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Multi-layered nanoparticles for penetrating the endosome and nuclear membrane via a step-wise membrane fusion process

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ABSTRACT

Efficient targeting of DNA to the nucleus is a prerequisite for effective gene therapy. The gene-delivery vehicle must penetrate through the plasma membrane, and the DNA-impermeable double-membraned nuclear envelope, and deposit its DNA cargo in a form ready for transcription. Here we introduce a concept for overcoming intracellular membrane barriers that involves step-wise membrane fusion. To achieve this, a nanotechnology was developed that creates a multi-layered nanoparticle, which we refer to as a Tetra-lamellar Multi-functional Envelope-type Nano Device (T-MEND). The critical structural elements of the T-MEND are a DNA-polycation condensed core coated with two nuclear membrane-fusogenic inner envelopes and two endosome-fusogenic outer envelopes, which are shed in stepwise fashion. A double-lamellar membrane structure is required for nuclear delivery via the stepwise fusion of double layered nuclear membrane structure. Intracellular membrane fusions to endosomes and nuclear membranes were verified by spectral imaging of fluorescence resonance energy transfer (FRET) between donor and acceptor fluorophores that had been dually labeled on the liposome surface. Coating the core with the minimum number of nucleus-fusogenic lipid envelopes (i.e., 2) is essential to facilitate transcription. As a result, the T-MEND achieves dramatic levels of transgene expression in non-dividing cells.

1. Introduction

For successful gene delivery, the gene carrier must efficiently enter the cells and subsequently pass through the intracellular space to the nucleus. In this travel, biological membranes such as the plasma membrane, the endosomal membrane and the nuclear membrane are significant barriers that must be overcome. In contrast to dividing cells, in which the nuclear membrane structure is temporally lost, transfection activity is severely diminished in non-dividing cells, since the nuclear permeation of DNA is restricted by virtue of its double-layered membrane structure [1–3]. To date, numerous attempts have been made to overcome this ultimate barrier. It is well known that the translocation of molecules between the cytosol and nucleus normally occurs via the nuclear pore complex (NPC) [4]. Certain viruses (i.e. adenovirus) have evolved mechanisms to co-opt the NPC in order to access the nucleus [5]. Such viral strategies have influenced the design of gene-targeting vectors, for example in models that add nuclear localization signal (NLS) peptides to either the gene carrier [6–8] or the plasmid DNA (pDNA) itself [9–11] to achieve the nuclear delivery of pDNA via the "NPC-dependent pathway". However, this particular strategy has limited success: the positively charged NLS can be neutralized by the anionic pDNA, and it is difficult to control the dimensions of the carrier, to maintain a sufficiently small size to permit it to pass through the nuclear pore (<39 nm). Here we introduce an original strategy for overcoming intracellular membrane barriers such as the endosomal membrane and the nuclear membrane via step-wise membrane fusions. In this strategy, pDNA is delivered to the nucleus via an 'NPC-independent pathway', which is completely different from those evolved by

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viruses. To achieve this, innovative nanotechnology is required to encapsulate DNA into two types of envelopes with different lipid compositions (nucleus-fusogenic and endosome-fusogenic lipids) in a stepwise manner.

We recently developed a Multi-functional Envelope-type Nano Device (MEND) based on a new packaging concept called "Programmed Packaging" [12–15], in which various functional devices that control intracellular trafficking are packed into single nanoparticles so as to allow them to function at the appropriate place and time. The MEND is composed of a condensed pDNA core which is covered with lipid membrane to which functional devices can be assigned to control intracellular trafficking as well as biodistribution. Octaarginine (R8), an artificially designed cell permeable peptide, can be introduced on the surface of the MEND (R8-MEND). The high density R8-MEND can induce macropinocytosis and escape lysosomal degradation, leading to transfection activities as high as that for adenovirus in dividing cells [16,17]. However, in non-dividing cells, the ultimate barrier, nuclear membrane must also be overcome. The R8-MEND cannot afford two kinds of envelopes with different compositions for different membrane fusions.

To solve this problem, an innovative nanotechnology was developed to construct a Tetra-lamellar Multi-functional Envelope-type Nano Device (T-MEND), in which a DNA/polycation core was coated with nuclear and endosomal membranes-fusogenic lipid envelopes in a step-wise manner to overcome these intracellular barriers via serial membrane fusion. Lipid compositions, which are adequate for fusion to the endosome and nuclear membrane were screened by monitoring the cancellation of fluorescence resonance energy transfer (FRET) between donor and acceptor fluorophores, modified on the surface of liposomes. The dually labeled liposomes were also useful probes for visualizing the intracellular fusion event, in which FRET activity can be monitored by means of spectral imaging. This technology has recently been used to evaluate intracellular fusion in endosomes [18] and mitochondria [19]. To verify the step-wise membrane fusion process, the inner or outer envelopes of T-MENDs were labeled by donor and acceptor, and the fusion of each envelope to the endosome and nucleus was then visualized by spectral imaging.

2. Materials and methods

Egg yolk phosphatidylcholine (EPC), 1,2-dioleoyl-*sn*-glycero-3-phosphatidylethanolamine (DOPE), 7-nitrobenz-2-oxa-1,3-diazole labeled DOPE (NBD-DOPE) and rhodamine labeled DOPE (Rho-DOPE) were purchased from Avanti Polar lipids (Alabaster, AL, USA). Cholesteryl hemisuccinate (5-cholesten-3-ol-3-hemisuccinate; CHEMS), cardiolipin (CL), phosphatidic acid (PA), phosphatidyl glycerol (PG), phosphatidyl inositol (PI), phosphatidyl serine (PS), and sphingomyelin (SM) were purchased form Sigma (St. Louis, MO, USA). Stearyl octaargine (STR-R8) was a generous gift from Prof. Futaki (Kyoto University, Japan).

2.1. Screening of lipid compositions for fusogenic activity to nuclear membranes by FRET analysis

Various compositions of liposomes labeled with both 1 mol% NBD-DOPE (Avanti, Alabaster, AL) and 0.5 mol% rhodamine-DOPE (Avanti) were prepared [20,21]. A 10 μL aliquot of labeled liposome (lipid concentration, 0.55 mM) was added to nuclei isolated from HeLa cells (corresponding to the 0.075 mM of phosphatidylcholine, determined by a Test Kit, Wako) in 90 μL of nuclear transport buffer (20 mM HEPES, 110 mM KOAc, 3 mM NaOAc, 2 mM MgOAc, 0.5 mM EGTA 4Na and 2 mM DTT), which was then incubated for 30 min at 37 °C. After incubation, energy transfer was assessed by measuring the fluorescence intensity (excitation at 470 nm and emission at 530 nm).

The maximum fluorescence is defined as the fluorescence of liposomes when dissolved in Triton X-100 (final concentration of 0.5%). Fusion activity (%) was estimated by the extent of recovery as follows:

$$\text{Fusion activity}(\%) = (F - F_0)/(F_{\max} - F_0) \times 100$$

where F, F_0 and $F_{\max}$ represent the fluorescence intensity of labeled liposome after incubation with nuclei, the fluorescence intensity of labeled liposome after incubation without nuclei, and the maximum fluorescence intensity after treatment with Triton X-100, respectively.

2.2. Delivery of GFP to isolated nuclei via nuclear membrane-fusogenic lipid

GFP was encapsulated in nuclear membrane-fusogenic (CL/DOPE = 1/1) or poorly fusogenic (EPC/Chol = 9/2) liposomes labeled with rhodamine-DOPE. Then, a 90 μL aliquot of GFP (0.125 mg/mL) encapsulated in liposomes was applied to 90 μL of isolated nuclei (0.076 mM of total lipid) to a final lipid concentration of 0.55 μM, followed by incubation for 30 min at 25 °C. Nuclei were stained with Hoechst 33342 (Sigma, St Louis, USA) and fluorescence images were captured with an LSM510 META (Carl Zeiss Co. Ltd., Jena, Germany) confocal laser scanning microscope equipped with an oil-immersion objective lens (Plan-Apochromat 63×/NA = 1.4).

2.3. Preparation of T-MEND

Lipid films composed of DOPE/CL = 1/1 (total lipid content: 0.55 μmol) were hydrated with 1 mL of 10 mM HEPES Buffer (pH 7.4) for 10 min at room temperature. The hydrated lipid film was then sonicated using a probe-type sonicator to form SUV. Condensed DNA particles were prepared by mixing 50 μL of DNA solution (0.1 mg/mL) with 75 μL of protamine solution (0.1 mg/mL) with vortexing (final -/+ ratio = 2.2). The suspended nuclear membrane-fusogenic liposomes and condensed DNA particles were then mixed at a ratio of 2:1 (v/v) to coat the condensed DNA particles with a double-lipid envelope as described previously [22]. A stearylated octaarginine (STR-R8) solution (20 mol% of total lipid) was added to the suspension of double-layered nuclear membrane-fusogenic particles. Then, this suspension of nuclear membrane-fusogenic particles was mixed with endosome-fusogenic liposomes at a ratio of 1:2 (v/v) to generate particles with a double endosome-fusogenic envelope, which we refer to as T-MEND. Stearylated octaarginine (STR-R8) solution (10 mol% of endosome-fusogenic lipid) was added to the suspension of T-MEND to modify the outer envelope with R8. When fusion-poor lipids were used to prepare the inner, outer and both of the envelopes, fusion among the neighboring SUVs was induced by the dropwise addition of 1 N HCl. Particle formation was confirmed by discontinuous sucrose density gradient ultracentrifugation, as reported previously [13].

2.4. Functional analysis of T-MEND

JAWS II cells derived from murine dendritic cells were purchased from the ATCC, and cultured in αMEM (Sigma) containing 20% FCS, 4 mM L-glutamine, 1 mM sodium pyruvate and 5 ng/mL of GM-CSF. The cells were trypsinized with 0.25% tripsin/0.53 mM EDTA for cell passage. For transfection, 8×10^4 cells of JAWS II cells were cultured in a 24-well dish for 1 day. The cells were washed with phosphate buffer saline (PBS), and the medium was then replaced with 0.5 mL of serum-free medium. T-MENDs containing 0.4 μg of pDNA were applied to the medium, and incubated for 3 h. Thereafter, medium was replaced with fresh medium containing 20% serum, and further incubated for 21 h. A subsequent luciferase assay was performed as reported previously [23] A cell counting experiment confirmed that cell growth was negligible at least within 2 days, when the cells were seeded in a sub-confluent condition (8×10^4 cells/24-well dish).

2.5. Phase-contrast transmission electron microscopy (TEM)

A suspension of T-MEND was dropped onto a copper grid coated with a carbon film. After carefully removing excess liquid with the tip of a filter paper, the sample was rapidly frozen in liquid ethane using a Leica rapid-freezing device (Leica EM CPC system). The grid with ice-embedded samples was transferred to the specimen chamber of a transmission electron microscope using a cryo-transfer system. The specimen chamber was cooled with liquid helium to reduce specimen damage caused by the electron beam. For observation, a JEOL JEM-3100FFC electron microscope with the HDC phase plate inserted into the back focal plane of the objective lens was operated at 300 kV [24,25].

2.6. Analysis of intracellular trafficking of pDNA and fusion events in living cells

For the imaging of fusion events, the outer (endosome-fusogenic) or inner membrane (nuclear membrane-fusogenic envelope) was labeled with 1 mol% NBD-DOPE (excitation at 460 nm and emission at 530 nm) and 0.5 mol% rhodamine-DOPE (excitation at 550 nm and emission at 590 nm) as a donor and acceptor of FRET, respectively. The cells were seeded on 35 mm glass base dishes (IWAKI, Tokyo, Japan), and the labeled T-MEND (0.3 μg pDNA/dish) was then incubated with JAWS II cells. To analyze for fusion between the outer envelope and the endosome, the T-MENDs were incubated with cells for 1 h, and the endosomes were then stained with 0.04 μM of LysoTracker DND-99 (ex: 570 nm, em: 600 nm) immediately before observation. Fluorescence images were captured with an LSM510 META equipped with an oil-immersion objective lens (Plan-Apochromat 63×/NA = 1.4) in phenol red-free DMEN/F-12 medium supplemented with 10% FCS, 4 mM L-glutamine and 10 mM HEPES. The NBD was then excited by light (488 nm) from an argon laser. The emitted light was filtered through a dichroic mirror