Efficient Cytoplasmic Protein Delivery by Means of a Multifunctional Envelope-type Nano Device

Ryosuke SUZUKI, Yuma YAMADA, and Hideyoshi HARASHIMA*

Laboratory for Molecular Design of Pharmaceutics, Faculty of Pharmaceutical Sciences, Hokkaido University; Kita-12, Nishi-6, Kita-ku, Sapporo 060–0812, Japan.

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The potential for protein therapy, such as the use of antibodies, and vaccines is now well accepted. However, it is difficult to enhance efficiency in protein therapy without a suitable delivery system for delivering proteins to target sites. Here we describe the development of protein delivery system, which is capable of cytoplasmic delivery as well as efficient packaging. The multifunctional envelope-type nano device (MEND), which was originally developed for the delivery of nucleic acids such as plasmid DNA and oligodeoxynucleotides, can also be applied to protein delivery. In this study, the green fluorescent protein (GFP), a model protein, was condensed with stearyl octaarginine (stearyl R8) to form a nano particle, which was then coated with a lipid membrane, thus permitting R8 to be introduced for efficient cellular uptake and controlled intracellular trafficking. The packaging efficiency of the MEND was significantly higher than that of conventional liposomes, because the GFP can be encapsulated a condensed form. According to confocal laser scanning microscopy, the MEND is internalized efficiently and escapes from the acidic compartment to efficiently release GFP into the cytosol. These results indicate that the MEND can serve as a useful cytoplasmic delivery system for protein therapy.

Key words non-viral vector; multifunctional envelope-type nano device; protein delivery; cytoplasmic delivery; stearyl octaarginine; packaging

Protein therapy is a new strategy in which proteins themselves are used as drugs and is recognized to have a high potential for curing diseases. Most reports regarding protein therapy have been restricted to target cell surface receptors as seen in cases of peptide hormones such as insulin, a monoclonal antibody for cancer therapy, etc. New opportunities would arise if proteins could be delivered intracellularly. For example, anticancer therapy by apoptosis inducing proteins (Bax, cytochrome c, caspase-3, etc.), antioxidative therapy to prevent oxidation due to reactive oxygen species by delivering superoxide dismutase proteins could be achieved by intracellular delivery of proteins. However, these strategies cannot be realized without an intracellular protein delivery system.

Liposomes are useful delivery carriers, which can target tumor tissue via enhanced permeability and retention effects, if their circulation is extended by PEGylation. Doxorubicin, a well known anticancer agent, can be efficiently encapsulated into sterically stabilized liposomes and this drug delivery system has already been used in clinical settings. However, there is no efficient method for encapsulating proteins into liposomes, and this has limited progress in protein therapy.

We recently developed a multifunctional envelope-type nano device (MEND) for the delivery of nucleic acids such as a plasmid DNA (pDNA) and an oligodeoxynucleotide (ODN). The MEND is composed of a condensed core of DNA with polycations, which is covered with lipid membranes. The MEND can encapsulate DNA or ODN efficiently. A MEND that is covered with a high density of octaarginine (R8) in the form of stearyl R8 (R8-MEND), can stimulate macropinocytosis and has been shown to be taken up efficiently.

In this study, we report on the development of a new delivery system by applying the MEND concept to protein delivery. The green fluorescent protein (GFP), a model protein, can be encapsulated in a MEND in a condensed form. Furthermore, the intracellular fate of the R8-MEND was followed by confocal laser scanning microscopy as well as Western blot analysis.

MATERIALS AND METHODS

Materials E. coli strain BL21 (DE3) (F−, ompT, hsdSB (rB mB), gal, dcm (DE3)) was purchased from Stratagene (La Jolla, CA, U.S.A.). pQBI 25 was purchased from Takara (Kusatsu, Japan). pTriEx-3 Neo Vector was purchased from Novagen (Madison, WI, U.S.A.). HisTrap HP column and HiTrap Desalting column were obtained from Amersham Biosciences Corp. (Piscataway, NJ, U.S.A.). Oligonucleotides were purchased from Sigma Genosys Japan (Ishikari, Japan) in purified form. Cholesteryl hemisuccinate (5-cholestene-3-ol 3-hemisuccinate; CHEMS) 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.). Egg yolk phosphatidycholine (EPC) was obtained from the Nippon Oil and Fats Co. (Tokyo, Japan). Octaarginine (R8) and stearyl R8 (R8) were generously supplied by Dr. S. Futaki (Kyoto University, Japan). HeLa human cervix carcinoma cells were obtained from the RIKEN Cell Bank (Tsukuba, Japan). Dulbecco’s modified Eagle medium (D’MEM) and fetal bovine serum (FBS) were purchased from Invitrogen Corp. (Carlsbad, CA, U.S.A.). LysoTracker Red DND-99 was purchased from Molecular Probes (Eugene, OR, U.S.A.). All other chemicals were commercially available reagent-grade products.

Protein Expression and Purification To prepare GFP for use as a model protein, we constructed a GFP expression vector (pTENG) to produce recombinant GFP protein. cDNA encoding GFP was amplified from pQBI 25 by the polymerase chain reaction with high-fidelity Pyrobest DNA polymerase (Takara, Otsu, Japan). The amplified cDNA was in-
serted into pTriEx3 Neo Vector between the EcoR I and Bgl II sites. This generated a cDNA encoding GFP with a (His)$_8$ tag fused to the C-terminus. The GFP fusion protein was expressed in E. coli strain BL21 (DE3), purified by HiTrap HP column chromatography, and further exchanged with 10 mM HEPES buffer (pH 7.4) by HiTrap Desalting column chromatography, according to the supplier instructions. The purified protein was analyzed by 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Protein concentrations were determined with a BCA Protein Assay Kit (Pierce; Rockford, IL, U.S.A.).

**Preparation of Condensed Protein Particles and Sedimentation Analysis by SDS-PAGE** GFP was gently mixed with the polycation as a condenser in 100 μl HB (pH 7.4), followed by incubation for 15 min at 25 °C to form condensed protein particles (CPP). Particles of the GFP/polyacation were formed using 12.5 μg GFP and R8, or stearyl R8 in a series of condenser/protein (C/P) molar ratios. Sedimentation analyses of GFP/polyacation interactions were performed using 12.5 μg GFP. The CPP were centrifuged for 15 min at 10000 x g and the supernatant removed. The pellet was washed twice in HB (pH 7.4), and then subjected to 10% SDS-PAGE.

**Construction of the MEND (GFP)** The GFP-encapsulated MEND (MEND (GFP)) was constructed by the lipid film hydration method, as reported previously. The procedure involved the following three steps. First, GFP, dissolved in HB (pH 7.4) (0.25 mg/ml), was mixed with a stearyl R8 solution (0.32 mg/ml) to condense GFP by gently pipetting at a C/P molar ratio of 25, followed by incubation for 15 min at 25 °C. Then, 0.25 ml of CPP was added to the lipid film, formed by the evaporation of a chloroform solution of 137.5 nmol lipids (EPC/CHEMS=9:2, or DOPE/CHEMS=9:2 (molar ratio)), on the bottom of a glass tube, followed by incubation for 15 min to hydrate the lipids. The glass tube was sonicated to produce the MEND (GFP) by coating the CPP with lipids for about 5 s in a bath-type sonicator (85 W; Aiwa Co., Tokyo, Japan) (125 ng/μl GFP concentration). To attach R8 to the surface of the carrier, a solution of stearyl R8 (5 mol% lipids) was incubated for 30 min at room temperature (R8-MEND (GFP)).

**Liposome Preparation** The GFP-encapsulated liposomes (LP (GFP)) were prepared as described below. The carrier was constructed using 137.5 nmol lipids (EPC/CHEMS=9:2 (molar ratio)) and 15.6 μg of GFP by a hydration method (125 ng/μl GFP concentration). A stearyl R8 solution (5 mol% lipids) was then added to the suspension, followed by incubation for 30 min at room temperature, to allow the R8 to become attached to the surface of the liposomes (R8-LP (GFP)).

**Measurement of Diameter and Zeta-Potential of Carriers** Particle diameter was measured by a quasi-elastic light scattering spectrophotometer (Zetasizer Nano ZS; Malvern Instruments, Herrenberg, Germany).

**Sucrose Density Gradient Centrifugation** MEND (GFP) and LP (GFP) containing rhodamine-labeled DOPE (1 mol% total lipids) were layered on a discontinuous sucrose density gradient (0 to 60%), and centrifuged at 160000 x g for 2 h at 4 °C. A 1 ml aliquot was collected from the top, and the amounts of GFP and lipid were then measured.

**Recovery Ratios of Protein and Lipid, and Condensation Efficiency** The amount of GFP present was determined with a BCA Protein Assay Kit. The BCA protein assay was performed after completely removing glucose by precipitating the protein with deoxycholate and trichloroacetic acid, as previously reported, because glucose interferes with the BCA protein assay. The amount of the lipid was determined by measuring the fluorescent intensity of the rhodamine-labeled lipids (excitation at 550 nm and emission at 590 nm) after treatment with 1% SDS. The recovery ratios and condensation efficiency were calculated as follows;

1. recovery ratio of protein (%)=recovered protein/applied protein x 100,
2. recovery ratio of lipid (%)=recovered lipid/applied lipid x 100,
3. condensation efficiency (%)=(recovered protein/recovered lipid)/ (applied protein / applied lipid) x 100.

**Protein Transduction Efficiency** HeLa cells (4 x 10⁴ cells/ml, 6 cm dish) were cultured in D’MEM with 10% FBS under 5% CO₂/air at 37 °C for 36 h. Samples containing GFP (12.5, 18.8, or 25 μg), suspended in 2 ml of serum-free medium, were added to the cells, followed by incubation for 1 h. The cells were next washed with phosphate-buffer saline (PBS (−)), and further incubated in medium with 10% serum for 2 h in the absence of samples. The cells were trypsinized and washed with PBS (−) to remove GFP bound to the cellular surface, twice with PBS (−) containing heparin (20 units/ml) to remove R8 modified carriers bound to the cell membrane, and then solubilized with reporter lysis buffer (Promega, Madison, WI, U.S.A.), which contained a protease inhibitor cocktail (Roche Ltd.; Basel, Switzerland). Protein concentrations of the cell lysate were determined using a BCA protein assay kit. GFP in the cell lysate was detected by Western blot analysis.

**Western Blot Analysis** A 2.5 μl of sample (0.3 mg of total cellular protein/ml) was subjected to 10% SDS-PAGE. After the electrophoresis, the proteins were electroblotted onto a Polyvinylidene Fluoride membrane (NIPPON Genetics Co., Ltd.; Tokyo, Japan). Antibodies against the N-terminus of GFP derived from rabbit (Sigma) were used at a 1 : 1000 dilution. GFP was further detected using secondary HRP-conjugated anti-rabbit antibodies (Amersham Biosciences Corp.) at a 1 : 1000 dilution.

**Intracellular Trafficking of MEND (GFP) by Confocal Laser Scanning Microscopy** HeLa cells (4 x 10⁴ cells/ml, 3.5 cm dish) were cultured in D’MEM with 10% FBS under 5% CO₂/air at 37 °C for 24 h. The cells were washed and then incubated in serum-free medium, which contained R8-MEND (GFP) (final lipid concentration, 22 μg), and further incubated in medium with 10% serum for 5 h in the absence of carriers. Ten minutes before the acquisition of the fluorescence images, LysoTracker Red DND-99 was applied to the medium at a final concentration of 100 nm to stain the acidic organelles (e.g., endosomes and lysosomes), and the cells were then washed three times with D’MEM. The cells were excited by 488 nm and 568 nm light from an Ar/Kr laser. A series of images were obtained by confocal laser scanning microscopy (LSM510; Carl Zeiss Co., Ltd., Jena, Germany).
using a water immersion objective lens (Achroplan 63×/NA=0.95) and a dichroic mirror (HFT488/568). The two fluorescence detection channels (Ch) were set to the following filters: Ch1: LP585 (red), Ch 2: BP 505—550 (green).

RESULTS AND DISCUSSION

Preparation of Condensed Protein Particles  The condensation of protein is an important step in the procedure, because the size and charge of the condensed protein particles (CPP) are critical factors in the construction of a MEND by the lipid hydration method.8,9 We therefore examined the effect of the molar ratio of protein and condenser on the diameter and the zeta potential of CPP and then determined the conditions for protein condensation, in which less than 150 nm and positively charged particles are produced. We chose GFP (M.W. 33 kDa, weak negative charge) as a model protein for intracellular observation using confocal laser scanning microscopy. In this experiment, we determined whether R8 and stearyl R8, which are good condensers of pDNA,13) were suitable as condensers for proteins.

When GFP was mixed with stearyl R8 (Fig. 1, closed circles), small positively charged particles were formed at C/P molar ratios higher than 10. In the region of C/P molar ratios below 0.1, small negatively charged particles were also formed. Aggregation occurred at a C/P molar ratio between 0.1 and 10. This suggests that aggregation resulted from the electrostatic neutralization of GFP by stearyl R8. This aggregation was also observed when condensed particles of DNA were prepared.8,9 In the case of R8 (Fig. 1, open circles), small positively charged particles were not formed, because aggregation (more than 2000 nm) occurred at C/P molar ratios higher than 10. When GFP was mixed with poly-L-lysine and poly-L-arginine, which contain only positive groups but no hydrophobic groups, aggregation was also observed at C/P molar ratios higher than 10 (data not shown). We concluded that it difficult to condense the protein by electrostatic interactions only, since proteins are different from DNA, a highly negatively charged molecule. We considered that formation of CPP requires two types of interactions such as electrostatic and hydrophobic. First, GFP and R8 interact with electrostatic force to form a complex which forms a relatively large particle. Hydrophobic residue of stearyl R8 can condense particle after neutralization between GFP and R8 to form a small particle. Therefore, it is important to have a hydrophobic moiety to obtain well condensed nano particles.

We next determined whether it was possible to condense GFP with stearyl R8 by sedimentation analyses (Fig. 2). Most of the GFP was detected as uncondensed GFP with the supernatant (uncondensed GFP) to the pellet fraction (condensed GFP) occurred between 0.1 and 1 C/P molar ratio.  S indicates the supernatant fraction. Protein shift from the supernatant (uncondensed GFP) to the pellet fraction (condensed GFP) occurred between 0.1 and 1 C/P molar ratios (lanes 2—5).

Fig. 2. Sedimentation Analysis of GFP and Stearyl R8 Interactions by SDS-PAGE

GFP (12.5 µg) was mixed with stearyl R8 in 100 µl HB (pH 7.4) for 15 min at 25°C at a series of C/P molar ratios. The samples were then centrifuged for 15 min at 10000 g, and then subjected to 10% SDS-PAGE. Lanes 1, GFP control (0.31 µg); lanes 2 and 3, particles of GFP/stearyl R8 at C/P molar ratio 0.1; lanes 4 and 5, ratio 1; lanes 6 and 7, ratio 10; lanes 8 and 9, ratio 25; lanes 10 and 11, ratio 50. P indicates the pellet fraction; S indicates the supernatant fraction. Protein shift from the supernatant (uncondensed GFP) to the pellet fraction (condensed GFP) occurred between 0.1 and 1 C/P molar ratios (lanes 2—5).

Construction of the MEND and Its Condensation Efficiency  We constructed a condensed core of GFP with stearyl R8, which is covered with a lipid bilayer (MEND (GFP)), and then calculated the condensation efficiencies. A suspension of condensed protein particles (CPP) were added to the lipid film containing the negatively charged lipid CHEMS, followed by incubation to hydrate the lipid and to induce electrostatic interactions. The CPP was then packaged in a lipid bilayer by sonication, and further incubated with a solution of stearyl R8 (5 mol% lipids) to produce the R8-MEND (GFP). As shown in Table 1, the diameter of the R8-MEND (GFP) was larger than CPP. The zeta potential of the R8-MEND (GFP) was positively charged, suggesting that R8 was modified with the surface of the MEND (GFP).

As shown in Fig. 3A, after purifying the MEND (GFP) by sucrose density gradient centrifugation, GFP and lipid were observed in the same fraction (#3, #6). Uncondensed GFP and empty liposomes were detected in fractions (#1—#3), re-
respectively, when each sample alone was purified (data not shown). Based on these results, it can be concluded that fraction #6 (Fig. 3A), in which the content of GFP was higher than that of the lipid, contained MEND (GFP) having both lipid and condensed GFP. On the other hand, in the case of liposomes encapsulating GFP (LP (GFP)) as a control, GFP and lipid were observed in the same fraction (#1—#3) as shown in Fig. 3B). These fractions contain LP (GFP), empty LP, and unencapsulated GFP. We compared the condensation efficiencies between MEND (GFP) and LP (GFP). The condensation efficiency of MEND (GFP) was 177.2% (Fig. 3A, #6), because MEND (GFP) can enhance condensation efficiency by forming condensed GFP particles (Table 2). The condensation efficiency for the MEND (GFP) was approximately 3-fold higher than that for LP (GFP). However, the recovery ratios for the MEND (GFP) were lower than that for LP (GFP) (Fig. 3B, #2). The calculated condensation efficiency and recovery ratios for LP (GFP) might be higher than the actual value, because unencapsulated GFP and empty liposomes were present in the same fraction (Fig. 3B, #2).

**Cytoplasmic Protein Delivery by R8-MEND** To determine whether the MEND could deliver protein intracellularly, protein transduction efficiency was evaluated by Western blot analysis, as shown in Fig. 4. It has been previously shown that high density R8 (5 mol%)-modified liposomes can stimulate macropinocytosis and are taken up efficiently. Therefore, all carriers were modified with a high density of R8 in this study. When GFP alone or R8-LP (GFP) was added to the cells, GFP bands were not detected at any applied dose (Fig. 4, lanes 2—7). On the other hand, in the case of R8-MEND (GFP), GFP bands were clearly observed, moreover, the intensities of the bands increased as a function of the applied dose, suggesting that GFP was successfully delivered intracellularly (Fig. 4, lanes 8—10). Since several bands were detected and the molecular weights of these bands were smaller than those of GFP (33 kDa), we concluded that these bands were GFP fragments, produced by cleavage by a protease(s) intracellularly. These results show that a high condensation efficiency increased protein transduction efficiency, indicating that the R8-MEND can function as a useful carrier for the cytoplasmic delivery of proteins.

We next examined the intracellular location of the R8-MEND by confocal laser scanning microscopy. When an R8-MEND composed of EPC/CHEMS (R8-MEND (EPC)) was added to cells (Figs. 5A—C), many green dots were observed in the cells, indicating that GFP was delivered intracellularly. However, most of the GFP was localized in acidic compartments and observed as yellow dots. In the case of the R8-MEND composed of DOPE/CHEMS (R8-MEND (DOPE)), which can fuse with the endosomal membrane, many green dots were observed outside the acidic compartments (Fig. 5D—F). This suggests that encapsulated GFP particles were released into the cytosol after membrane fusion between R8-MEND (DOPE) and the endosomal membrane. We consider that GFP is expected to be released from GFP particles due to replacement with cytoplasmic proteins after endosomal escape, since GFP particles can release GFP in freshly prepared homogenates in vitro (data not shown). Collectively, the above findings demonstrate that the R8-MEND is...
MEND (DOPE) can achieve the efficient cytoplasmic delivery of protein.

In conclusion, we succeeded in developing an efficient protein packaging method by producing a condensed protein core, covered with a lipid membrane. The MEND showed a higher condensation efficiency than conventional liposomes. Furthermore, the R8-MEND (DOPE) successfully delivered protein to the cytosol. This strategy will be useful for delivering proteins to the cytosol.

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