Photo-enhancement of Transfection Efficiency with a Novel Azobenzene-based Cationic Lipid

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The transfection efficiency of a novel photoresponsive cationic lipid having an azobenzene structure can be controlled by UV irradiation. After DNA complexes pass through the cell membrane by endocytosis, trans to cis isomerization of the azobenzene structure would destabilize the vesicle membrane.

Lysosomal degradation is the most common fate of internalized drug unless specific release mechanisms have been incorporated to facilitate drug escape from endocytic vesicle. Light, as stimulation source, is superior in terms of temporal and spatial manipulability, and if light irradiation is able to destabilize endocytic vesicle membranes to improve escape of an agent from the vesicles and delivery of the agent into the cytosol, then an ideal intracellular delivery system could be developed. To date, numerous studies have investigated the regulation of membrane transport of ions[1] and therapeutic agents[2] using photochromic lipids. Furthermore, it has been reported that using photocleavable lipid, plasmalogens,[3] and photopolymerizable phospholipids,[4] fusion with endocytic vesicles was successfully accelerated by light irradiating the membrane structure to destabilize it.

On the other hand, in order to improve the transfection efficiency of nonviral vectors, nonviral vectors must overcome several intracellular barriers before mRNA transcription.[5] With lipoplexes (cationic lipid/DNA complexes),[6] the biggest obstacle is the escape of DNA from endocytic vesicles after passing through cell membrane by endocytosis.[7]

Prasmickaite et al.[8] used a phthalocyanine derivative as a photosensitizer, and by destabilizing the membrane of endocytic vesicles with light irradiation, they successfully facilitated the escape of an exogenous gene and improved transfection efficiency. However, this method is highly toxic,[9] and although the transfection efficiency of polyplexes is improved, that of lipoplexes is reduced. With the exception of this type of photochemical internalization using photosensitizers, no studies have investigated the delivery of exogenous genes into the cytoplasm using photoresponsive system. In the hope of facilitating the escape of a gene from endocytic vesicles and improving transfection efficiency, we have investigated photoresponsive gene carriers using UV irradiation during gene delivery in order to destabilize endocytic vesicle membranes and to facilitate membrane fusion. In the present study, we synthesized a novel cationic lipid having a photoisomerizable azobenzene structure, and successfully improved the transfection efficiency by UV irradiation.

Figure 1 shows the chemical structure of **KAON12**, a novel photoresponsive cationic lipid.[10] The azobenzene structure served as the photoresponsive region because UV induces marked structural and dipolar changes. **KAON12** has a lysine residue (basic hydrophilic group) and a didodecylamide structure

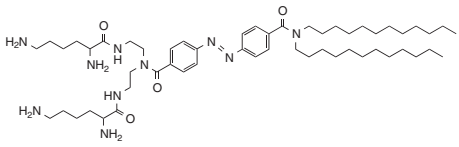

Figure 1. Structure of **KAON12**.

(hydrophobic region). When preparing liposomes, small unilamellar vesicles (SUVs) were produced using 100% *trans*-**KAON12** (stored in darkness) according to conventional methods.[11]

Self-assembling behavior of **KAON12** was examined by transmission electron microscopy (Figure 2). With *trans*-azobenzene, SUVs with a diameter of approximately 20 nm were seen. However, with UV (365 nm) irradiation, the azobenzene structure underwent trans to cis isomerization, and lamellar structures other than SUVs appeared (arrows), thus suggesting membrane fusion. This trans to cis isomerization of the azobenzene structure within SUVs was also confirmed on absorption spectra. At photostationary state, 57% of azobenzene structures underwent trans to cis isomerization, and the average particle size, measured by dynamic light scattering, changed from 28.8 to 209 nm. In other words, this UV-induced membrane fusion was also supported in terms of changes in particle size, thus agreeing with the results of the transmission electron microscopy. Gel shift assay using prepared liposomes and pGL3-control (Promega, Madison, WI, USA) plasmids was performed in order to assess the stability of the resulting DNA complexes.[12] When polyethyleneimine (PEI, Mw: 25000, Aldrich) was used as comparison, no free DNA was observed at a charge ratio of 0.8. On the other hand, no free DNA was detected at 0.6 and 1.6, respectively, with either of 100% and 43% *trans*-azobenzene. These results showed that the affinity of 100% *trans*-**KAON12** towards DNA was higher than that of PEI, but this decreased as a result of UV-induced membrane fusion.

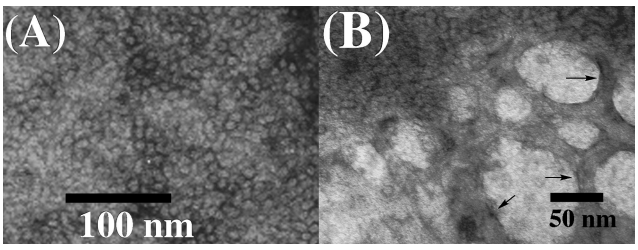

Figure 2. Transmission electron micrograph of **KAON12** membrane before (A) and after (B) UV irradiation for 5 min. The membrane is negatively stained with uranyl acetate.

In a transfection experiment using the cationic lipid, all procedures were carried out in darkness.[13] Table 1 shows the

Table 1. Transfection Efficiency of DNA Complexes with Cationic Lipids

Gene carrier	Transfection efficiency (RLU/mg protein)	
	UV: −[a]	UV: +[a]
KAON12	$(2.27 \pm 0.42) \times 10^5$	$(4.70 \pm 0.35) \times 10^5$
Lipofectin	$(1.00 \pm 0.02) \times 10^5$	$(1.25 \pm 0.10) \times 10^5$

[a]− or + represent the transfection without or with UV irradiation, respectively.

results of the transfection experiment. In this experiment, Lipofectin, a commercially available cationic lipid genetransfecting agent, was used for comparison. Even without UV irradiation, the transfection efficiency of **KAON12** was more than twice that of Lipofectin, and UV irradiation further improved the transfection efficiency of **KAON12**.

Furthermore, the intracellular trafficking of added DNA/lipids complex was examined by confocal fluorescence microscopy. First of all, by transfecting the pEGFP-Endo gene (which expresses a green fluorescent protein on the lipid membrane of cytoplasmic vesicles such as endosomes) into COS-1 cells, the lipid membrane of endocytic vesicles was visualized green.[14] Next, DNA/lipids complex, where *trans*-**KAON12** was mixed with 1% rhodamine-modified phosphotidylethanolamine, was prepared and added to cells. As was the case with transfection, after three hours of incubation, the cells were fixed and examined by confocal fluorescence microscopy (data not shown). The result showed that the localization of the red fluorescence for administered carrier lipids mostly matched green fluorescence. This finding confirms not only the existence of the DNA complex in endocytic vesicles but also the internalization of the DNA complex by endocytosis.

In conclusion, we confirmed that the transfection efficiency of this novel photoresponsive cationic lipid having an azobenzene structure can be controlled by UV irradiation. After vesicles pass through the cell membrane by endocytosis, trans to cis isomerization of the azobenzene structure would destabilize the vesicle membrane, thus accelerating membrane fusion. This aids the escape of genes from the endocytic vesicles, thus improving transfection efficiency.

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10 Selected data for **KAON12**; mp 133.5–136 °C; IR (Neat) ν_{max} 3308 (NH) and 1715–1640 (C=O) cm^{-1}; δ (DMSO-d_6; 130 °C, 400 MHz): 0.86 (t, $J = 6.8$ Hz, 6 H, CH_3), 1.23–1.87 (m, 52 H, CCH_2C), 2.83 (dt, $J = 7.6$ Hz, 4 H, $H_3N\underline{CH_2}$), 3.31 (t, $J = 7.6$ Hz, 4 H, $OCH_2CON\underline{CH_2}$), 3.40–3.78 (m, 8 H, $N\underline{CH_2CH_2}N$), 3.89 and 4.27 (s(br) × 2, 2 H, CH), 4.86 (s, 2 H, $\underline{OCH_2}$), 7.11 (d, $J = 8.8$ Hz, 2 H, *o*-ArH to OCH_2), 7.84 (d, $J = 8.8$ Hz, 2 H, *m*-ArH to CO), 7.87 (d, $J = 8.8$ Hz, 2 H, *m*-ArH to OCH_2), 8.09 (d, $J = 8.8$ Hz, 2 H, *o*-ArH to CO), 8.70 (s(br) × 2, 2 H, CONH); FAB-MS(+) m/z 977.9 $[M + H]^+$; calcd for $C_{55}H_{97}N_{10}O_5$, 977.77; Anal. Calcd for $C_{55}H_{96}N_{10}O_5 \cdot 4HCl \cdot 3H_2O$: C, 56.11; H, 9.08; N, 11.90%. Found: C, 56.41, H, 9.01; N, 11.93%.

11 **KAON12** was dissolved in a chloroform/MeOH (50/50) solution, and by the use of an evaporator, a thin membrane was formed along the wall of a glass tube. Then, by the use of a voltex, the thin membrane was subjected to ultrasound at 50 °C and dispersed in a Tris buffer (pH: 7.5) to attempt lipid aggregate formation.

12 Various amounts of cationic compounds were mixed to fixed amount (150 ng) of pGL3-control in order to obtain charge ratio (+/−) ranging from 0 to 3.2. After 30 min incubation at 37 °C, the complexes were electrophoresed through a 0.8% agarose gel for 45 min at 100 V. DNAs were stained by ethidium bromide.

13 DNA complexe was formed using 1 mM lipid dispersion (7.7 μmL) and pGL3-control plasmid encoding luciferase (1 μmg). After that, transfection was carried out for three hours using COS-1 cells in a 24-well plate, and the activity of luciferase in lysed cell solutions was measured 48 h later to assess transfection efficiency. The luciferase assay was carried out using a Steady-Glo luciferase assay kit (Promega, Madison, WI, USA). The intensity of chemiluminescence was measured with a microplate luminometer (Fluoroskan Ascent-FL, Thermo Labsystems, Vantaa, Finland). In order to investigate the effects of photo irradiation on transfection, after allowing cells to come in contact with complexes for three hours, UV (365 nm, 3.5 mW/cm^2) was irradiated for zero or ten minutes, and the results were compared. The measurement of transfection efficiencies was performed in triplicate.

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