Research Article

Nucleic Acid Delivery System with DOP-DEDA, a Charge-Reversible Lipid Derivative

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Abstract

We have developed dioleoylglycerophosphate-diethylenediamine (DOP-DEDA) for nucleic acid delivery using lipid nanoparticles (LNPs). DOP-DEDA is a charge-reversible lipid derivative in which the net charge of the head group changes from -1 to +2 depending on pH, and can be applied to the delivery of small interfering RNA (siRNA) and mRNA. Here, we introduce siRNA delivery using DOP-DEDA-based LNP (DOP-DEDA LNP). siRNA-encapsulated DOP-DEDA LNP prepared by micromixing were shown to be uniform spherical particles having a particle size of about 100 nm. DOP-DEDA LNP showed high dispersibility and formed uniform particles without the use of polyethylene glycol (PEG) lipids because of the amphipathic property of DOP-DEDA. The surface charge of DOP-DEDA LNP was almost neutral at physiological pH and cationic under acidic conditions. DOP-DEDA LNP showed high pH responsiveness, suggesting high endosomal escapability. Potent gene silencing effects were observed in cancer cells transfected with siRNA encapsulated in DOP-DEDA LNP, even at low siRNA concentration. Our findings indicate that DOP-DEDA is a pH-responsive lipid derivative having characteristics different from those of general ionizable lipids, and is expected to contribute the development of nucleic acid drugs and mRNA vaccines.

Keywords: dioleoylglycerophosphate-diethylenediamine (DOP-DEDA), lipid nanoparticle (LNP), DOP-DEDA LNP, charge-reversible lipid derivative, nucleic acid delivery system

Introduction

In recent years, nucleic acid drugs have received a great deal of attention as a new modality in drug discovery. With the progress of chemical modification of nucleic acid and drug delivery system (DDS) technologies, RNA interference drugs and mRNA vaccines have come into practical use. ONPATTRO®, the first RNA interference drug in the world, developed by Alnylam Pharmaceuticals Inc. is a lipid nanoparticle (LNP) formulation of a small interfering RNA (siRNA). LNP technology is also used in the mRNA vaccine "mRNA-1273" for coronavirus disease 2019 (COVID-19) developed by Moderna, Inc. and the vaccine "COMIRNATY®" developed by BioNTech SE and Pfizer Inc. Encapsulation of siRNA or mRNA in LNP significantly improves the stability and the transfection efficiency of RNA *in vivo*, resulting in the effects of RNA interference drugs and mRNA vaccines, respectively. In general, the lipid component of LNP includes a pH-responsive lipid derivative that is essential for encapsulation of nucleic acids, their delivery into cells, and their endosomal escape. DLin-MC3-DMA, SM-102, and ALC-0315 are pH-responsive lipid derivatives formulated in ONPATTRO, mRNA-1273, and COMIRNATY, respectively (1). The common structure of these lipid derivatives is that they have a tertiary amine on their head group, and the head group is ionized and positively charged only under acidic conditions. Therefore, these lipid derivatives are called an ionizable lipid. The performance of LNP in nucleic acid delivery depends on the performance of the pH-responsive lipid derivative. To achieve safe and highly efficient nucleic acid delivery, it is necessary to appropriately design the pH-responsive lipid derivative and then optimize the LNP formulation such as the lipid composition. We have studied the nucleic acid delivery system and developed original pH-responsive lipid derivatives for LNP formulation (2, 3). Recently, in collaboration with Nippon Fine Chemical Co., Ltd., we have newly designed dioleoylglycerophosphate-diethylenediamine (DOP-DEDA),

an amphipathic charge-reversible lipid derivative with high pH responsiveness and prepared DOP-DEDA-based LNP (DOP-DEDA LNP) for efficient nucleic acid delivery (4, 5). Here, we introduce the nucleic acid delivery system using DOP-DEDA LNP.

Structural features of DOP-DEDA

The structural formula of DOP-DEDA is shown in **Fig. 1**. The basic skeleton of DOP-DEDA is very similar to that of natural glycerophospholipids. In DOP-DEDA, two oleic acids and phosphoric acid are bound to the glycerol skeleton, and DEDA is bound via phosphoric acid. DOP-DEDA is a lipid derivative in which the net charge of the head group changes from -1 to +2 depending on pH. Under acidic conditions, the head group of DOP-DEDA is positively charged and therefore electrostatically interacts with negatively charged nucleic acids (siRNA, mRNA, etc.). Therefore, by mixing the component lipids including DOP-DEDA and nucleic acids under acidic condition in a microchannel, DOP-DEDA LNP encapsulating nucleic acids can be prepared (**Fig. 2**). The surface charge of DOP-DEDA LNP encapsulating nucleic acids is almost neutral at physiological pH and positively charged under acidic conditions. In addition, since it is negatively charged under alkaline conditions, DOP-DEDA LNP is an LNP that has the property of charge inversion depending on pH. DOP-DEDA is a state in which the head group is ionized even at physiological pH, and the positive and negative charges are offset so that the net charge is almost zero (**Fig. 3**). Since the head group of general ionizable lipids containing a tertiary amine is not ionized at physiological pH, the authors consider that it is not appropriate to classify DOP-DEDA as an ionizable lipid. Therefore, we refer it to as a charge-reversible lipid derivative or pH-responsive lipid derivative. The properties of DOP-DEDA, which does not show cationicity under physiological conditions and behaves as a neutral lipid, are considered to be advantageous in terms of safety in the same manner as general ionizable lipids.

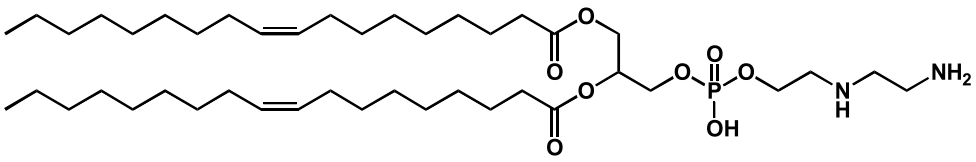

Figure 1. Structure of DOP-DEDA

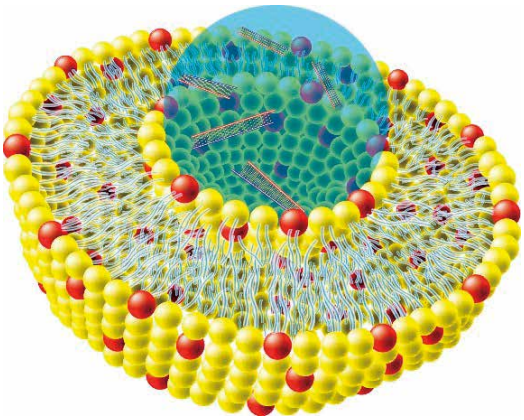

Figure 2. Schematic image of DOP-DEDA LNP

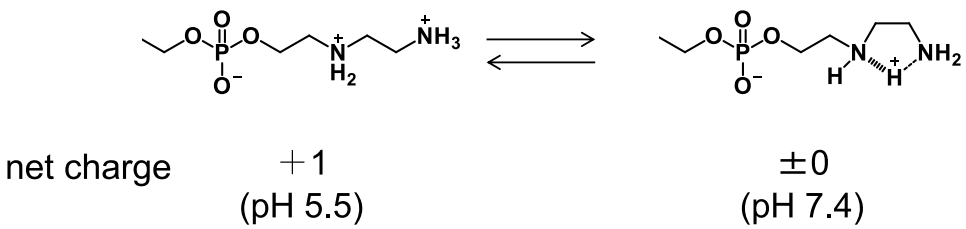

Figure 3. Change in the net charge of DOP-DEDA depending on pH

DOP-DEDA has the characteristic of having physicochemical properties similar to amphipathic glycerophospholipids at physiological pH, which is different from tertiary amine-containing ionizable lipids. Since the head group of the ionizable lipid at physiological pH is not ionized, the polarity of the head group is lower than that of general amphipathic phospholipids. On the other hand, since DOP-DEDA behaves as an amphipathic phospholipid at physiological pH, DOP-DEDA LNP has excellent dispersibility and stability in an aqueous solution. Therefore, even if the lipid composition of DOP-DEDA LNP does not contain polyethylene glycol (PEG)-lipids, it is stable in the aqueous solution. LNPs containing ionizable lipids generally contain less than a few mole percent of PEG-lipids relative to total lipids, and it is known that these PEG-lipids contribute to stable particle formation. In fact, the authors tried to prepare LNPs containing commercially available ionizable lipids without PEG-lipids, we could not obtain uniform particles due to lipid aggregation. In addition, it was reported that ionizable lipid-based LNPs were not formed due to aggregation unless PEG-lipid was added (6). A cryogenic transmission electron microscopy (cryo-TEM) image of DOP-DEDA LNPs encapsulating siRNA is shown in **Fig. 4**. The LNPs are formed with a composition of DOP-DEDA / dipalmitoylphosphatidylcholine (DPPC)/cholesterol = 45/10/45 (molar ratio). This LNP formulation contains no PEG-lipids. We believe that DOP-DEDA can be differentiated from conventional ionizable lipids in that it can form highly dispersible LNPs without the use of PEG-lipids. Concerns about PEG-lipids have been raised in the side effects of mRNA vaccines (LNP preparations) (7). The fact that DOP-DEDA does not require PEG-lipids for LNP formation may be an important advantage.

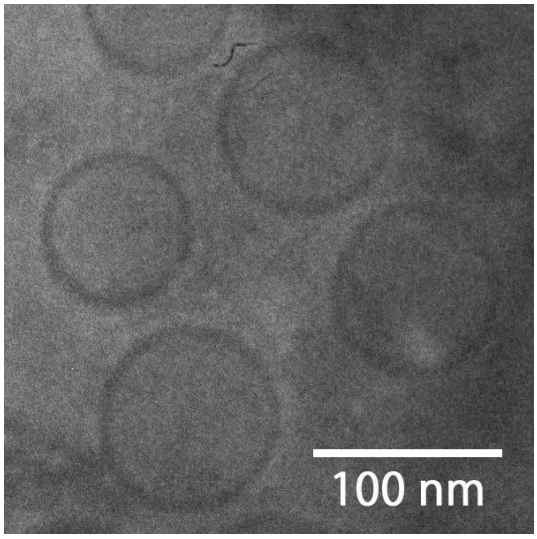

Figure 4. Cryo-TEM image of DOP-DEDA LNPs encapsulating siRNA
DOP-DEDA/DPPC/cholesterol = 45/10/45 (molar ratio), total lipids/siRNA = 7000/1 (molar ratio)

Preparation of DOP-DEDA LNP

DOP-DEDA LNP can be prepared by a method using a microchannel like LNP containing an ionizable lipid. It can be prepared by mixing a mixed lipid dissolved in alcohol and RNA dissolved in an aqueous solvent in a microchannel, and then removing the alcohol by dialysis. We first prepared a freeze-dried mixture of DOP-DEDA and helper lipids, and added alcohol to the lyophilized mixture to dissolve the mixed lipids. The alcohol we used is *tert*-butanol or ethanol. Because the solubility of mixed lipids in alcohol is higher with *tert*-butanol than with ethanol, *tert*-butanol is useful when a high concentration lipid solution needs to be prepared. However, it is considered preferable to dissolve the mixed lipids with ethanol for practical use.

We use 1 mM citric acid solution (pH 4.5) as the aqueous solvent for dissolving siRNA or mRNA. The concentration of citric acid is one of the factors that affect the formation of LNP. When mixed lipids containing DOP-DEDA dissolved in ethanol and siRNA or mRNA dissolved in an aqueous citric acid solution are mixed in the microchannel, intermediate particles of DOP-DEDA LNP are formed. The intermediate particles are transferred in a dialysis membrane and dialyzed in water to remove ethanol. The final DOP-DEDA LNPs are formed by dialysis.

The particle size and polydispersity index (PdI) of DOP-DEDA LNP can be controlled by adjusting preparation conditions such as alcohol/water solvent

ratio and lipid concentration. The optimal conditions for adjusting the LNP to the desired particle size should be determined using the microchannels that you use. We usually prepare DOP-DEDA LNP with the average particle size of about 100 nm and the PdI of less than 0.100. **Table 1** shows our results of the particle size, PdI, ζ- potential, and siRNA encapsulation efficiency of DOP-DEDA LNP. The encapsulation efficiency of siRNA and mRNA in DOP-DEDA LNP can be calculated using the RiboGreen® reagent. When the encapsulation efficiency of siRNA or mRNA into DOP-DEDA LNP was examined by this method, it was more than 95%.

Table 1. Particle size, PDI, ζ-potential, and siRNA encapsulation efficiency of DOP-DEDA LNP

Particle size (nm)	PdI	ζ-Potential (mV)	Encapsulation efficiency (%)
98.3 ± 7.20	0.08 ± 0.02	+0.8 ± 0.2	97.8

DOP-DEDA/DPPC/cholesterol = 45/10/45 (molar ratio), total lipids/siRNA = 7000/1 (molar ratio)
DOP-DEDA LNP was diluted with 10 mM Tris-HCl buffer (pH 7.4) for the measurement of ζ-potential.

pH responsiveness of DOP-DEDA LNP

To deliver nucleic acids into the cytoplasm using LNPs, it is necessary for LNPs taken up by the cell via endocytosis and escape from the endosome. LNPs with high escapability from endosomes efficiently deliver nucleic acids into the cytoplasm, resulting in beneficial effects. After pH-responsive LNPs are endocytosed into cells, the pH-responsive lipids of LNPs are protonated under acidic condition in endosomes (pH 5-6), and LNPs become positively charged. As protons are absorbed by LNP, protons and anions flow into the endosome from the outside, increasing the salt concentration and osmotic pressure in the endosome, and destabilizing the endosome. In addition, because positively charged LNPs easily interact with the inner membrane of endosomes, the escape of LNPs from endosomes is promoted. Therefore, pH responsiveness of LNPs is a determinant of the efficiency of nucleic acid delivery.

One of the methods for evaluating the pH responsiveness is an assay using 2-(*p*-Toluidino)-naphthalene-6-sulfonic acid (TNS). **Figure 5** shows the results of evaluating the pH responsiveness of DOP-DEDA LNP encapsulating siRNA by the TNS assay. The fluorescence intensity of TNS increases as the pH decreases, indicating that the surface charge of DOP-DEDA LNPs become more positive with decreasing pH. As mentioned above, the net charge of DOP-DEDA varies from -1 to +2. On the other hand, the LNPs used as a control in the TNS assay contain ionizable lipids, the net charge change of which is 0 to +1. Reflecting this difference, DOP-DEDA LNPs have a greater change in fluorescence intensity in response to pH changes. The apparent pKa of DOP-DEDA was calculated from the titration curve to be about 6.5. When DOP-DEDA LNPs were incubated with bovine erythrocytes, they induced hemolysis at pH 5.5, endosome pH, but did not induce hemolysis at pH 7.4 (5). This result suggests that DOP-DEDA LNPs do not affect the barrier capacity of the membrane under physiological pH condition, but destabilize the membrane under endosomal pH condition. The time-lapse imaging using a confocal laser scanning microscope for analyzing the intracellular dynamics of the siRNA showed that siRNA transfected with DOP-DEDA LNPs diffused throughout the cytoplasm (5). From these results, it is considered that DOP-DEDA LNP having high pH responsiveness has excellent endosomal escapability and is useful as a carrier for delivering nucleic acids into the cytoplasm.

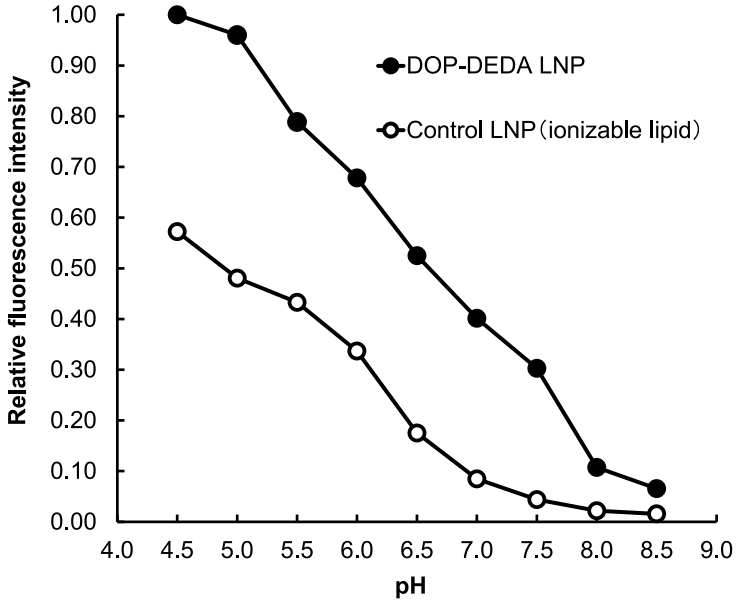

Figure 5. Titration of DOP-DEDA LNP by the TNS assay
DOP-DEDA/DPPC/cholesterol = 45/10/45 (molar ratio)
Control LNP: control ionizable lipid /DSPC/cholesterol/DMG-PEG2000 = 50/10/38.5/1.5 (molar ratio)
Concentration of DOP-DEDA and control LNPs: 20 μM as a total lipid concentration
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siRNA delivery system using DOP-DEDA LNP

DOP-DEDA LNPs encapsulating siRNA targeting polo-like kinase 1 (siPLK1), a cell cycle control protein, were prepared to evaluate their gene silencing effects on MDA-MB-231 human breast cancer cells. The expression of PLK1 mRNA was measured by RT-PCR 72 h after the transfection of MDA-MB-231 cells with siPLK1. DOP-DEDA LNPs encapsulating siPLK1 significantly induced gene silencing at a low siRNA concentration of 3 to 15 nM in a dose dependent manner (**Fig. 6a**). In contrast, DOP-DEDA LNP encapsulating scrambled siRNA did not induce any gene silencing effect. The amount of PLK1 protein was examined by western blotting 72 h after the transfection of MDA-MB-231 cells with siPLK1. Consistent with the results of RT-PCR, western blotting data showed that DOP-DEDA LNPs encapsulating siPLK1 suppressed the expression of PLK1 protein at a low siRNA concentration (**Fig. 6b**). Damage of DOP-DEDA LNPs to cell membrane was examined by lactate dehydrogenase (LDH) assay. The results showed that DOP-DEDA LNPs encapsulating siPLK1 can induce significant gene silencing without membrane damage (data not shown). Gene silencing using DOP-DEDA LNP encapsulating siRNA was also confirmed with other cancer cell lines such as HT1080 human fibrosarcoma cells and immune cells such as macrophages. The transfection efficiency of DOP-DEDA LNPs differs depending on the cells used, but the reason has not been clarified at present. If we can clarify the kind of cells that are targetable by DOP-DEDA LNPs and the mechanism of delivery with them in future studies, it may lead to cell-selective treatment. In the case of LNPs containing ionizable lipids, the cellular uptake mechanism mediated by the apolipoprotein E (ApoE) receptor is well known (8). In addition, the enhanced expression of ApoE receptor on MDA-MB-231 cells was reported (9). Therefore, we investigated whether DOP-DEDA LNPs were also taken up by the same mechanism. It was shown that DOP-DEDA LNPs were taken up by MDA-MB-231 cells in an ApoE dose-dependent manner (5). However, there are still many unclear points about the cellular uptake mechanism of DOP-DEDA LNP.

One of the features of DOP-DEDA LNP is that it forms a stable LNP even in the absence of PEG-lipids. However, it is also possible to modify DOP-DEDA LNP with PEG to improve blood retention. In fact, PEGylated DOP-DEDA LNPs injected intravenously accumulated in solid tumors due to enhanced permeability and retention (EPR) effects in tumor-bearing mice (4). In addition, the silencing of the target gene was confirmed in the solid tumors after the intravenous injection of siRNA encapsulated in DOP-DEDA LNPs (4).

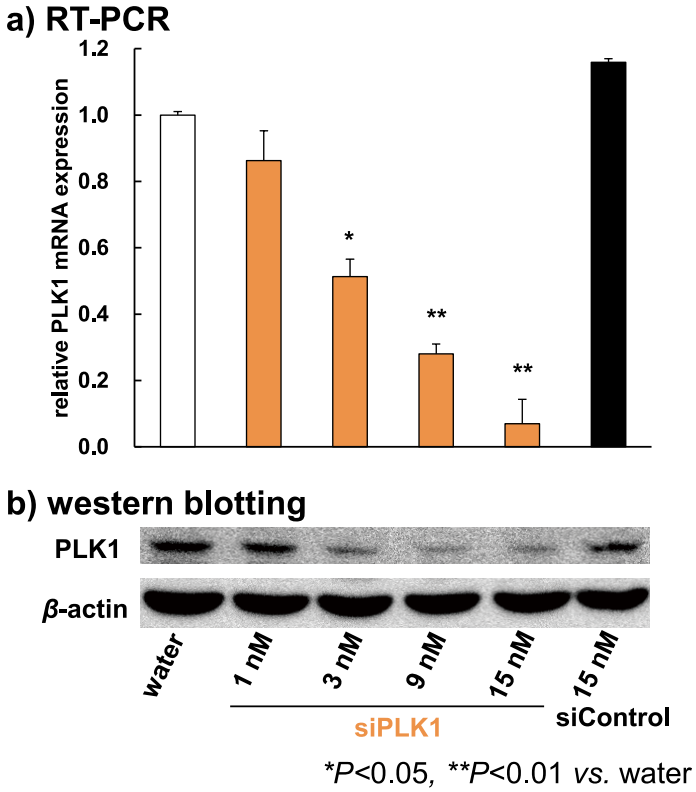

Figure 6. Gene silencing in MDA-MB-231 cells transfected with siRNA-encapsulated DOP-DEDA LNPs
The figure is reused and modified from (5) with permission of Elsevier.

Conclusion

The medical application of lipid-based nanoparticles began with the liposome formulations such as AmBisome® and Doxil®, which were developed in the 1990s. In recent years, LNP formulations such as ONPATTRO and COVID-19 vaccine (mRNA-1273, COMIRNATY) had a great impact on drug and vaccine developments. It has been proven by history that lipid-based nanoparticles are useful and practical in the medical application of nano-DDS. Since small vesicles (exosomes, organelles, etc.) surrounded by lipid membranes have various functions in living organisms, the range of applications of artificially created lipid vesicles is expected to expand more. In particular, nano-DDS, which mimics the physiological transport system, is considered to be a promising approach for innovative drug development. Lipid-based DDS is an old but new technology.

Here, we introduced a siRNA delivery system using DOP-DEDA LNP, but our study also showed that DOP-DEDA LNP can also be applied to mRNA delivery. In addition, it may be applicable to protein delivery. Since DOP-DEDA is a glycerophospholipid derivative having characteristics different from those of ionizable lipids, it is considered that there may be applications that utilize their own characteristics and advantages. We hope that our DOP-DEDA technology contributes to the development of nucleic acid drugs and mRNA vaccines.

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Related Product

DOP-DEDA	50mg	D5882

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