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**A pH-sensitive cationic lipid facilitates the delivery of liposomal siRNA and gene silencing activity *in vitro* and *in vivo***

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## Abstract

Modification of liposomal siRNA carriers with polyethylene glycol, i.e., PEGylation, is a generally accepted strategy for achieving *in vivo* stability and delivery to tumor tissue. However, PEGylation significantly inhibits both cellular uptake and the endosomal escape process of the carriers. In a previous study, we reported on the development of a multifunctional envelope-type nano device (MEND) for siRNA delivery and peptide-based functional devices for overcoming the limitations and succeeded in the efficient delivery of siRNA to tumors. In this study, we synthesized a pH-sensitive cationic lipid, YSK05, to overcome the limitations. The YSK05-MEND had a higher ability for endosomal escape than other MENDs containing conventional cationic lipids. The PEGylated YSK05-MEND induced efficient gene silencing and overcame the limitations followed by optimization of the lipid composition. Furthermore, the intratumoral administration of the YSK05-MEND resulted in a more efficient gene silencing compared with MENDs containing conventional cationic lipids. Collectively, these data confirm that YSK05 facilitates the endosomal escape of the MEND and thereby enhances the efficacy of siRNA delivery into cytosol and gene silencing.

## Key words

Multifunctional envelope-type nano device (MEND), siRNA delivery, pH-sensitive cationic lipid, intracellular trafficking, endosomal escape

## Abbreviations

cDNA	Complementary DNA
CHE	Cholesteryl hexadecyl ether
Chol	Cholesterol
DMEM	Dulbecco's modified Eagle medium
DMG	Dimyristoyl-sn-glycerol
DSG	Distearoyl-sn-glycerol
DODAP	1,2-Dioleoyl-3-dimethylammonium propane
DOPC	1,2-Dioleoyl-sn-glycero-3-phosphatidylcholine
DOPE	1,2-Dioleoyl-sn-glycero-3-phosphoethanolamine

DOTAP	1,2-Dioleoyl-3-trimethylammonium propane
DSPC	1,2-Distearoyl-sn-glycero-3-phosphatidylcholine
EPR	Enhanced permeability and retention
FBS	Fetal bovine albumin
LF2k	Lipofectamine 2000
LNPs	Lipid nanoparticles
MEND	Multifunctional envelope-type nano device
MMPs	Matrix metalloproteases
mRNA	messenger RNA
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PEG	Polyethyleneglycol
PLK1	Polo-like kinase 1
POPE	1-Palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine
RACE	Rapid amplification of cDNA ends
RBC	Red blood cell
RISC	RNA-induced silencing complex
RNAi	RNA interference
siRNA	Short interference RNA
SNALP	Stable nucleic acid lipid nanoparticles
SOPC	1-Stearoyl-2-oleoyl-sn-glycero-3-phosphatidylcholine
TNS	6-(p-Toluidino)-2-naphthalenesulfonic acid

## 1. Introduction

RNA interference (RNAi) can be used as novel therapeutic procedure through the specific *in vivo* silencing of therapeutically relevant target genes [1,2]. Small interfering RNA (siRNA) duplexes are promising candidates for therapeutic molecules that might be capable of achieving the sequence-specific inhibition of objective genes such as for oncogenes in carcinomas. The most significant issue for bringing out the potency of siRNAs is an efficient delivery system, because the high molecular weight (~13 kDa) and negative charge of siRNA molecules are serious limitations to the passive diffusion across the plasma membrane of most cells and susceptibility to enzymatic degradation in an *in vivo* environment [3,4]. Therefore, although delivering siRNA without a carrier may be possible in some cases [5,6], systemic delivery to various tissues, including tumors, demands a carrier to stabilize and transport siRNA to the target cells. Moreover, a carrier system is required, which can allow siRNA to avoid endosomal/lysosomal degradation and to be localized in the cytoplasm, where the RNAi machinery is located. Thus, a number of attempts to develop various nano carrier systems have been reported [7-12]. We also recently reported on the development of a unique siRNA delivery system, described as a multifunctional envelope-type nano device (MEND) [13,14].

It is generally accepted that prolonging the circulation time of a nano carrier will facilitate tumoral accumulation via the enhanced permeability and retention (EPR) effect [15]. Sterically stabilization of a lipid based nano carrier by poly(ethyleneglycol) (PEG) is the most popular method and is widely used to enhance circulation time by reducing nonspecific interaction between positively charged nano carriers and negatively charged serum components, leading to severe aggregation and rapid clearance from circulation by the reticuloendothelial system [16]. However, it is well-known that PEGylation leads to a severe decline of cellular uptake via endocytosis and the endosomal escape process of nano carriers, which results in the loss of efficacy for delivering siRNA into the cytoplasm [17,18]. In other words, PEGylation improves the pharmacokinetics but decreases the intracellular trafficking of the nanocarrier, a situation that we refer to as the 'PEG-dilemma' [19].

To resolve the PEG-dilemma, many groups have attempted to use functional devices, such as a cleavable PEG-lipid that is affected by an acidic

or reducing condition [20,21]. We previously reported on the production of a functional PEG-lipid, PPD, that is affected by matrix metalloproteinases (MMPs), which are secreted by various tumor cells and succeeded in silencing marker gene expression in tumor tissue by the systemic administration of PPD modified MEND [22,23]. In addition, we reported that GALA, a pH-sensitive fusogenic peptide, modified MEND could be applied to an *in vivo* intratumoral injection model and shGALA, a new shorter version of GALA to improve the pharmacokinetics of the MEND, which was modified to achieve gene silencing in tumor tissue followed by systemic administration [23-26]. As other strategy, Semple S.C. et. al. [27] reported that the pharmacokinetics of the lipid nanoparticles (LNPs) with an ionizable aminolipid, which is largely neutral but changes to a cationic form under acidic conditions, was improved compared to that with a conventional cationic lipid. The LNPs system also succeeded in tumor specific reporter gene expression, and gene silencing in orthotopic and subcutaneous tumors [28,29].

In the present study, we report on the development of a new pH-sensitive cationic lipid, YSK05, for improving the efficient intracellular trafficking and consequently to improve the gene silencing activity of a MEND both *in vitro* and *in vivo* instead of peptide-based functional devices (PPD, GALA and shGALA). Our results suggest that by the suitable manipulation of intracellular trafficking, the successful delivery of siRNA can be achieved, both *in vitro* and *in vivo*.

## 2. Materials and Methods

### 2.1 Materials

Anti-luciferase siRNA (sense: 5'-CCG UCG UAU UCG UGA GCA AdTdT-3'; antisense: 5'-UUG CUC ACG AAU ACG ACG GdTdT-3') was purchased from Sigma (Ishikari, Japan). Anti-PLK1 siRNA (sense: 5'-AGA uCA CCC uCC UUA AAu AUU-3'; antisense: 5'-UAU UUA AGG AGG GUG AuC UUU-3', 2'-OMe-modified nucleotides are in lowercase.) and Cy5-labeled anti-luciferase siRNA (sense: 5'-Cy5-GCG CUG CUG GUG CCA ACC CdTdT-3'; antisense: 5'-GGG UUG GCA CCA GCA GCA GCC CdTdT-3') were purchased from Hokkaido System Science Co., Ltd. (Sapporo, Japan). Protamine was purchased from CalbioChem (San Diego, CA, USA). 1,2-dioleoyl-3-trimethylammonium propane (DOTAP), 1,2-dioleoyl-3-dimethylammonium propane (DODAP), 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE), 1-Stearoyl-2-oleyl-*sn*-glycero-3-phosphatidylcholine (SOPC), 1,2-Dioleoyl-*sn*-3-phosphatidylcholine (DOPC) and cholesterol were purchased from Avanti Polar Lipid (Albaster, AL, USA). 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (POPE), 1,2-Distearoyl-*sn*-3-phosphatidylcholine (DSPC), 1,2-dimyristoyl-*sn*-glycerol, methoxyethyleneglycol 2000 ether (PEG-DMG) and 1,2-distearoyl-*sn*-glycerol, methoxyethyleneglycol 2000 ether (PEG-DSG) were purchased from NOF Corporation (Tokyo, Japan). <sup>3</sup>H-labeled cholesteryl hexadecyl ether (<sup>3</sup>H-CHE) was purchased from PerkinElmer Life Science (Tokyo, Japan). 6-(p-Toluidino)-2-naphthalenesulfonic acid (TNS) was purchased from Wako chemicals (Osaka, Japan). Ribogreen was purchased from Molecular Probes (Eugene, OR, USA). Lipofectamine 2000 (LF2k) and TRIzol reagent were purchased from Invitrogen (Carlsbad, CA, USA). Dual-Luciferase Reporter Assay Reagent was purchased from Promega Corporation (Madison, WI, USA). HeLa human cervical carcinoma cells were purchased from RIKEN Cell Bank (Tsukuba, Japan). OS-RC-2 human renal cell carcinoma cells were kindly provided by K. Hida (Hokkaido University, Sapporo, Hokkaido, Japan).

### 2.2 Experimental animals

Male ICR mice and BALB/cAjl nude mice were purchased from Japan SLC (Shizuoka, Japan) and CLEA (Tokyo, Japan), respectively. The

experimental protocols were reviewed and approved by the Hokkaido University Animal Care Committee in accordance with the guidelines for the care and use of laboratory animals.

### 2.3 MEND formulations

All MENDs were prepared using a cationic lipid, a phospholipid, cholesterol and PEG-DMG using a *t*-BuOH dilution procedure. The initial MENDs had a component molar ratio of 30/40/30/3 (DOTAP, DODAP or YSK05/DOPE/cholesterol/PEG-DMG) and optimized YSK05 MEND had 50/25/25/3 (YSK05/POPE/cholesterol/PEG-DMG). PEG-DMG was used to stabilize the lipid membrane during the formulation process and for preservation. Typically, 1.5 mM lipids were dissolved in 90% *t*-BuOH solution. siRNA was complexed with protamine at a nitrogen/phosphate ratio of 1.1 in 1 mM citrate buffer (pH4.5) and was titrated slowly to lipid solution under vigorous mixing to avoid low local concentration of *t*-BuOH and diluted quickly with citrate buffer to final <20% *t*-BuOH. Ultrafiltration was performed to remove *t*-BuOH, replacing external buffer with phosphate buffered saline (PBS, pH7.4) and concentrating the MENDs. To incorporate PEG-DSG, the MENDs were incubated at 45°C for 45 minutes with PEG-DSG at 5.0 mol% total lipid under 10 v/v% ethanol conditions. Again, ultrafiltration was performed against PBS to remove EtOH and for concentration. An empty MEND with the same lipid composition was prepared by a similar procedure, with the exception that an equivalent volume of 1 mM citrate buffer was titrated to the lipid solution instead of siRNA/protamine complex. Radiolabeled MENDs were prepared by adding a trace amount of <sup>3</sup>H-CHE to the lipid-*t*-BuOH solution prior to mixing with the siRNA/protamine complex. The average diameter and zeta-potential of MENDs were determined using a Zetasizer Nano ZS ZEN3600 (MALVERN Instrument, Worchestershire, UK).

### 2.4 Ribogreen assay

To determine siRNA encapsulation efficiency and its concentration, Ribogreen fluorescence assay was performed. MENDs were diluted in 10 mM hepes buffer at pH7.4 containing 20 µg/mL dextran sulfate and Ribogreen in the presence or absence of 0.1 w/v% TritonX-100. Fluorescence was measured by Varioskan Flash (Thermo scientific) with  $\lambda_{ex}=500$  nm,  $\lambda_{em}=525$

nm. siRNA concentration was calculated from siRNA standard curve. siRNA encapsulation efficiency was calculated by comparing siRNA concentration in the presence and absence of TritonX-100.

## 2.5 TNS assay

Thirty  $\mu\text{M}$  of MEND lipid and 6  $\mu\text{M}$  of TNS were mixed in 200  $\mu\text{L}$  of 20 mM citrate buffer, 20 mM sodium phosphate buffer or 20 mM Tris-HCl buffer, containing 130 mM NaCl, at a pH ranging from 3.0 to 9.0. Fluorescence was measured by a Varioskan Flash set up with  $\lambda_{\text{ex}}=321$  nm,  $\lambda_{\text{em}}=447$  nm at 37°C. The pKa values were measured as the pH giving rise to half-maximal fluorescent intensity.

## 2.6 Stability of MENDs in mouse serum

Fresh mouse serum was collected from a male ICR mouse and mixed with free siRNA, a mixture of siRNA/protamine complex and the empty MEND, and MEND formulating siRNA followed by incubation at 37°C for various periods. For disruption of the lipid bilayer of the MEND, 0.05 w/v% TritonX-100 was added to incubation mixture. At selected time points, aliquots were frozen at -80°C to stop siRNA degradation. Afterward, the samples were thawed at r.t. and siRNA was immediately extracted with a phenol-chloroform-isoamylalcohol mixture. The aqueous phase, containing the siRNA, was run on 20% polyacrylamide gels. After 2 hours of electrophoresis at 150 V, siRNA was stained and visualized with 1  $\mu\text{g}/\text{mL}$  ethidium bromide.

## 2.7 Cell culture and *in vitro* luciferase gene silencing

HeLa cells stably expressing Firefly and Renilla luciferase (HeLa-dluc) were cultured in cell-culture dishes (Corning) containing DMEM supplemented with 10% FBS, penicillin (100 U/mL), streptomycin (100 mg/mL) and G418 (0.4 mg/mL) at 37°C in 5% CO<sub>2</sub>. For luciferase gene silencing, HeLa-dluc cells were seeded at a density of 5,000 cells per well in 96-well plates in growth medium 24 hours prior to transfection and incubated overnight at 37°C in 5% CO<sub>2</sub> to allow cells to become attached. For transfection, media containing MENDs at the indicated doses of siRNA were added to cells after aspiration of the spent media. LF2k, as a control, was used according to the manufacturer's protocol. The MENDs were allowed to

incubate with cells for 24 hours prior to analysis for luciferase expression. To evaluate whether the gene silencing activities of the MENDs depend on endosomal acidification, MEND suspension in media containing various concentrations of chloroquine or  $\text{NH}_4\text{Cl}$  were added to the cells after aspiration of the spent media. The MENDs were incubated for 4 hours with cells, and new media were added after aspiration of the spent media and further incubated for 20 hours prior to analysis for luciferase expression. Firefly and Renilla luciferase activities were analyzed using the Dual-Glo assay (Promega) according to manufacture protocol. Luminescence was measured using a luminometer (Luminescencer-PSN, ATTO, Japan). For data analysis, Firefly luciferase activity was normalized by Renilla luciferase activity and treated samples were compared to untreated samples to determine degree of luciferase silencing.

## **2.8 Cellular uptake**

Twenty-four hours prior to transfection, HeLa-dluc cells were seeded at a density of  $1 \times 10^5$  cells per well in 6-well plates in growth medium and incubated overnight at  $37^\circ\text{C}$  in 5%  $\text{CO}_2$  to allow cells attachment. For transfection, media containing  $^3\text{H}$ -labeled MENDs were transferred to cells after aspiration of spent media. MENDs were allowed incubation with cells from 1 to 24 hours. At selected time points, cells were washed twice with PBS containing heparin (20 U/mL) and lysed with passive lysis buffer (Promega). Radioactivity was determined using a LSC-6100 (ALOKA) scintillation counter. The amount of cellular uptake was represented as the % of total transfected radioactivity.

## **2.9 Hemolysis assay**

Fresh red blood cells (RBC) were collected from an ICR mouse and suspended in PBS. The RBC suspension was mixed with each quantity of empty MEND, and incubated at  $37^\circ\text{C}$  for 30 minutes. After the incubation, the absorbance at 545 nm of supernatant was measured after centrifugation ( $4^\circ\text{C}$ , 400 g, 5 minutes). The samples incubated with 0.5 w/v% TritonX-100 as a positive control and without MENDs as a negative control were also measured. The %hemolysis was represented as the % of the absorbance of positive control.

## **2.10 Assessment of intracellular trafficking of MEND-siRNA using confocal microscopy**

Twenty-four hours prior to transfection, HeLa-dluc cells were seeded at a density of  $1 \times 10^5$  cells per dish in a glass based dish in growth medium and incubated overnight at 37°C in 5%CO<sub>2</sub> to allow cells attachment. For transfection, media containing 100 nM of Cy5-labeled siRNA formulated in optimized YSK05-MEND or DODAP-MEND were added to cells and incubated for 1 hour after aspiration of the spent media. The incubation was stopped by removing the media followed by washing the dishes three times with cold PBS. For the sample for the 6 hour time point, fresh media was then added and cells were incubated at 37°C for a further 6 hours and washed in cold PBS. After washes, cells were fixed with 4% paraformaldehyde (Wako Chemicals) for 10 minutes at room temperature. The cells were washed in PBS, then stained with nuclear marker Hoechst33342 (Wako Chemicals) for 10 minutes to identify individual cells. After several washes, cells were viewed using a Nikon A1 (Nikon Co. Ltd., Tokyo, Japan) to assess the intracellular pattern of Cy5-labeled siRNA. Images were captured with x60 objective following excitation with a 633 nm laser.

## **2.11 *In vivo* gene silencing activity of MENDs in tumor tissue**

OS-RC-2 cells were cultured in cell culture dishes (corning) containing RPMI1640 supplemented with 10% FBS, penicillin (100 U/mL) and streptomycin (100 mg/mL) at 37°C in 5%CO<sub>2</sub>. Tumor bearing mice were prepared by the subcutaneous injection of male BALB/c nude mice with OS-RC-2 cells ( $1 \times 10^6$  cells/mouse). When the size of the tumor reached around 100 mm<sup>3</sup>, MENDs were administrated to tumor tissue at a dose of 10 µg siRNA. At 24 hours after administration, tumor tissues were collected, and approximately 30 mg of tumor tissue was homogenized using a PreCellys 24 (Bertin technologies, France). The resulting tumor homogenates were centrifuged at 15,000 rpm for 10 minutes at 4°C to obtain the supernatants. Total RNA (1 µg) was isolated with RNeasy (Qiagen) and reverse transcribed using a High Capacity RNA-to-cDNA kit (ABI) according to manufacturer's protocol. A quantitative PCR analysis was performed on 2 ng of cDNA using Fast SYBR Green Master Mix (ABI) and Lightcycler480 system II (Roche). All reactions were performed at a volume of 15 µL. The

primers for human PLK1 were (forward) 5'-AAT AAA GGC TTG GAG AAC CC-3' and (reverse) 5'-ACC TCA CCT GTC TCT CGA AC-3' and for human GAPDH were (forward) 5'-CCT CTG ACT TCA ACA GCG AC-3' and (reverse) 5'-CGT TGT CAT ACC AGG AAA TGA G-3'.

## 2.12 5' rapid amplification of cDNA ends-PCR (5' RACE PCR)

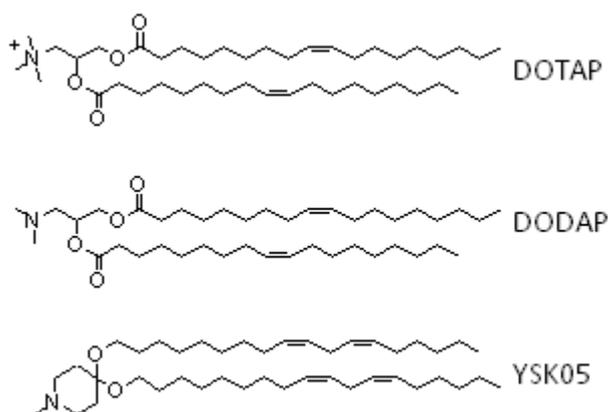
Total RNA was isolated from *in vitro* cultured cell by lysis in TRIzol. For *in vivo* tumor samples, approximately 30 mg of tumor tissue was homogenized in 500 µL TRIzol, then processed to isolate total RNA. Five µg of total RNA was heated to 65°C for 5 minutes and snap-cooled to 4°C prior to ligation. RNA ligation was performed at 37°C for 1 hour in 1 x ligation buffer, T4 RNA ligase (TaKaRa, Japan) and 1.2 µg RNA adaptor (5'-NH<sub>2</sub>-CGA CUG GAG CAC GAG GAC ACU GAC AUG GAC UGA AGG AGU AGA AA-3'). Samples were then purified by phenol-chloroform-isoamylalcohol extraction and ethanol precipitation. The RNA ligation product (10 µL) was reverse transcribed using SuperScript III (Invitrogen) and PLK1 gene specific primer (5'-GGA CAA GGC TGT AGA ACC CAC AC-3'). After heat denaturalization at 65°C for 5 minutes followed by snap cooling at 4°C, reverse transcription was carried out at 50°C for 1 hour followed by inactivation at 70°C for 15 minutes and snap-cooling at 4°C. 5' RACE PCR was performed using forward primer (Ad1) in the RNA adaptor and reverse primer (Rev1) in the 3' end of PLK1 mRNA. PCR primer sequences were Ad1 5'-CGA CTG GAG CAC GAG GAC ACT GA-3' and Rev1 5'-GCT TGT CCA CCA TAG TGC GG-3'. PCR was performed using a 2720 Thermal Cycler (ABI) using touchdown PCR conditions of 94°C for 2 minutes (1 cycle), 94°C for 30 seconds and 72°C for 40 seconds (5 cycles), 94°C for 30 seconds and 70°C for 40 seconds (5 cycles), 94°C for 30 seconds and 66°C for 30 seconds and 72°C for 40 seconds (25 cycles), and 72°C for 10 minutes (1 cycle). Nested PCR was then performed using the forward primer (Ad2) in the RNA adaptor 3' to the Ad1 and reverse primer (Rev2) in the PLK1 mRNA 5' to the Rev1. PCR primer sequences were Ad2 5'-GGA CAC TGA CAT GGA CTG AAG GAG TA-3' and Rev2 5'-TCC TTG CAG CAG CCG TAC TC-3'. Nested PCR was performed using the same instrument with PCR conditions of 94°C for 2 minutes (1 cycle), 94°C for 30 seconds and 64°C for 30 seconds and 72°C for 40 seconds (20 cycles), and 72°C for 10 minutes (1 cycle). PCR products were run on a 2% TBE Agarose gel and stained and visualized with 1 µg/mL

ethidium bromide.

### 3. Results

#### 3.1 Synthesis of YSK05

DOTAP and DODAP are commercially available cationic lipids for transfection. The former is a quaternary ammonium derivative, resulting in cationic properties that are independent of the pH environment (Fig. 1). In contrast, the latter has a structure similar to DOTAP except that it contains a tertiary amine instead of a quaternary ammonium group resulting in pH-sensitive properties and becomes cationic at an acidic pH and is almost neutral at physiological pH. It is known that unsaturated carbon chains cause the formation of cone shaped molecules which promote transfection efficiency through facilitating the process of endosomal escape. Attempts to design new lipid structures based on this well known knowledge and in consideration of the ease of synthesis resulted in the successful synthesis of YSK05 as described. YSK05, newly synthesized in this laboratory, also contains one tertiary amine which confers pH-sensitive properties, and long, unsaturated carbon chains for emphasizing a cone shaped structure (Fig.1). The yield was 62% (Supplementary methods).



**Fig. 1. Chemical structures of cationic lipids incorporated into MENDs**

DOTAP and DODAP are conventional and available cationic lipids which have quaternary ammonium, indicating cationic without depending on pH, and tertiary amine, indicating cationic at acidic pH and neutral at physiological pH, respectively. YSK05 is a cationic lipid that contains a tertiary amine group for pH-sensitivity.

#### 3.2 Characteristics of the prepared MENDs

To prepare small and uniform particles and efficiently encapsulate siRNA reproducibly, MENDs were formulated by a *t*-BuOH dilution process as described. Particle size, zeta-potential, siRNA encapsulation and apparent pKa were assessed and the data are shown in Table 1. Diameters of all of the MENDs were approximately the same (115 - 125 nm). Three MENDs containing the pH-sensitive cationic lipid were approximately neutral at a pH of 7.4 and showed a high siRNA encapsulation efficiency (> 90%), on the other hand, only the DOTAP-MEND showed high cationic properties and a somewhat low encapsulation efficiency. The apparent pKa of MENDs containing the pH-sensitive cationic lipid was measured using the fluorescent probe, TNS. It was observed that the apparent pKa of YSK05-MEND was around 6.5, while that of DODAP-MEND was lower than 6.0 (Supplementary Fig. S1).

**Table 1. Physical properties of MENDs**

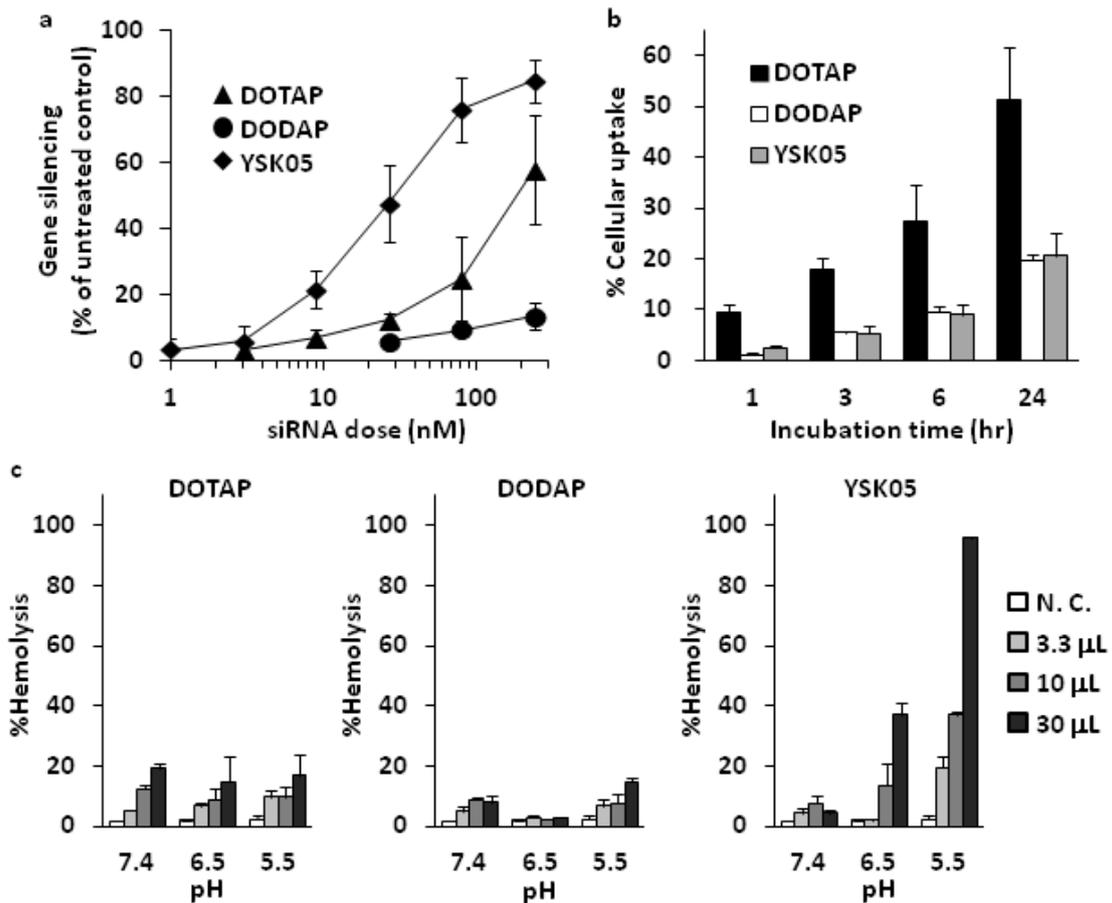
MENDs	DOTAP	DODAP	YSK05	Optimized YSK05
Diameter (nm)	125 ± 1	119 ± 6	115 ± 5	116 ± 5
Polydispersity	0.16 ± 0.02	0.14 ± 0.02	0.15 ± 0.01	0.09 ± 0.02
Zeta potential (mV)	+20.3 ± 1.8	-1.6 ± 1.3	+4.5 ± 2.1	+4.5 ± 2.1
siRNA encapsulation (%)	67 ± 16	90 ± 5	90 ± 6	93 ± 4
Apparent pKa	-	5.8	6.6	6.4

Particle diameter, polydispersity and zeta-potential were measured using a Malvern Zetasizer. Percentage of siRNA encapsulation was determined by Ribogreen fluorescence assay to measure the amount of siRNA relative to total siRNA present. Apparent pKa value was determined by TNS fluorescence assay as a pH giving rise to half-maximal fluorescent intensity. Data points are expressed as the mean ± SD (n=3).

### 3.3 YSK05-MEND indicates highest membrane disrupt potency and gene silencing activity *in vitro*

The ability of MENDs containing each cationic lipids to induce gene silencing in HeLa-dluc cells was evaluated. It was found that YSK05-MEND indicated higher gene silencing activity than DOTAP-MEND and its IC<sub>50</sub> (that is, the dose to achieve 50% gene silencing) was around 30 nM, while DODAP-MEND failed to induce clear gene silencing (Fig. 2a). The amount of

cellular uptake of each formulation was next measured with MENDs with incorporated  $^3\text{H}$ -labeled CHE (Fig. 2b). The cellular uptake of the MEND containing DOTAP, pH-insensitive cationic lipid, was highest, while that of MENDs containing each pH-sensitive cationic lipid, almost neutral at physiological pH, were relatively low and similar to one another. The ability of MENDs to induce membrane disruption with RBC membranes was evaluated to judge the potency for escape from the endosomal compartment via membrane fusion. As shown in Fig. 2c, it was observed that YSK05-MEND induced membrane disruption depending on the dose of MENDs and decline of pH, while other two MENDs induced only minor membrane disruption even at low pH.



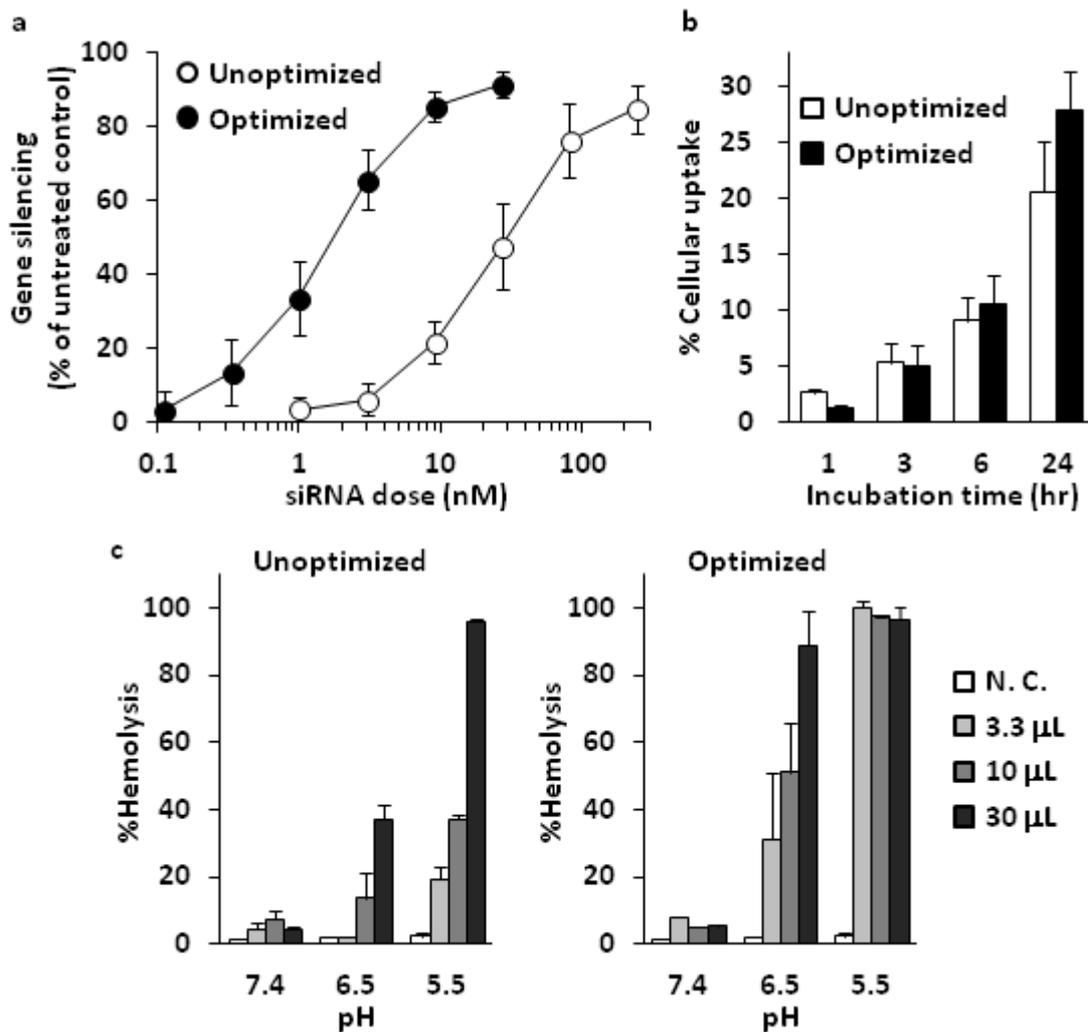
**Fig. 2. Comparison of MENDs containing each cationic lipid *in vitro***

(a) MEND mediated gene silencing *in vitro*. HeLa-dluc cells were treated with MENDs containing anti-luciferase siRNA for 24 hours. (b) Cellular uptake of MEND. HeLa-dluc cells were treated with MENDs containing  $^3\text{H}$ -labeled CHE for

various times. The cells were washed to remove unincorporated MENDs prior to measurement of  $^3\text{H}$ -CHE. (c) MEND mediated hemolysis *in vitro*. RBC suspension was mixed with empty DOTAP- (left), DODAP- (center) or YSK05- (right) MEND and incubated for 30 minutes at  $37^\circ\text{C}$ . The absorbance due to hemoglobin was measured after removal of intact RBCs by centrifugation. Data points are represented as the mean  $\pm$  SD (n=3). N.C.; negative control.

### 3.4 Optimization of YSK05-MEND lipid composition

To enhance the potency of the YSK05-MEND, the influence of phospholipid on gene silencing activity was evaluated. We used three phosphatidyl choline and two phosphatidyl ethanolamine derivatives as candidate phospholipids. It was observed that the YSK05-MEND containing any phosphatidyl choline (PC) failed to induce high gene silencing activity, while phosphatidyl ethanolamine (PE) succeeded (Supplementary Fig. S2). Additionally, we found that YSK05-MEND containing POPE had a higher gene silencing potency than DOPE and the highest stability in mouse serum of all tested phospholipids. Moreover, we found the optimal ratio of lipid combination through the screening of the lipid composition of YSK05-MEND, containing YSK05, POPE and cholesterol. The resulted YSK05-MEND, represented as optimized YSK05-MEND (YSK05/POPE/cholesterol=50/25/25), indicated similar physical properties compared to the unoptimized one (Table 1) and the  $\text{IC}_{50}$  of 2 nM, approximately 15 times lower than unoptimized one (Fig. 3a). The cellular uptake of optimized YSK05-MEND was almost similar to that for the unoptimized one (Fig. 3b), while the hemolytic activity of the former was significantly higher than the latter (Fig. 3c). Namely, it was revealed that the escalation of gene silencing activity through the optimization resulted from the escalation of the endosomal escape ability but not cellular uptake. We also evaluated the durability of gene silencing of the optimized YSK05-MEND (Supplementary Fig. S4). The findings indicate that the durability of gene silencing was dependent on the dose of siRNA, and the gene silencing rate decreased to 50% or below on day 4 at a dose of 10 nM siRNA and on day 7 at a dose of 100 nM siRNA.

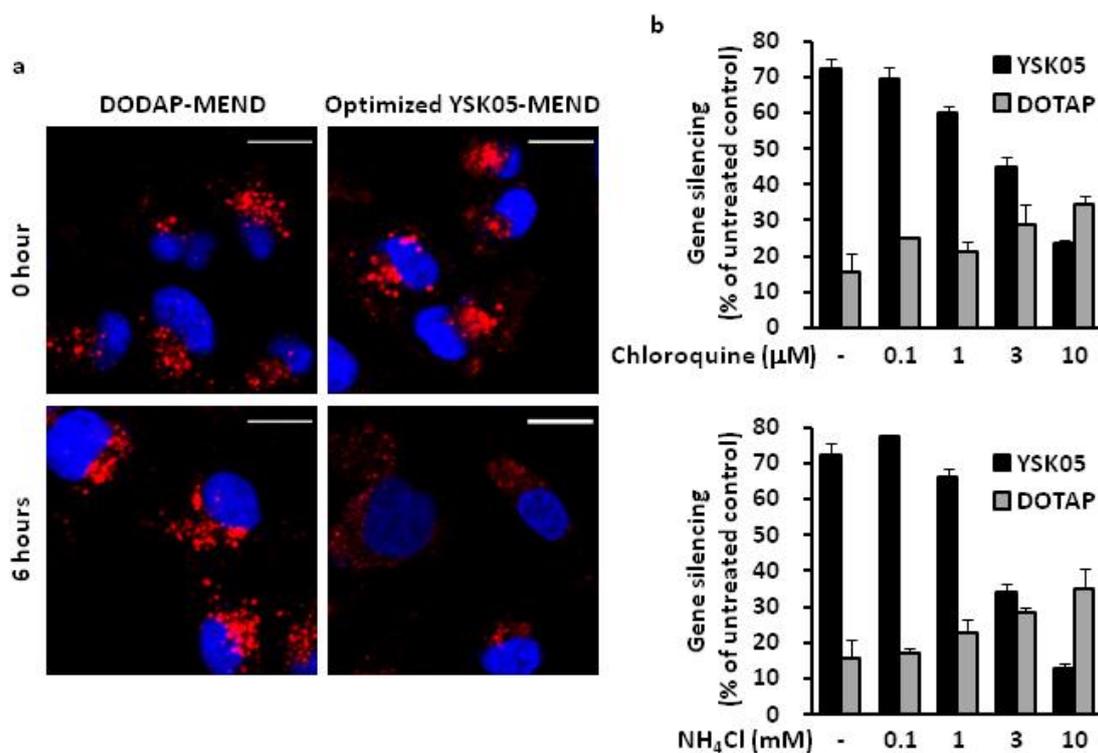


**Fig. 3. Comparison of YSK05-MENDs before and after optimization of lipid composition**

(a) MEND mediated gene silencing *in vitro*. HeLa-dluc cells were treated with MENDs containing anti-luciferase siRNA for 24 hours (n=3-6). (b) Cellular uptake of MEND. HeLa-dluc cells were treated with MENDs containing  $^3\text{H}$ -labeled CHE for various times. The cells were washed to remove unincorporated MENDs prior to measurement of  $^3\text{H}$ -CHE (n=3). (c) MEND mediated hemolysis *in vitro*. RBC suspension was mixed with empty unoptimized (left) or optimized (right) YSK05-MEND and incubated for 30 minutes at  $37^\circ\text{C}$ . The absorbance from hemoglobin was measured after removal of intact RBCs by centrifugation (n=3). Data points are represented as the mean  $\pm$  SD. N.C.; negative control.

### 3.5 YSK05-MEND internalizes via endocytosis and escapes depends on endosomal acidification

To further investigate the intracellular fate of siRNA formulated in the YSK05-MEND, Cy5-labeled siRNA was encapsulated in DODAP-MENDs and optimized YSK05-MENDs and observed in HeLa-dluc cells using confocal microscope (Fig. 4a and Supplementary Fig. S3). Most of the Cy5 fluorescence of both formulations exhibited a punctuate pattern at the 0 hour time point, suggesting that both formulations were internalized into cells via endocytosis (Fig. 4a, upper). However, at the 6 hour time point, a formulation dependent difference was observed in the intracellular distribution pattern of Cy5-labeled siRNA in HeLa-dluc cells (Fig. 4a, lower). Cy5-labeled siRNA formulated in DODAP-MENDs exhibited a punctuate pattern at the 6 hour time point, the same as the 0 hour time point. In contrast, most Cy5-labeled siRNA formulated in optimized YSK05-MENDs exhibited a diffuse distribution at the 6 hour time point. This result suggests that the optimized YSK05-MEND delivered siRNA into the cytoplasm through escaping lysosomal degradation following internalization via endocytosis. To confirm that the release of siRNA in the cytoplasm by the YSK05-MEND was dependent on endosomal acidification, the gene silencing activity of the optimized YSK05-MEND was evaluated in the presence of an inhibitor of endosomal acidification (Fig. 4b). Two low molecular weight drugs, chloroquine and ammonium chloride ( $\text{NH}_4\text{Cl}$ ), were used for buffering acidic vesicles, resulting in the inhibition of lysosomal enzymes and possibly altering normal lysosomal trafficking. DOTAP-MEND was used as a control of pH-independent carrier. The gene silencing activity of DOTAP-MEND slightly increased depending on drug concentration, while that of YSK05-MEND was significantly suppressed by both drugs. From these results, it was confirmed that optimized YSK05-MEND was taken up into cells via endocytosis and escaped from endosome significantly depending on endosomal acidification followed by inducing gene silencing.



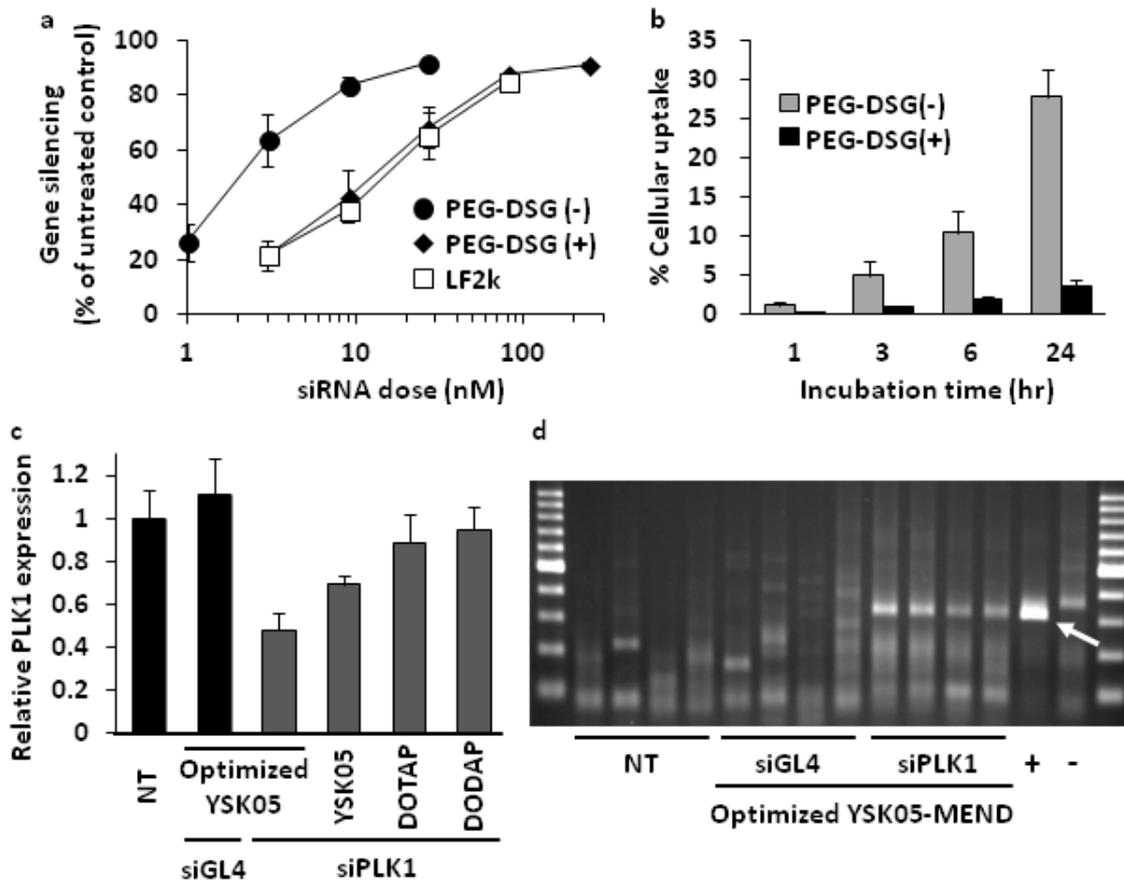
**Fig. 4. Confirming the endosomal escape of optimized YSK05-MEND**

(a) The intracellular pattern and distribution of siRNA. HeLa-dluc cells were treated with Cy5-labeled siRNA formulated in DODAP- (left) or optimized YSK05- (right) MEND at a dose of 100 nM siRNA for 1 hour. Unincorporated MENDs were washed out and siRNA distribution was visualized after 0 hour (upper) or 6 hours (lower) using a confocal microscopy. Scale bars indicate 20  $\mu$ m. Full-size pictures are presented in Supplementary Fig. S3. (b) MEND mediated gene silencing in the presence of the chemicals buffering endosome. HeLa-dluc cells were treated with anti-luciferase siRNA formulated in DOTAP-MEND (150 nM) and optimized YSK05-MEND (27 nM) in the presence of chloroquine (upper) or NH<sub>4</sub>Cl (lower) for 4 hours. The cells were further incubated for 20 hours after medium change. Data points are represented as the mean  $\pm$  SD (n=3).

### 3.6 PEGylated optimized YSK05-MEND induces gene silencing both *in vitro* and *in vivo*

The incorporation of PEG-DSG into optimized YSK05-MENDs was carried out to provide stability under an *in vivo* environment. It is known that PEG lipid with a long acyl chain, such as PEG-DSG, is retained strongly by a lipid membrane while PEG lipid with short acyl chain, such as PEG-DMG,

dissociated more readily from a membrane [28]. After the incorporation of PEG-DSG, the stability of the optimized YSK05-MEND in serum was significantly increased, as expected (Supplementary Fig. S7). Next, to evaluate gene silencing activity in tumor tissue, anti-polo-like kinase 1 (PLK1) siRNA (siPLK1) formulated in PEG-DSG modified MENDs with each different lipid composition were topically administrated on OS-RC-2 subcutaneous tumors. Although MENDs containing DOTAP and DODAP failed to induce gene silencing, the unoptimized and optimized YSK05-MENDs succeeded approximately 30% and 50% gene silencing was found, respectively (Fig. 5c). To confirm that the gene silencing was clearly the result of RNAi, we detected the RNAi-specific PLK1 mRNA cleavage product by the 5' RACE PCR method. Active PLK1 mRNA cleavage was detected only after administration of siPLK1 formulated in optimized YSK05-MEND but not the non-treatment control or the anti-luciferase siRNA (siGL4) control, clearly demonstrating that a sequence specific RNAi occurred in tumor tissues (Fig. 5d).



**Fig. 5. Gene silencing activity of PEGylated optimized YSK05-MEND *in vitro* and *in vivo***

(a) MEND mediated gene silencing *in vitro*. HeLa-dluc cells were treated with MENDs and Lipofectamine 2000 (LF2k) incorporating anti-luciferase siRNA (siGL4) for 24 hours (n=3). (b) Cellular uptake. HeLa-dluc cells were treated with MENDs containing <sup>3</sup>H-labeled CHE for various times (n=3). (c) MEND mediated gene silencing *in vivo*. OS-RC-2 tumor bearing mice were topically administrated with MENDs at a dose of 10 µg siPLK1 or siGL4 into tumors. After 24 hours, mice were euthanized and PLK1 gene expression was measured (n=4). (d) 5' RACE PCR analysis. RNAi specific PLK1 mRNA cleavage products were detected after 24 hours topical administration of MENDs. Positive control from *in vitro* OS-RC-2 cell lysates treated with siPLK1 indicated by plus sign and negative control indicated by minus sign. RACE PCR detects the 5' cleavage product of human PLK1 mRNA from tumors. Each lane represents different tumor tissues. The predicted 321-bp PLK1 product is indicated by an arrow. Data points are represented as the mean ± SD.

#### 4. Discussion

There are several obstacles to the delivery of siRNA. These include its safe delivery to the target cell population, internalization into the cells and escape from the endosomal compartment for protecting from lysosomal degradation and localization in the cytoplasm, where siRNAs located and functions. PEGylation enhances the stability and half-life of nano carriers in blood circulation by avoiding recognition and clearance by phagocytic cells of the reticuloendothelial system and is widely used in *in vivo* applications of nano carriers. As the conventional MEND was composed of a cationic lipid, such as DOTAP, it was necessary to modify the conventional cationic MEND with high amounts of PEG lipids to suppress non-specific electrostatic interactions with internal components, such as negatively charged proteins [22,23,26]. However, it is also known that PEGylation results in a significant loss of gene silencing efficiency. We previously showed that the two intracellular processes, cellular uptake and endosomal escape, are suppressed by PEGylation. The outer PEG layer sterically hinders interaction between a carrier and cell surface membrane, and results in a decrease in cellular uptake via endocytosis. After uptake, the PEG layer similarly inhibits endosomal escape via membrane fusion between the carrier membrane and the endosomal membrane. In order to solve this problem, it is necessary to remove or reduce the level of the PEG moiety. Strategies for overcoming the PEG-dilemma are outlined below. The first is acceleration of steps that are inhibited, such as cellular uptake and endosomal escape, by introducing functional devices into highly PEG-modified carriers. As shown previously, more than 10 mol% of PEG is necessary to achieve sufficient blood circulation in the case of a cationic DOTAP-MEND [22]. Therefore, the highly PEGylated DOTAP-MEND was modified with enzymatically cleavable PEG [23] or a shorter version the GALA peptide [26] to achieve both sufficient blood circulation and efficient silencing activity. The second approach is decreasing the level of the PEG moiety by introducing charge changeable devices. Huang L and colleagues [30] proposed the concept of pH-sensitive liposomes, that release encapsulated drugs following an acid treatment. Judge AD and colleagues [31] prepared stable nucleic acid lipid particles (SNALP) and succeeded in achieving gene silencing in tumor tissue after the systemic administration of low amounts of PEG lipid used in the modification process. SNALP is

comprised of an ionizable amino lipid and it was reported that the chemical structure of the ionizable amino lipid has a significant influence on the intracellular fate of SNALP [32,33]. In this study, we synthesized a new pH-sensitive cationic lipid, YSK05, for the second purpose.

YSK05-MEND induced higher gene silencing activity than other MENDs containing each conventional cationic lipid, DOTAP and DODAP, a pH-insensitive and a pH-sensitive cationic lipid, respectively (Fig. 2). Despite its comparable cellular uptake, the YSK05-MEND showed a significantly higher gene silencing activity than DODAP-MEND. There are two reasons for this, described below. One reason is that the YSK05-MEND with an apparent pKa of around 6.6, can rapidly become a cationic species in response to endosomal acidification and thus avoid lysosomal degradation, while the DODAP-MEND with an apparent pKa < 6.0, cannot accomplish this, at least not rapidly (Table 1). The other reason is that YSK05-MEND has a considerably higher hemolytic activity than the DODAP-MEND, indicating that YSK05-MEND can more easily escape from endosomes via membrane fusion (Fig. 2c). It was verified, using confocal microscopy, that YSK05-MEND escapes from endosomes followed by releasing siRNA into cytosol (Fig. 4a). Also, the YSK05-MEND indicated higher gene silencing activity than DOTAP-MEND in spite of the fact that the amount of cellular uptake of the former was lower than that of the latter (Fig. 2a and c). This result also can be explained from the observation that the YSK05-MEND has a significantly higher potency for membrane fusion than the DOTAP-MEND, as evidenced by a hemolysis assay (Fig. 2c).

Additionally, the lipid composition significantly influenced the gene silencing activity of YSK05-MEND. YSK05-MENDs containing two phosphatidyl ethanolamines, DOPE and POPE, induced gene silencing at a dose of 9 nM, while the YSK05-MENDs containing all of the phosphatidyl cholines failed (Supplementary Fig. S2b). The cause of this result might be the shape of the phospholipid molecules. It is generally accepted that phosphatidyl ethanolamine is classified as an inverted lipid capable accelerating membrane fusion by inducing structural changes in the lipid membrane from a lamellar to a inverted hexagonal ( $H_{II}$ ) phase, while phosphatidyl choline induced the formation of a cylindrical lipid capable stabilizing a lamellar phase [34]. Although there is no direct evidence to show that the YSK05-MEND containing POPE has a higher gene silencing

activity than DOPE, the excellent stability in serum of the former may account for this (Supplementary Fig. S2a). We next, carried out the screening of lipid composition of YSK05-MEND, containing YSK05, POPE and cholesterol, and represented the resulting MEND as the optimized YSK05-MEND. The physical parameters of the optimized YSK05-MEND were similar to the unoptimized preparation. The potential for endosomal escape but not cellular uptake was enhanced through optimization of the lipid components and resulted in the escalation of gene silencing activity (Fig. 3). We then evaluated the durability of gene silencing of the optimized YSK05-MEND using HeLa-dluc cells. The durability of gene silencing was extended by increasing the dose of siRNA from 10 nM to 100 nM. This result would result from a situation where the excess siRNAs in the cytosol were incorporated into the RNA-induced silencing complex (RISC) one after another following the degradation of siRNAs incorporated into RISC. Although the durability of gene silencing is dependent on the cell division rate, the stability of siRNA and so on, the span and pattern was similar to that reported in our previous study [34].

The result of confocal microscopy experiments confirmed that the optimized YSK05-MENDs were taken up via endocytosis and were able to release siRNA into the cytoplasm by endosomal escape after a 6 hour chase (Fig. 4a). In contrast, siRNA formulated in the DODAP-MEND showed a punctuate pattern even in the case of a 6 hour chase, indicating that the DODAP-MEND failed to escape from the endosomal compartments. This result corresponds to the failure of both gene silencing and hemolysis. To elucidate the dependence on pH decline for endosomal escape and further gene silencing of the YSK05-MEND, transfection in the presence of chloroquine and  $\text{NH}_4\text{Cl}$  was examined. Chloroquine and  $\text{NH}_4\text{Cl}$  are known to buffer the pH of endosomes and lysosomes and to improve the transfection efficiency of vectors which are unable to escape from the lysosomal trafficking pathway [36-39]. The gene silencing activity of DOTAP-MEND, a pH-insensitive carrier, gradually increased depending on the concentration of each compound, indicating that the endosomal escape efficacy of the DOTAP-MEND was increased by avoiding trafficking to degradative lysosomes. In contrast, the gene silencing activity of the optimized YSK05-MEND dramatically decreased, indicating that the endosomal escape

process of the YSK05-MEND strongly depends on the acidification of endosomes as we expected (Fig. 4b).

Finally, we evaluated the gene silencing activity of the MENDs in tumor tissue. In this study, we chose the amount of PEG modification as 5 mol% of the total lipid, because the blood circulation of DODAP-MEND with 3.5 or more mol% of PEG was saturated, indicating that a 5 mol% PEG modification is sufficient in terms of preventing nonspecific interactions between DODAP-MEND, a pH-sensitive cationic system, and serum components (Supplementary Fig. S5). Actually, an attempt to induce gene silencing in OS-RC-2 subcutaneous tumor tissue by the intratumoral administration of optimized YSK05-MEND without incorporated PEG-DSG resulted in failure (data not shown). Although the reason for this is not clear, there are two possibilities. One possibility is that the stability of the optimized YSK05-MEND has too low to tolerate the *in vivo* environment. Another possibility is that the optimized YSK05-MEND was taken up by other cells in tumor tissue, such as tumor associated macrophages, rather than tumor cells. Therefore, the surface of the MEND would have been shielded by the incorporation of PEG-DSG, increasing its physical stability and reducing non-specific cellular uptake. In an *in vitro* examination, the cellular uptake of the optimized YSK05-MEND with 5 mol% of PEG-DSG incorporated was severely reduced (Fig. 5a). This indicates that 5 mol% PEG modification is sufficient to prevent nonspecific interaction between the optimized YSK05-MEND and biomembranes at physiological pH. However, the gene silencing activity of the optimized YSK05-MEND with PEG-DSG incorporated was similar to that for LF2k, a commonly used transfection reagent (Fig. 5b), indicating that it escaped efficiently from endosomes. This result can be explained as follows. DOTAP-MEND, a cationic system, could be shielded by the 5 mol% PEG and more than 10 mol% PEG was necessary for shielding its surface sufficiently (Supplementary Fig. S5), indicating that 5 mol% PEG was not sufficient to shield the highly cationic surface of the MEND. Once the optimized YSK05-MEND is internalized via endocytosis, YSK05 (50 mol% of total lipid) was immediately protonated in the acidified interior of the endosome. Therefore, only 5 mol% PEG could not sufficiently prevent the interaction between the protonated (cationic) YSK05 of the MEND lipid bilayer and anionic lipids of the endosomal membrane, resulting in efficient endosomal escape. This suggests that YSK05 is sufficiently

potent to permit it to overcome the PEG dilemma, compared to the use of PPD and GALA, as we previously reported [21,22,24]. PEG-DSG incorporated MENDs containing various lipid compositions were topically administrated on OS-RC-2 subcutaneous tumors and the gene silencing activity of each MEND was evaluated *in vivo*. Only the MEND containing YSK05 was able to induce gene silencing, indicating that YSK05 is also able to overcome the PEG dilemma *in vivo*, as well (Fig. 5c). Furthermore, it was revealed that the PEGylation of the YSK05-MEND was necessary to induce a gene silencing in tumor tissues, because the PEGylation resulted in increasing the stability of the YSK05-MEND or changing the intratumoral trafficking of the YSK05-MEND. The result for 5' RACE PCR suggests that the PLK1 gene silencing in tumor tissue surely results from a sequence specific RNAi mechanism (Fig. 5d). It is well-known that the suppression of PLK1 function or gene expression leads to apoptosis or the suppression of cell proliferation in most cancer cell lines. Given this fact, we evaluated PLK1 gene silencing and cytotoxicity on both OS-RC-2 cells and HeLa-dluc cells *in vitro* (Supplementary Fig. S7). The findings revealed that gene silencing by the optimized YSK05-MEND was induced more easily in OS-RC-2 cells than in HeLa-dluc cells (Supplementary Fig. S7a). However, cytotoxicity as the result of PLK1 silencing was not observed on OS-RC-2 cells, but was clearly observed in HeLa-dluc cells (Supplementary Fig. S7b, c). These findings, therefore, suggest that PLK1 gene silencing on OS-RC-2 subcutaneous tumor tissue by the topical administration of optimized YSK05-MEND would fail to induce an antitumor effect. To obtain an antitumor effect, it will be necessary to examine other target genes that are capable inducing apoptosis or suppressing the cell proliferation of OS-RC-2 cells through its gene silencing.

## 5. Conclusion

The results of present study indicate that the new pH-sensitive cationic lipid YSK05 improves the intracellular trafficking of non-viral vectors. In an *in vitro* study, the gene silencing efficiency of YSK05-MEND was significantly greater than that of DOTAP- and DODAP-MENDs. It was confirmed that YSK05-MEND efficiently escaped from endosomes and the process was strongly dependent on endosomal acidification. Furthermore, the PEG-DSG incorporated optimized YSK05-MEND showed a gene silencing activity that was similar to LF2k, suggesting that YSK05 overcame the suppression of endosomal escape by PEGylation. In the *in vivo* study, the optimized YSK05-MEND indicated most efficient gene silencing of all MENDs and RNAi mediated gene silencing was confirmed by the detection of sequence specific mRNA cleavage products using the 5' RACE PCR method. Collectively, YSK05 effectively enhances siRNA delivery both *in vitro* and *in vivo*.

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### Table caption

#### **Table 1. Physical properties of MENDs**

Particle diameter, polydispersity and zeta-potential were measured using a Malvern Zetasizer. Percentage of siRNA encapsulation was determined by Ribogreen fluorescence assay to measure the amount of siRNA relative to total siRNA present. Apparent pKa value was determined by TNS fluorescence assay as a pH giving rise to half-maximal fluorescent intensity. Data points are expressed as the mean  $\pm$  SD (n=3).

## Figure captions

### Fig. 1. Chemical structures of cationic lipids incorporated into MENDs

DOTAP and DODAP are conventional and available cationic lipids which have quaternary ammonium, indicating cationic without depending on pH, and tertiary amine, indicating cationic at acidic pH and neutral at physiological pH, respectively. YSK05 is a cationic lipid that contains a tertiary amine group for pH-sensitivity.

### Fig. 2. Comparison of MENDs containing each cationic lipid *in vitro*

(a) MEND mediated gene silencing *in vitro*. HeLa-dluc cells were treated with MENDs containing anti-luciferase siRNA for 24 hours. (b) Cellular uptake of MEND. HeLa-dluc cells were treated with MENDs containing <sup>3</sup>H-labeled CHE for various times. The cells were washed to remove unincorporated MENDs prior to measurement of <sup>3</sup>H-CHE. (c) MEND mediated hemolysis *in vitro*. RBC suspension was mixed with empty DOTAP- (left), DODAP- (center) or YSK05- (right) MEND and incubated for 30 minutes at 37<sup>0</sup>C. The absorbance due to hemoglobin was measured after removal of intact RBCs by centrifugation. Data points are represented as the mean ± SD (n=3). N.C.; negative control, P.C.; positive control.

### Fig. 3. Comparison of YSK05-MENDs before and after optimization of lipid composition

(a) MEND mediated gene silencing *in vitro*. HeLa-dluc cells were treated with MENDs containing anti-luciferase siRNA for 24 hours (n=3-6). (b) Cellular uptake of MEND. HeLa-dluc cells were treated with MENDs containing <sup>3</sup>H-labeled CHE for various times. The cells were washed to remove unincorporated MENDs prior to measurement of <sup>3</sup>H-CHE (n=3). (c) MEND mediated hemolysis *in vitro*. RBC suspension was mixed with empty unoptimized (left) or optimized (right) YSK05-MEND and incubated for 30 minutes at 37<sup>0</sup>C. The absorbance from hemoglobin was measured after removal of intact RBCs by centrifugation (n=3). Data points are represented as the mean ± SD. N.C.; negative control, P.C.; positive control.

### Fig. 4. Confirming the endosomal escape of optimized YSK05-MEND

(a) The intracellular pattern and distribution of siRNA. HeLa-dluc cells were treated with Cy5-labeled siRNA formulated in DODAP- (left) or optimized

YSK05- (right) MEND at a dose of 100 nM siRNA for 1 hour. Unincorporated MENDs were washed out and siRNA distribution was visualized after 0 hour (upper) or 6 hours (lower) using a confocal microscopy. Scale bars indicate 20  $\mu$ m. Full-size pictures are presented in Supplementary Fig. S3. (b) MEND mediated gene silencing in the presence of the chemicals buffering endosome. HeLa-dluc cells were treated with anti-luciferase siRNA formulated in DOTAP-MEND (150 nM) and optimized YSK05-MEND (27 nM) in the presence of chloroquine (upper) or  $\text{NH}_4\text{Cl}$  (lower) for 4 hours. The cells were further incubated for 20 hours after medium change. Data points are represented as the mean  $\pm$  SD (n=3).

**Fig. 5. Gene silencing activity of PEGylated optimized YSK05-MEND *in vitro* and *in vivo***

(a) MEND mediated gene silencing *in vitro*. HeLa-dluc cells were treated with MENDs and Lipofectamine 2000 (LF2k) incorporating anti-luciferase siRNA (siGL4) for 24 hours (n=3). (b) Cellular uptake. HeLa-dluc cells were treated with MENDs containing  $^3\text{H}$ -labeled CHE for various times (n=3). (c) MEND mediated gene silencing *in vivo*. OS-RC-2 tumor bearing mice were topically administrated with MENDs at a dose of 10  $\mu$ g siPLK1 or siGL4 into tumors. After 24 hours, mice were euthanized and PLK1 gene expression was measured (n=4). (d) 5' RACE PCR analysis. RNAi specific PLK1 mRNA cleavage products were detected after 24 hours topical administration of MENDs. Positive control from *in vitro* OS-RC-2 cell lysates treated with siPLK1 indicated by plus sign and negative control indicated by minus sign. RACE PCR detects the 5' cleavage product of human PLK1 mRNA from tumors. Each lane represents different tumor tissues. The predicted 321-bp PLK1 product is indicated by an arrow. Data points are represented as the mean  $\pm$  SD.