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Construction of a multifunctional envelope-type nano device by a SUV*-fusion method

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Abstract

A novel assembly method "SUV*-fusion method" was developed for the construction of a small and homogenous multifunctional envelope-type nano device (MEND) by utilizing a detergent-rich small unilamellar vesicle (SUV*). The method consists of three steps: (1) DNA condensation with a polycation, (2) electrostatic interaction of the SUV* with the DNA/polycation complex (DPC) and (3) lipid coating of DPC by SUV* fusion via removal of the detergent. We confirmed the construction of the MEND by sucrose density gradient centrifugation, and isolated the MEND only from the boundary between 25% and 40% sucrose. The isolated MEND had a small diameter (155 nm), was negatively charged (−24 mV), and encapsulated 30% of the total DNA. The MEND was formed by only SUV*, not by a lipid/detergent micelle. This confirms that a small and homogenous MEND can be constructed by the SUV*-fusion method. Furthermore, we confirmed that a transferrin-modified MEND could deliver a gene into a cell through receptor-mediated endocytosis. Consequently, we report on the successful construction of a small and homogenous MEND by a novel SUV*-fusion method.

Keywords: Gene delivery; Packaging; Multifunctional envelope-type nano device; SUV*-fusion

1. Introduction

For the efficient gene delivery to the nucleus of a target cell, non-viral vectors must overcome several barriers such as the plasma membrane, the endosomal membrane and the nuclear membrane (Harashima et al., 2001; Kamiya et al., 2003; Akita et al., 2004).

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Thus, to overcome such barriers, the gene delivery system must be equipped with various functional devices such as ligands for specific receptors, pH-sensitive fusogenic peptides for endosomal escape and a nuclear localization signal (NLS) for nuclear targeting (Kamiya et al., 2003). However, it is difficult to integrate all functional devices into a nano-size structure by simple mixing to exert each function at the appropriate time and the correct place. Therefore, we propose a new packaging concept, "Programmed Packaging", for constructing an ideal vector, which fulfils all these requirements. This concept consists of three components: (1) programming based on a strategy to overcome all barriers; (2) design of functional devices to overcome the barriers and their assignment into a nano-size structure; (3) nano-technology for assembling all devices into the nano-size structure. Thus, an advanced assembly technique is required to realize Programmed Packaging.

Several assembly methods for non-viral gene delivery system have been reported to date (Gregoriadis et al., 1999; Wheeler et al., 1999; Perrie and Gregoriadis, 2000; Shi and Pardridge, 2000; Shi et al., 2001; Fenske et al., 2002). Gregodiadis and co-workers developed a dehydration–rehydration procedure, in which a negatively charged free plasmid DNA is mixed with preformed empty cationic liposomes, and the mixture is dehydrated by freeze-drying, followed by rehydration in an aqueous buffer (Gregoriadis et al., 1999; Perrie and Gregoriadis, 2000). The liposome encapsulating DNA contained a fusogenic lipid dioleolylphosphatidylethanolamine (DOPE) for endosomal escape. Similarly, Pardridge and co-workers entrapped free plasmid DNA by freeze-thawing a cationic liposome/DNA mixture (Shi and Pardridge, 2000; Shi et al., 2001). Their carrier was equipped with polyethylene glycol (PEG) conjugated to a peptidemimeric mAb for targeting the transporting receptor on the blood–brain barrier. On the other hand, Cullis and co-workers developed a detergent dialysis method, and constructed stabilized plasmid–lipid particles (SPLP) (Wheeler et al., 1999; Fenske et al., 2002). The principle of their method involves the encapsulation of free plasmid DNA into a cationic liposome as the result of detergent removal from the lipid/detergent mixed micelles. The SPLP was equipped not only with the fusogenic lipid DOPE for endosomal escape but also with PEG for stabilization and in vivo administration. Thus, these three assembly methods are effective for the encapsulation of free plasmid DNA into cationic liposomes, and the carriers can be equipped with various devices to overcome the plasma membrane and the endosomal membrane. However, these carriers lack a device for nuclear targeting, i.e., the free plasmid DNA must be equipped with devices for nuclear targeting except for direct modification, because the direct conjugation of a NLS peptide to linearized double-stranded DNA does not enhance nuclear entry (Tanimoto et al., 2003).

We concluded that the condensation of DNA by polycations is advantageous not only for protection from nucleases and efficient encapsulation, but also for the enhancement of nuclear delivery by using nuclear targetable polycations. Thus, it is necessary to develop a novel assembly method for the encapsulation of condensed DNA into liposomes. We recently succeeded in the development of a novel non-viral vector multifunctional envelope-type nano device (MEND) by the lipid film hydration method based on "Programmed Packaging" (Kogure et al., 2004). MEND is composed of a condensed DNA core and a lipid envelope. The condensed DNA and lipid layer can be equipped with various functional devices such as an NLS and ligands for specific receptors to optimize the system. In addition, the lipid layer can also control the topology of the equipped devices (Kamiya et al., 2003; Khalil et al., 2004; Kogure et al., 2004). The lipid hydration method is very simple and easy, and the encapsulation efficiency of DNA was 70%. However, the MEND was relatively large (around 300 nm), and showed a heterogenous distribution on sucrose density gradient fractionation (Kogure et al., 2004). Furthermore, the sonication process has the potential to damage the delicate components.

Thus, it is necessary to solve the problems of size, heterogeneity and a sonication process for the construction of an ideal MEND. Furthermore, a novel assembly technique is required to equip the MEND with PEG for protection from serum nucleases and a ligand for selective targeting. Thus, we turned our attention to small unilamellar vesicle (SUV*) for the construction of a smaller and homogenous MEND which is equipped with PEG and a targeting ligand. SUV* is a detergent-rich small unilamellar vesicle, i.e., the detergent concentration of SUV* is near to but lower than CMC

(Ueno et al., 1997). An SUV* is temporarily formed in the vesicle formation process from a micelle by detergent removal. An SUV* is small (20–30 nm) and homogenous (Ueno and Akechi, 1991), and can fuse with each other turning into a large unilamellar vesicle (LUV) by complete removal of detergent (Ueno et al., 1997). Thus, we attempted to utilize the self-fusion ability of an SUV* to construct a smaller and homogenous MEND.

In the present study, we succeeded in constructing a MEND by the SUV*-fusion method. A small and positively charged DNA/polycation complex (DPC) was initially prepared by DNA condensation with poly-L-lysine (PLL). The DPC was then incubated with the negatively charged SUV* containing a large amount of non-ionic detergent octylglucopyranoside, and the detergent was removed for lipid coating. We confirmed the successful construction of a MEND by sucrose density gradient centrifugation, and verified that it can deliver a gene into a cell through receptor-mediated endocytosis.

2. Materials and methods

2.1. Materials

Dioleoyl phosphatidylethanolamine (DOPE), distearyl phosphatidylethanolamine–polyethylenglycol 2000 (DSPE–PEG2000), 4-nitrobenzo-2-oxa-1,3-diazolyl-DOPE (NBD–DOPE) and lissamine rhodamine B-DOPE (Rho-DOPE) were purchased from AVANTI Polar Lipids Inc. (Alabaster, AL, USA). Poly-L-lysine (PLL, MW 27,400), dicetylphosphate (DCP), *n*-octyl β-D-glucopyranoside (OGP), human holo-transferrin (Tf), 3-(2-pyridyldithio)propionic acid *N*-hydroxysuccinimide ester (SPDP) and dithiothreitol (DTT) were obtained from Sigma–Aldrich Co. (St. Louis, MO, USA). Fluorescein Label IT labeling kit and CX-Rhodamine Label IT labeling kit were purchased from MIRUS Co. (Madison, WI, USA). Plasmid DNA pCMV-luc (8454 bp) encoding luciferase were prepared by EndFree Plasmid Mega Kit (Qiagen GmbH, Hilden, Germany). K562 cells, human chronic myelogenous leukemia cells, were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum in a humidifier incubator (5% CO_2) at 37 °C.

2.2. Preparation of MEND by SUV*-fusion method

The procedure for the preparation of MEND by SUV*-fusion method involves three steps as follows (schematically shown in Fig. 1):

(i) *DNA condensation with PLL*: DNA and PLL were dissolved in 5 mM HEPES buffer (pH 7.4). Labeling of plasmid DNA with fluorescein isothiocyanate (FITC) or rhordamine was performed using a Label IT labeling kit. To condense the plasmid DNA, the DNA solution (0.1 mg/ml) containing FITC (or rhodamine)-labeled DNA (20% of total DNA) was added to a PLL solution (0.1 mg/ml) under vortexing at room temperature. The final DNA concentration of the DNA/PLL complex (DPC), prepared at a nitrogen/phosphate (N/P) ratio of 2.4, was 0.05 mg/ml.

(ii) *Electrostatic interaction of charged SUV* with positively charged DPC*: A diluted DPC suspension (0.25 mg/ml) was added to mixture of lipid (DOPE–NBD (or rhodamine)-labeled DOPE/DCP/DSPE/PEG2000 = 77:5:8:10 (molar ratio)) and detergent OGP containing 5 mM HEPES buffer (pH 7.4). The final concentrations of DNA, lipid and detergent were 0.023 mg/ml, 0.49 μM and 18 mM, respectively. Under these conditions, an SUV* is formed, and the SUV* is bound to the surface of the DPC.

(iii) *Lipid coating of the DPC due to the fusion of SUV* by detergent removal*: The SUV*/DPC suspension was dialyzed six times against 1 l of 5 mM HEPES buffer (pH 7.4) for over 4 h to remove the detergent using regenerated cellulose membrane tubes (Spectra/Por MWCO15000, SpectrumLabs Com., Rancho Dominguez, CA), with a molecular weight cut off size of 15,000 Da. The dialyzed sample was subjected to discontinuous sucrose density gradient ultra-centrifugation for the isolation of the MEND.

2.3. Isolation of MEND by discontinuous sucrose density gradient ultra-centrifugation

A suspension of the MEND was layered on a discontinuous sucrose density gradient (0–40%), and centrifuged at 160,000 × *g* for 2 h at 20 °C. A 1-ml aliquot

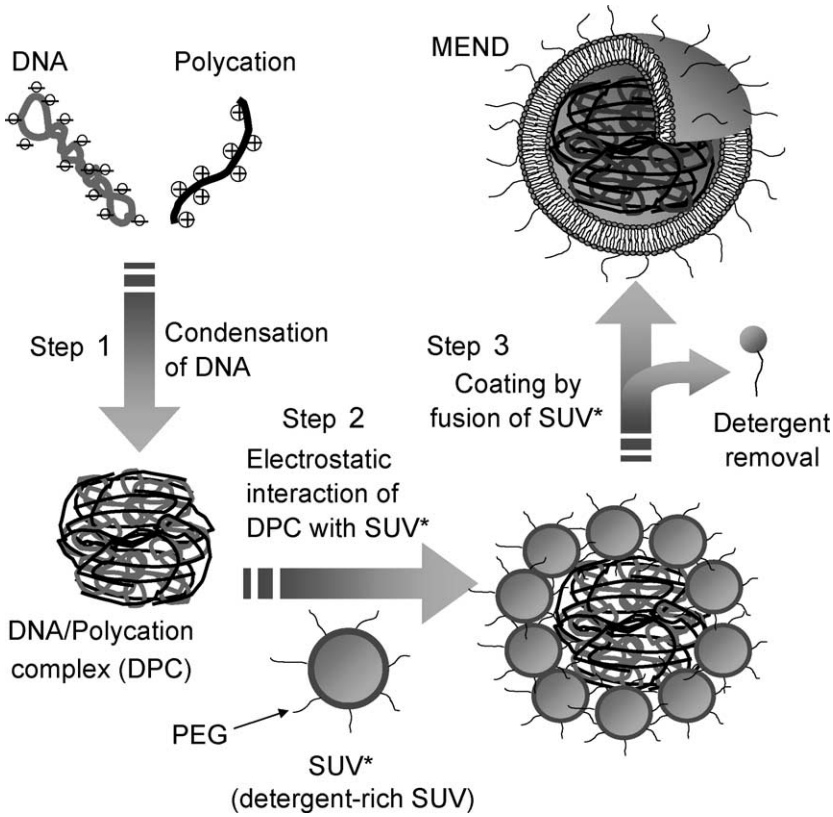

Fig. 1. Three steps involved in constructing MEND by a novel SUV*-fusion method: (1) DNA condensation with a polycation, (2) electrostatic interaction of the charged SUV* with a DNA/polycation complex (DPC) and (3) lipid coating of the DPC due to fusion of the SUV* by detergent removal.

was collected from the top and the fluorescence intensities were measured. The collected fraction containing MEND was then dialyzed three times against 1 l of 5 mM HEPES buffer (pH 7.4) for over 3 h to remove the sucrose. The hydrodynamic diameter was measured by a quasi-elastic light scattering method and the zeta-potential was analyzed electrophoretically by means of an electrophoretic light scattering spectrophotometer (ELS-8000, Otsuka electronics, Japan).

2.4. Electron microscopy of MEND

Samples were rapidly frozen at −196 °C, and fractured at −120 °C with a Freeze replica apparatus (FR-7000B, Hitachi, Japan). An electric discharge was applied to deposit Pt/C, then C on the surface at angles of 45° and 90°. The replicas, mounted on 300-mesh Ni grids, were observed by electron microscopy (JEM200 CX, JEOL, Tokyo, Japan).

2.5. Modification of MEND by transferrin

Modification of the MEND by Tf was performed according to the method reported previously (Hatakeyama et al., 2004). Tf (final concentration of 62.5 μM) was treated with SPDP (final concentration of 66 μM) for 30 min at room temperature. The resulting 3-(2-pyridinedithio)propionyl (PDP)-Tf was separated from the unreacted SPDP by gel filtration on a Sephadex G-25 Fine column equilibrated with phosphate buffered saline (PBS). The PDP-Tf was then reduced with 50 mM DTT in H_2O for 30 min at room temperature to yield 3-mercaptopropyl-Tf, which was purified on a Sephadex G-25 Fine col-

umn. Disulfide cross-linking between Tf and MEND was then conducted by treating 3-mercaptopropyl-Tf with the MEND containing 1 mol% of maleimidic DSPE–PEG2000 at a 1:20 lipid molar ratio at 4 °C overnight. To separate the unreacted Tf, the reaction mixture was centrifuged at 160,000 × *g* for 2 h at 4 °C. Tf-modified MEND was equilibrated with 50 mM $Fe_2(SO_4)_3$–EDTA (pH 7.4, final Fe^{3+} concentration of 100 μM) for Tf resaturation with Fe^{3+}.

2.6. Cellular uptake and intracellular trafficking of Tf-modified MEND

A Tf-modified MEND was prepared using rhodamine-labeled DNA and NBD–DOPE, for functional study, by confocal laser scanning microscope. A suspension of Tf-modified MEND containing 0.5 μg DNA was added to K562 cells (5×10^4 cells/50 μl) suspended in RPMI1640 without serum and antibiotics, and incubated for 3 h at 37 °C in the presence of $Fe_2(SO_4)_3$–EDTA (final Fe^{3+} concentration of 100 μM) as reported previously (Kakudo et al., 2004). Then, 1 ml of RPMI1640 containing 10% fetal calf serum was added to the cells, followed by a further incubation for 15 h. For staining the cells with endosome/lysosome probe Lysosensor (Molecular Probes, Eugene, OR, USA), 1 mM lysosensor (final concentration of 1 μM) was added to the cell suspension of the Tf-modified MEND, and incubated for a further 30 min. The cells were then washed twice with PBS at 4 °C and analyzed by confocal laser scanning microscopy (LSM510, Carl Zeiss, Germany) (Kamiya et al., 2002).

3. Results and discussion

3.1. A novel assembly strategy for construction of MEND: SUV*-fusion method

The SUV*-fusion method consists of three steps as shown in Fig. 1, i.e., (1) DNA condensation with a polycation, (2) electrostatic interaction of the charged SUV*, which has a weak negative charge, with a positively charged DNA/polycation complex and (3) lipid coating of the DPC due to fusion of the SUV* by detergent removal. In the first step, plasmid DNA is condensed electrostatically with a polycation under vortexing at room temperature as described previously (Kogure et al., 2004). In the next step, positively charged DPC is surrounded electrostatically by an excessive amount of negatively charged SUV*. Removal of the detergent by dialysis then induces a lipid coating on the DPC due to membrane fusion of an adjacent SUV*. This SUV*-fusion method has several advantages, i.e., it is quick, simple and mild. Furthermore, the lipid used for the SUV*-fusion method is not restricted.

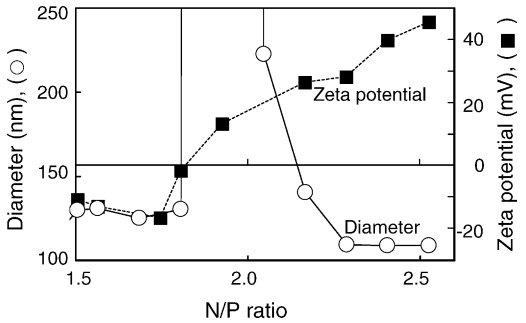

Fig. 2. Optimum conditions for DNA condensation. DPC was prepared at various N/P ratios due to the addition of the plasmid DNA solution (0.1 mg/ml) to the PLL solution (0.1 mg/ml), and the diameter (open circle) and zeta potential of each sample were measured (closed square).

3.2. Optimum condition for DNA condensation

DNA condensation with a polycation is an important step, because the size and charge of the DPC are critical factors in the construction of a MEND by the SUV*-fusion method. Furthermore, a smaller size is preferable for the cellular uptake of a MEND through receptor-mediated endocytosis. We first attempted to determine the optimal conditions for DNA condensation to obtain a small and positively charged DPC. In the present study, we prepared a DPC at various nitrogen/phosphate (N/P) ratios by the addition of a plasmid DNA solution to a polycation poly-L-lysine (PLL) solution, and measured the diameter and zeta potential of each preparation. As shown in Fig. 2, the diameter and zeta potential of the DPC were around 100 nm and positive (40 mV), respectively, above an N/P ratio of 2.3. However, aggregation was observed at N/P ratios between 2.3 and 1.8. The zeta potential of the DPC was neutral at an N/P

ratio 1.8, suggesting that this ratio is the apparent isoelectrostatic point of PLL and DNA under these conditions. The diameter and zeta potential were around 130 nm and −15 mV, respectively, at N/P ratios below 1.8. From these results, a small and positively charged DPC is obtained when the N/P ratios are higher than 2.3. However, an excess of PLL may prevent the electrostatic interaction of the DPC with the SUV* at N/P ratios above 2.4. Thus, small and positively charged DPC should be prepared at an N/P ratio of 2.4.

3.3. Lipid coating of DPC by SUV*-fusion

A negatively charged SUV*, which contains 18 mM of neutral detergent OGP, was prepared for electrostatic binding with positively charged DPC by dilution of a DOPE/DCP/PEG-lipid/OGP mixed micelle, since SUV* is formed during the vesicle formation process from the micelle by the decrease in detergent concentration (Ueno et al., 1997). The mixture of DPC and SUV* was then dialyzed to remove detergent to permit fusion of the SUV*. After detergent removal, we analyzed the distribution of DNA and lipid by discontinuous sucrose density gradient ultra-centrifugation. As shown in Fig. 3, fraction #9, which is the boundary between 25% and 40% of sucrose, contained a high amount of DNA and a small amount of lipid, suggesting that DPC coated with lipid (MEND) is present in this fraction. No complex of free DNA with lipids would exist in the fraction #9, because negatively charged DNA could not interact electrostatically with the lipids containing negatively charged dicetylphosphate. On the other hand, in fractions #1–3, high amounts of lipid and low contents of DNA were also observed. These are probably empty liposomes and uncondensed free DNA. An uncoated DPC was observed at the bottom fraction (fraction #12). The DNA content of the isolated MEND in fraction #9 was 30% of the total DNA. The diameter and zeta potential of the isolated MEND were 155 nm and −24 mV, respectively. Since the zeta potential of the MEND was contrary to that of the DPC, this indicates that the positively charged DPC was coated with negatively charged lipids. Furthermore, the MEND was analyzed by electron microscopy. The MEND appears to be spherical with a diameter of around 150 nm (Fig. 3),

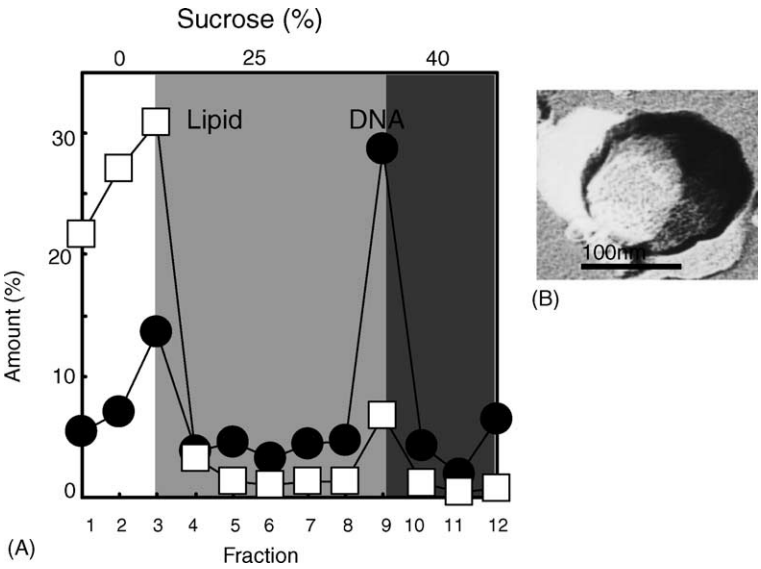

Fig. 3. Purification of a MEND by discontinuous sucrose density gradient ultra-centrifugation. (A) Distribution of DNA and lipids after discontinuous sucrose density gradient ultra-centrifugation of MEND. Amounts of DNA (closed circle) and lipids (open square) were estimated by measuring the fluorescence intensities of FITC and rhodamine in each fraction, respectively. (B) An electronmicroscopic image of isolated MEND. The MEND was isolated in fraction #9 by sucrose density gradient ultra-centrifugation. The fraction was then dialyzed to remove sucrose, and rapidly frozen for freeze-fracture electron microscopy at −196 °C.

which corresponds to diameters measured by quasi-elastic light scattering. It became clear from these results that we succeeded in the construction of a MEND. In the present study, lipid coating by SUV*-fusion occurs during the detergent removal process. The fusion of the SUV* is likely caused easily, because SUV* binding to the DPC surface is very close to each other and detergent removal from the outer leaflet of the SUV* makes the liposomal membrane unstable. The construction of a MEND failed on several conditions in which the PEG lipid was not present and without removal of the detergent from the mixture of DPC and SUV*, because co-existence of lipid and DNA was not recognized in the fraction #9 after sucrose density centrifugation. From these results, it was also confirmed that PEG lipid and detergent removal are essential.

3.4. MEND constructed by only SUV*, not micelle

Cullis and co-workers (Wheeler et al., 1999; Fenske et al., 2002) previously reported on an assembly method for non-viral gene delivery systems by detergent removal from a mixture of DNA and a lipid/detergent micelle. Thus, as well as in our case, there is a possibility that a MEND might be prepared from not only SUV* but also a micelle. We examined the effect of the concentration of detergent and lipid on the construction of a MEND to confirm that only SUV*, but not the micelle, is able to form a MEND. The constructed MEND was evaluated by sucrose density gradient centrifugation for each condition (Fig. 4). The construction of the MEND failed at detergent concentrations higher than 25 mM, which is the critical micellar concentration (CMC) of OGP (Ueno, 1989), because lipid and DNA did not co-exist in the fraction #9, which contained MEND as shown in Fig. 3. Under these conditions, lipids should be solubilized completely and form lipid/detergent mixed micelles (Ueno and Akechi, 1991). However, the construction of the MEND was successful only at concentrations of detergent lower than 20 mM. In these conditions, DNA and lipid were observed in the fraction #9 after sucrose density gradient centrifugation. It is noteworthy that the highest DNA encapsulation efficiency was achieved only under conditions of a lipid concentration of 0.49 μM and a detergent concentration of 18 mM (Fig. 4, closed circles). Since SUV* is formed only at detergent concentration

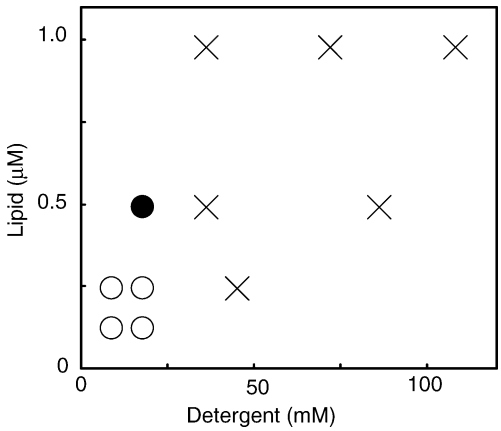

Fig. 4. Optimal condition for SUV*-fusion method. Construction of MEND was examined on the conditions with various concentrations of detergent and lipid. A success (circle) or failure (cross) of construction of MEND was evaluated by sucrose density gradient centrifugation method on each condition. MEND with the highest DNA encapsulation efficiency was obtained only on conditions of a lipid concentration of 0.49 μM, and a detergent concentration of 18 mM (a closed circle).

near to but lower than the CMC (Ueno et al., 1997), this proves that only an SUV*, not a micelle, forms a MEND, and that the SUV*-fusion method differs from the methods of Cullis et al. (Wheeler et al., 1999; Fenske et al., 2002). Moreover, the physicochemical properties of the detergent is a very important factor in the SUV*-fusion method. For example, an SUV* containing negatively charged detergent, such as sodium cholate, would not be suitable to induce fusion because of potent repulsion by the high negative charge of each SUV* and the shielding of the positively charged DPC by the detergent (Ueno et al., 1997). In addition, a well known non-ionic detergent TritonX-100 would not be suitable for the SUV*-fusion method, because the control of the state of the SUV* would be very difficult due to its low CMC (0.25 mM). Thus, a non-ionic detergent OGP which has a high CMC is the best detergent for the SUV*-fusion method.

3.5. MEND can deliver a gene into a cell

We next prepared a transferrin (Tf)-modified MEND containing rhodamine-labeled DNA and NBD-labeled lipid (Tf-MEND) to confirm whether the MEND is capable of delivering a gene into a cell by receptor-mediated endocytosis. The Tf-MEND

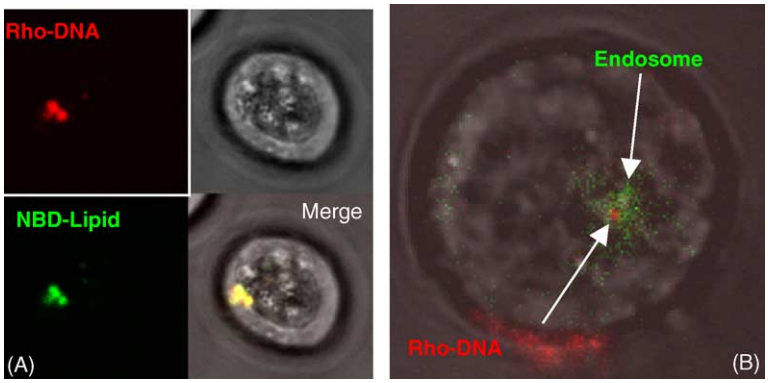

Fig. 5. Confocal laser scanning microscopy of a MEND delivering a gene into a cell via endocytosis. (A) Confocal laser scanning microscopic images of human leukemia K562 cells incubated with Tf-MEND, which contained rhodamine-labeled DNA (Rho-DNA, red) and NBD-labeled lipid (NBD-Lipid, green), for 18 h at 37 °C. (B) A CLSM image of lysosensor (Endosome, green)-stained K562 cells incubated with Tf-MEND containing rhodamine-labeled DNA (Rho-DNA, red) on the same conditions as (A).

was incubated for 18 h with human leukemia cells K562, which contain a large amount of Tf receptor (Klausner et al., 1983), and we observed the intracellular trafficking of MEND by conforcal laser scanning microscopy. As shown in Fig. 5A, rhodamine-labeled DNA (red) and NBD-labeled lipid (green) were clearly observed in the cell, and the merged image showed the co-localization of DNA and lipid in the cytoplasmic space, indicating that DNA encapsulated in lipid was successfully internalized. Furthermore, rhodamine-labeled DNA (red) was co-localized with the endosomal compartment (green), indicating that the Tf-MEND was present in the endosome (Fig. 5B), which is consistent with our previous experiment (Kakudo et al., 2004). In conclusion, we succeeded in the construction of a MEND by a novel SUV*-fusion method, by which a small and homogenous MEND can be constructed. The MEND, equipped with Tf on the surface, is capable of delivering a condensed plasmid DNA via Tf-receptor-mediated endocytosis. Functional optimization is now under investigation for efficient transfection using Tf-MEND.

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