(Prot) showed a 70% silencing effect in the transformed cells. These results suggest that the MEND(Prot) has less heterogeneity in transfection, while the MEND(PLL) and the MEND(STR-R8) have large heterogeneities. These results demonstrate that MEND(Prot) is a promising gene delivery system for siRNA expression plasmids with less heterogeneity associated with the transfection.


*Keywords:* Non-viral gene delivery system; Multifunctional envelope-type nano device; siRNA plasmid; Protamine

## 1. Introduction

RNA interference (RNAi) by short interfering RNA (siRNA) is known to be a means for post-transcriptional gene silencing (Kawasaki et al., 2004). Therefore, gene

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silencing by a synthetic siRNA holds some promise for the gene therapy of various diseases such as cancer and viral infections (Caplen, 2004). However, a sufficient amount of synthetic siRNA must be delivered to cells to show a RNAi effect, although siRNA is recycled in the form of a RNA-induced silencing complex (RISC) (Scherer and Rossi, 2003). An siRNA expression plasmid DNA was constructed (Brummelkamp et al., 2002; Miyagishi and Taira, 2002) to supply a large amount of siRNA and for a sufficient period in the cells. Therefore, a non-viral gene delivery system, which has a high nuclear transfer ability, is required, since the siRNA expression plasmid DNA must be delivered to the nucleus for its transcription.

For efficient gene delivery to the nucleus, non-viral vectors need to overcome several barriers such as the plasma membrane, the endosomal membrane and the nuclear membrane. To overcome these obstacles, it is necessary to equip the delivery system with various functional devices such as ligands for specific receptors, devices for endosomal escape and nuclear localization signals (NLS) for enhanced nuclear delivery (Kamiya et al., 2003). However, it is difficult to package all these functional devices into a single system to exert each of their functions at the appropriate time and at the correct location. In a previous report, we proposed a new packaging concept, "Programmed Packaging" (Sasaki et al., 2005). This concept consists of three components: (1) a program to overcome all barriers. (2) Development of functional devices and their three-dimensional assignment. (3) Nano-technology for assembling all devices into a nano-size structure.

We recently reported on the successful development of an original non-viral vector, multifunctional envelope-type nano device (MEND) based on "Programmed Packaging" (Kogure et al., 2004; Sasaki et al., 2005). As shown in Fig. 1, the MEND is composed of a condensed core of plasmid DNA with polycations and a lipid envelope modified by an octaarginine (R8) peptide, a membrane penetrating peptide (Futaki et al., 2003; Khalil et al., 2004). The transfection activity of MEND was as high as that of adenovirus, and no toxicity was observed (Khalil et al., submitted for publication). The cellular uptake of the MEND, directed by R8 peptide, was found to be mainly by macropinocytosis, which was responsible for the high transfection activity by virtue of its escaping lysosomal degradation (Khalil et al., submitted for publication). Therefore, the MEND could be a suitable gene delivery system of siRNA expression plasmid into the nucleus.

In the present study, we constructed a MEND encapsulating anti-luciferase siRNA plasmid condensed by poly-L-lysine (PLL), and evaluated its silencing effect in vitro. In the co-transfection experiment of the siRNA plasmid and a luciferase plasmid, luciferase activity was almost completely reduced by the MEND(PLL). While in the luciferase transformed cells, the

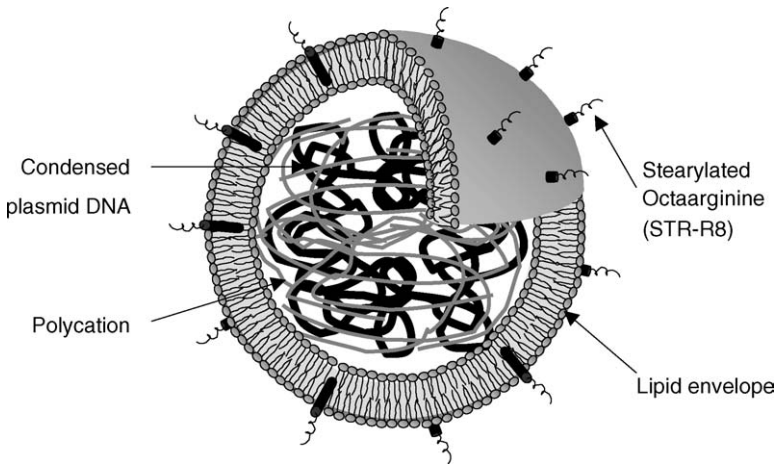


Fig. 1. Schematic representation of a multifunctional envelope-type nano device (MEND) modified with stearylated octaarginine (STR-R8). MEND consists of a polycation-condensed plasmid DNA core and a lipid envelope structure based on a new packaging concept, "Programmed Packaging". The lipid envelope is modified with the membrane penetrating peptide STR-R8 to improve cellular uptake and intracellular trafficking.

MEND(PLL) did not show any significant silencing effect. We then optimized the condensing polycation to improve the RNAi effect of the MEND, and a 70% silencing effect was achieved by this optimization.

## 2. Materials and methods

### 2.1. Materials

siRNA expression plasmid DNAs having the human U6 promoter were constructed from piGENE hU6 vector (iGENE Therapeutics, Tsukuba, Japan) according to a previous report (Miyagishi and Taira, 2002). The plasmid pU6-stem21 transcribes a single-stranded RNA 5′-GUG CGU UGU UGG UGU UAA UCC UUC AAG AGA GGG UUG GCA CCA GCA GCG CAC UUU U-3′, which forms stem-loop-structured siRNA, targeted to firefly luciferase$^{+}$ mRNA (targeted sequence: GTG CGC TGC TGG TGC CAA CCC), with loop sequences of UUCAA-GAGA (Brummelkamp et al., 2002). As a non-specific siRNA expression plasmid, piGENE hU6 vector, which transcribes non-related sequences of RNA 5′-GUG AGC AGG UGU AAA GCC ACC AUG GAA GAC ACC UGC CAA CUU UU-3′ with partial duplex formation, was used. Dioleoyl phosphatidylethanolamine (DOPE) was purchased from AVANTI Polar Lipids Inc (Alabaster, AL). Poly-L-lysine (MW 27,400) and cholesteryl hemisuccinate (CHEMS) was obtained from Sigma–Aldrich Co. (St. Louis, MO). Stearyl octaarginine (STR-R8) was synthesized as described previously (Futaki et al., 2001). Protamine sulfate salmon milt was purchased from Merck KGaA (Darmstadt, Germany). Lipofectamine 2000 was obtained from Invitrogen Co. (Carlsbad, CA). Plasmid DNA pCMV-luc (7037 bp) encoding luciferase mentioned below and the siRNA-expression plasmid DNA (2954 bp) were prepared by EndFree Plasmid Mega Kit (Qiagen GmbH, Hilden, Germany). COS7 cells were obtained from the American Type Culture Collection (Manassas, VA).

### 2.2. Preparation of MEND

MEND was prepared by the lipid film hydration method described in a previous report (Kogure et al., 2004) as follows; DNA dissolved with 10 mM HEPES buffer (pH 7.4) (0.1 mg/ml) was mixed with a PLL solution (0.1 mg/ml) to condense the plasmid DNA under vortexing at a nitrogen/phosphate (N/P) ratio of 2.4. Then, 0.25 ml of the condensed DNA suspension was added to the lipid film containing 137.5 nmol lipids (DOPE/CHEMS = 9:2 (molar ratio)), followed by incubation for 10 min to hydrate lipids. The final concentration of lipid was 0.55 mM. The hydrated mixture was sonicated to construct MEND by coating the condensed DNA with lipids for 1 min in a bath-type sonicator (85 W, Aiwa Co., Tokyo, Japan). An STR-R8 solution (5 mol% of lipids) was added to the suspension to attach R8 peptide to the envelope of MEND, and the mixture was incubated for 30 min at room temperature. The diameter and zeta-potential of the MEND were measured by an electrophoretic light scattering spectrophotometer (ELS-8000, Otsuka electronics, Japan).

### 2.3. Preparation of luciferase transfomant cells

To establish stable luciferase transfected cell lines, COS7 cells were transfected with pGL3-control (Promega Co., Madison, WI) along with selectable marker pRSVneo (ATCC 37198), conferring G418 resistance. G418-resistant colonies were identified by treatment with 400 μg/ml G418 for 14 days. The doubling time was about 16 h in the experimental conditions, and the morphological image of the transformed cells was the same as that of non-treated COS7 cells. These cells express luciferase activity constantly in the order of $10^5$ RLU/mg protein ($4 \times 10^3$ cells).

### 2.4. Transfection assay

Samples containing 0.4 μg of DNA suspended in 0.25 ml of DMEM without serum and antibiotics was added to $4 \times 10^4$ or $4 \times 10^3$ COS7 cells, and incubated for 3 h at 37 °C. Next, 1 ml of DMEM containing 10% fetal calf serum was added to the cells, followed by a further incubation for 45 or 21 h. The cells were then washed, and solubilized with reporter lysis buffer (Promega Co.). Luciferase activity of the cell lysate was measured in the presence of lusiferase assay reagent (Promega Co.) by means of a luminometer (Luminescencer-PSN, ATTO, Japan). The amount of protein was determined using a BCA protein assay kit (PIERCE, Rockford, IL). The data were shown as the mean ± standard deviation ($n = 3$).

### 2.5. Electrophoresis

For agarose gel electrophoresis of MENDs, samples containing 0.25 μg DNA (20 μg/ml) were treated with 1% Triton X-100 and various concentrations of poly-aspartic acid to release the plasmid DNA. Electrophoresis was performed on 1% agarose gel at 100 V for 30 min, and the gel was then stained with ethidium bromide.

## 3. Results

We constructed a MEND in which both anti-luciferase siRNA plasmid and luciferase encoding plasmids condensed by poly-L-lysine were encapsulated, referred to as MEND(PLL). As shown in Fig. 2(a), the luciferase activity of the control cells, which were co-transfected by the MEND(PLL) encapsulating luciferase plasmid and a non-specific siRNA plasmid, was around $10^9$ RLU/mg protein. On the other hand, the luciferase activity of cells transfected by the MEND(PLL) encapsulating anti-luciferase siRNA plasmid was significantly lower than that of the control cells (96%), indicating that anti-luciferase siRNA was transcribed from the siRNA plasmid introduced by the MEND(PLL). We compared the RNAi effect of the MEND(PLL) with the commercially available lipoplex-type non-viral vector Lipofectamine 2000 (Dalby et al., 2004). As shown in Fig. 2(b), the RNAi effect of the MEND(PLL) was comparable to Lipofectamine 2000. The protein content of cells treated with the MEND(PLL) was the same as that of non-treated cells (1.1 mg/ml); however, a 40% inhibition was observed in the case of Lipofectamine 2000, indicating the significant cytotoxicity of the Lipofectamine 2000. We then examined the dilution effect of the siRNA plasmid on the RNAi effect (Fig. 3). The silencing effect of MEND(PLL) decreased with increasing dilution of the siRNA plasmid, but, the silencing effect remained at 64%, even at a 100-fold dilution. These findings confirm that the U6-siRNA expression plasmid is an efficient siRNA expression system.

We next examined the silencing effect of the MEND(PLL) in luciferase transformed cells, which showed a constant luciferase activity in the order of $10^5$ RLU/mg protein. However, MEND(PLL) showed no significant silencing effect (10%) (data not shown). In this experiment, we used PLL as condensing agent of DNA (ratio of nitrogen/phosphate (N/P) = 2.4). How-

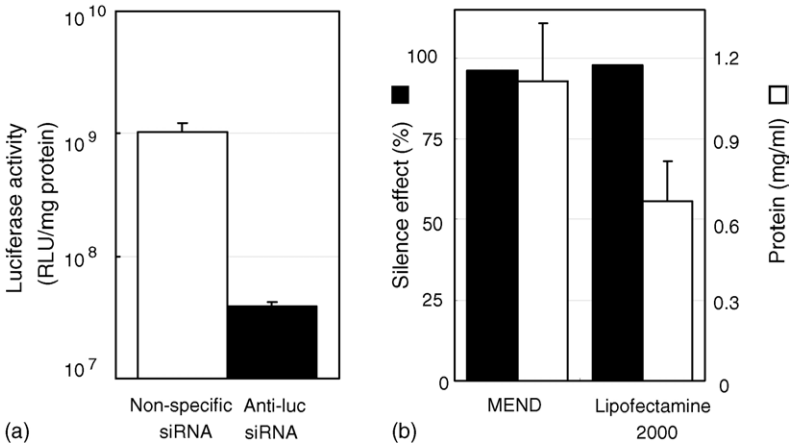


Fig. 2. MEND(PLL) shows a high RNAi effect, comparable to Lipofectamine 2000 with no cytotoxicity in the co-transfection study. (a) Luciferase activities of COS7 cells co-transfected by MEND encapsulating luciferase plasmid and non-specific (open column) or anti-luciferase (closed column) siRNA plasmid. (b) Comparison of the silenced percentage of luciferase activity (closed column) and amount of protein (open column) in the cells transfected by MEND(PLL) with those by Lipofectamine 2000. Sample containing 0.4 μg DNA (molar ratio: siRNA plasmid/luciferase plasmid = 2) was added to $4 \times 10^4$ COS7 cells. Luciferase activities were measured as described in Section 2 48 h after transfection, and are expressed as relative light units (RLU) per mg of protein. Percentages of the silence effect were calculated as the difference of luciferase activities between control and anti-luciferase siRNA-transfected cells divided by the luciferase activity of control cells, multiplied by 100.

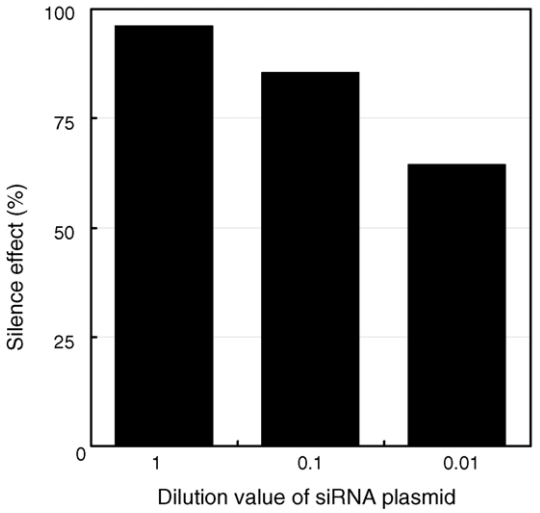


Fig. 3. MEND(PLL) shows a significant RNAi effect even at dilution of 1/100 in the co-transfection study. The molar ratio of total siRNA plasmid to target plasmid in MEND was fixed 2. Non-specific siRNA plasmid was added to compensate difference of the amount of anti-luciferase siRNA plasmid. Other experimental conditions were the same as shown in Fig. 2.

ever, we recently reported that stearylated octaarginine (STR-R8) was effective for condensing DNA, and the condensed DNA showed a high transfection activity (Khalil et al., 2004). On the other hand, spermatozoal peptide protamine (PRRRRSSSRPVRRRRRPRVSRRRRRRGGRRRR) is known to be a good DNA-condensing agent (Sorgi et al., 1997; Brewer et al., 1999), because the peptide contains a high content of arginine residues, and exists in the spermatozoal nucleus as a DNA/protamine complex. Therefore, to improve the RNAi effect of the MEND on luciferase transformed cells, we prepared MENDs in which STR-R8-condensed DNA (N/P = 2.9) and protamine sulfate-condensed DNA (N/P = 2.2) were encapsulated. In this study, MENDs encapsulating PLL-, STR-R8- and protamine-condensed DNA cores are referred to as MEND(PLL), MEND(STR-R8) and MEND(Prot), respectively. The diameters and zeta-potentials of these MENDs are summarized in Table 1. No significant differences were found for these parameters.

Table 1
No significant difference exists among the diameters and zeta-potentials of the three types of MENDs

| Condensing agent | Diameter (nm) | Zeta-potential (mV) |
|---|---|---|
| MEND(PLL) | 294 ± 39 | 53.1 ± 7.6 |
| MEND(STR-R8) | 315 ± 97 | 55.9 ± 5.7 |
| MEND(Prot) | 354 ± 57 | 45.8 ± 6.6 |

We then examined the effect of the co-transfection of the anti-luciferase siRNA plasmid and luciferase plasmid by the three MENDs on luciferase activity. As shown in Fig. 4(b), the silencing effect of luciferase activity by the three MENDs were almost the same (around 95%), although the luciferase activities in the co-transfected control cells were different among these

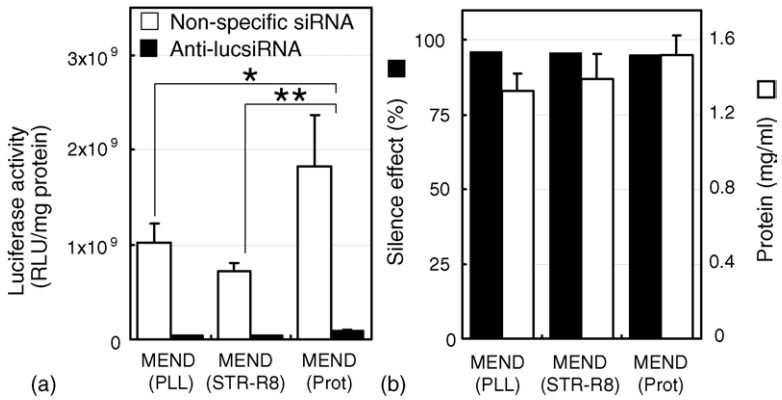


Fig. 4. MEND(STR-R8) and MEND(Prot) also show a strong silencing effect in the co-transfection study. (a) Luciferase activity of cells co-transfected by MEND encapsulating luciferase plasmid and non-specific (open column) or anti-luciferase (closed column) siRNA plasmid. $^{*}P < 0.05$, $^{**}P < 0.01$. (b) Silenced percentages of luciferase activity (closed column) and amount of protein (open column) in the cells co-transfected by MENDs encapsulating PLL-, STR-R8- and protamine-condensed cores, referred to as MEND(PLL), MEND(STR-R8) and MEND(Prot). Experimental conditions were the same as described in Fig. 2.

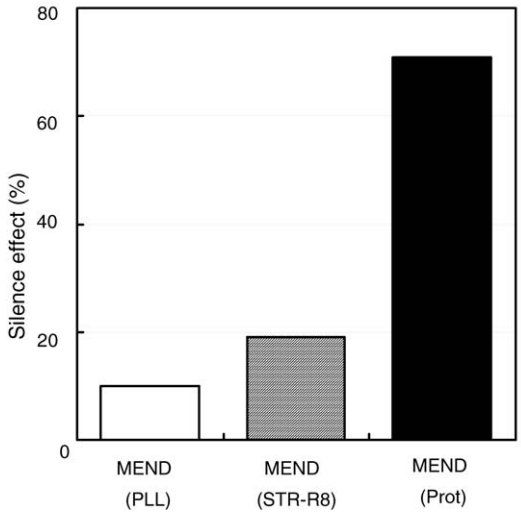


Fig. 5. RNAi effect of MEND(Prot) is significantly higher than MEND(PLL) and MEND(STR-R8) in the transformed cells. Suspension of MENDs containing 0.4 μg DNA was added to $4 \times 10^3$ transformed COS7 cells, and luciferase activities were measured 24 h after the transfection.

MENDs. The protein contents of cells treated with MEND(STR-R8) and MEND(Prot) were almost the same as that of MEND(PLL)-treated cells (Fig. 4b), indicating no cytotoxicity of MEND(STR-R8) and MEND(Prot) like MEND(PLL). We then examined the silencing effect of the three MENDs in luciferase transformed cells (Fig. 5). The condensing agent had a dramatic effect on the silencing effect of the MENDs. STR-R8 increased the effect slightly more than PLL, while protamine enhanced the RNAi effect from 10 to 70%. This silencing effect of MENDs with different condensing agents did not correlate with their transfection activities (Fig. 4a).

In order to confirm the properties of the condensed DNA core of each MEND, we examined the effect of poly-aspartic acid (pAsp) as a decondensing agent on the migration patterns of the condensed DNA in MEND(PLL), MEND(STR-R8) and MEND(Prot) in the presence of the detergent TritonX-100 by agarose gel electrophoresis (Fig. 6). The condensed DNA of MEND(PLL) was observed at the origin in the absence of pAsp. Clear two bands were observed at the same position as naked plasmid DNA (left end) by the addition of 50 μg/ml pAsp, indicating that the condensed DNA was decondensed with the polyanion. In addition, the brightness of the unmigrated DNA increased with increasing pAsp concentration. Similarly, condensed DNA of MEND(STR-R8) was decondensed with pAsp in a concentration-dependent manner. Only one band was observed and unmigrated DNA was not observed at the origin, unlike the migration patterns of MEND(PLL). In the case of the MEND(Prot), a faint band was observed in both presence and absence of pAsp, and the fluorescent intensity of the band was independent of the concentration of pAsp.

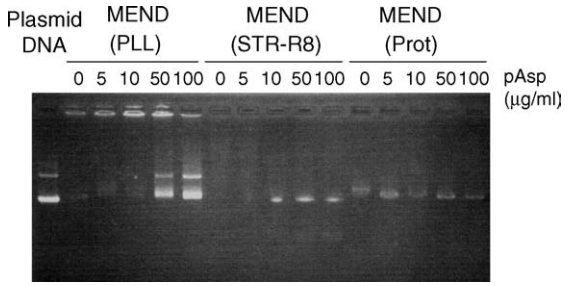


Fig. 6. Prot/DNA complex migrates on an agarose gel without any decondensing agent. The image shows migration patterns for the agarose gel electrophoresis of MEND(PLL), MEND(STR-R8) and MEND(Prot). For the electrophoresis, samples containing 0.25 μg DNA (20 μg/ml) were treated with 1% Triton X-100 and various amounts of polyaspartic acid to release DNA from the MENDs. Electrophoresis was performed on a 1% agarose gel at 100 V for 30 min.

## 4. Discussion

In the present study, the novel non-viral vector MEND encapsulating an siRNA expression plasmid showed a strong RNAi effect. The RNAi effect of MEND(PLL) was comparable to that of Lipofectamine 2000 on the co-transfection of a luciferase plasmid and an anti-luciferase siRNA plasmid (Fig. 2b). Surprisingly, even at a 100-fold dilution of the siRNA plasmid, the MEND(PLL) showed a significant silencing effect. A single strand of siRNA, complementary to the target mRNA, is incorporated into a distinct nuclease complex RISC, which exerts the site specific cleavage of mRNA, and the RISC is recycled (Scherer and Rossi, 2003). Thus, a small amount of siRNA can be used to cleave a large amount of target mRNA, because siRNA can be reused in the form of RISC. Furthermore, MEND(PLL) showed no cytotoxicity, although Lipofectamine 2000 was highly toxic (Fig. 2b). The reason

for the MEND(PLL) showed no cytotoxity could be due to little cationic components and/or its unique entrance mechanism via macropinocytosis with membrane penetrating peptide octaarginine presented on the surface of MEND (Khalil et al., submitted for publication).

It is interesting to note that the silencing effects of MEND(PLL), MEND(STR-R8) and MEND(Prot) were almost the same in co-transfection experiments (Fig. 4), while in the transformed cells only MEND(Prot) showed a significantly high silencing effect (Fig. 5). In the co-transfection experiment, it seems reasonable that the same silencing effect would be exerted among the three MENDs, since the target plasmid and siRNA plasmid were packaged together within the same MEND. However, in the transformed cells, the silencing effect may depend on the amount of siRNA generated in each cell, since all cells should have the same levels of target mRNA. The MEND(PLL) can produce only a 10% inhibition while 70% was achieved by the MEND(Prot), which is not the case for the co-transfection study where the three MENDs showed almost the same silencing effects (Fig. 4b). There are some differences in the transfection activities among these MENDs as shown in Fig. 4(a); however, the differences were not sufficient to explain the differences found in the transfomed cells (Fig. 5). A four-order of magnitude difference in luciferase activities was observed between the co-transfection study (Fig. 4) and transformed cell study (Fig. 5). In the co-transfection study, the higher transfection activities around $10^9$ could be determined by the small fraction (10%) of highly transfected cells in the MEND(PLL) or MEND(STR-R8), which also explains the 10–20% of silencing effect in transformed cells. In case of the MEND(Prot), it was suggested that the MEND(Prot) can transfect cells more homogenously than the MEND(PLL) or the MEND(STR-R8), since 70% inhibition was observed in transformed cells with MEND(Prot). Then, why MEND(Prot) can transfect homogenously?

We estimated the condensation state of the polycation/DNA complex core based on the electrophoretic migration patterns of the three MENDs. In the case of PLL/DNA complexes, fluorescence was observed at the origin, which indicated that there is some space for EtBr to enter between DNA and PLL. The decondensation of PLL/DNA was dependent on the concentration of pAsp and 50 μg/ml was required to decondense the complex. The decondensed DNA was almost similar to that of naked DNA. These results indicate that electrostatic interactions between DNA and PLL were strong but the complex was not so tight and reversibly dissociate by the addition of pASp. In the case of STR-R8/DNA complexes, no fluorescence was observed at the origin in the presence or absence of pAsp, which suggests that STR-R8 and DNA formed a very tight complex. This result was also consistent with the result by atomic force microscopy (Khalil et al., 2004). There is a concentration dependency of pAsp in the decondensation of the complex and the decondensation starts at lower concentrations of pAsp (10 μg/ml). These results indicate that STR-R8 is able to highly condense DNA but allows it to dissociate more readily than PLL, which serves to explain the enhanced siRNA inhibition compared to PLL in transformed cells (Fig. 5). In the case of Prot/DNA complexes, no fluorescent intensity was observed at the origin in the presence or absence of pAsp as in the case of STR-R8/DNA. However, a slight fluorescence was observed at the supercoil position even in the absence of pAsp. No concentration dependent decondensation of DNA was observed. The faint fluorescence at the position of super coiled DNA could be a complexed form of Prot/DNA, which is well condensed but sufficiently flexible to move in the agarose gel. These results indicate that the Prot can form flexible (soft) particles with DNA, which can be efficient in nuclear delivery as well as nuclear transcription.

The enhanced siRNA effect of the MEND(Prot) can also be explained by the following specific mechanisms. Nucleoplasmin, which is a member of the histone chaperone group, decondenses Prot/DNA complexes in the nucleus, since it is known that sperm chromatin condensed with protamine is decondensed by nucleoplasmin during fertilization (Ruiz-Lara et al., 1996). Protamine has also been proposed to be evolved from histone H1 (Lewis et al., 2004), and plays the same role as histone in the sperm nucleus, and another histone chaperone nucleosome assembly protein (NAP)-1 transfers histone H2A/H2B from the cytoplasm into the nucleus (Loyola and Almouzni, 2004). These findings indicate that protamine can enhance the nuclear delivery of condensed DNA, which is consistent with the report that protamine contains an NLS-like sequence (Sorgi et al., 1997) and increased nuclear localization of cationic liposome/DNA complex (Noguchi et al., 2002).

We have no direct evidence on the nuclear delivery of DNA in the form of MEND, in the form of a condensed particle, or in the form of a released (naked) DNA. However, based on the above discussion, the following hypothesis is proposed: after internalization of the MEND by macropinocytosis (Nakase et al., 2004; Wadia et al., 2004; Khalil et al., submitted for publication), Prot/DNA may be released from the MEND into cytoplasm in the form of a condensed particle. The Prot/DNA particles can be efficiently transported to the nucleus by NAP-1, where the Prot/DNA particles can be decondensed by the nucleoplasmin and be transcribed. PLL/DNA and STR-R8/DNA have no such active mechanisms for nuclear delivery or nuclear decondensation. Further study of this issue is now underway in our laboratory.

In conclusion, the findings herein show that the novel non-viral vector MEND(Prot) is a useful gene delivery system for siRNA expression plasmid DNA. The silencing effect of MEND(PLL) was comparable to that of Lipofectamine 2000 without any detectable cytotoxicity. The MEND(PLL) showed a significant silencing effect, even when diluted by 100-fold. Furthermore, the optimized MEND(Prot) showed a 70% silencing effect in transformed cells, possibly due to efficient nuclear translocation as well as nuclear decondensation. Thus, the MEND(Prot) is a promising gene delivery system for siRNA expression plasmids.

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