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Pharm Res (2014) 31:2685–2695
DOI 10.1007/s11095-014-1366-7

RESEARCH PAPER

Insight into Mechanisms of Cellular Uptake of Lipid Nanoparticles and Intracellular Release of Small RNAs

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Received: 13 November 2013 / Accepted: 21 March 2014 / Published online: 17 April 2014

ABSTRACT

Purpose Understanding mechanisms of cellular uptake and intracellular release would enable better design of nanocarriers for delivery of nucleic acids such as siRNA and microRNA (miRNA).

Method In this study, we investigated cellular pharmacokinetics of siRNA by co-encapsulating fluorescently labeled siRNA and molecular beacon (MB) in four different formulations of cationic lipid nanoparticles (LNPs). A miRNA mimic was also used as a probe for investigating cellular pharmacokinetics, which correlated well with RNAi activities.

Results We tried to find the best LNP formulation based on the combination of DOTMA and DODMA. When the DOTMA/DODMA ratio was at 5/40, the LNP containing a luciferase siRNA produced the highest gene silencing activity. The superior potency of DOTMA/DODMA could be attributed to higher uptake and improved ability to facilitate siRNA release from endosomes subsequent to uptake.

Conclusions Our findings may provide new insights into RNAi transfection pathways and have implications on cationic LNP design.

KEY WORDS intracellular trafficking · lipid nanoparticles · miRNA · molecular beacon · siRNA

INTRODUCTION

RNA-based therapeutics such as small interfering RNA (siRNA) and microRNA (miRNA) hold great promise to treat a variety of human diseases by silencing target genes in a specific manner (1–4). Delivery is the key to their clinical translation. In designing a delivery system, degradation avoidance, efficient cellular uptake, timely endosomal escape and efficient loading into the RNA-induced silencing complex (RISC) are all critical for the successful induction of gene silencing by small RNAs. Tremendous progress has been made in the development of safe and efficient delivery systems for the clinical application of RNAi therapeutics (2,5–7). Lipid nanoparticles (LNPs) are recognized as one of the most promising delivery systems for RNAi

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because of their relative safety and simplicity. Both permanently and conditionally ionized cationic lipids have been extensively utilized in LNP formulations for siRNA or miRNA delivery (5–10). However, these formulations are still far from optimal.

The potency of an LNP formulation depends on both their cellular uptake and intracellular trafficking processes (2,10–13). Fundamental understanding of these mechanisms and their correlations are very important for improving formulation design. Despite extensive research, few general conclusions can be drawn on cellular uptake kinetics, uptake mechanism, and intracellular trafficking pathways of the LNPs. Although fluorescently-labeled siRNAs have been widely used to investigate their intracellular trafficking (5,7–10,14–21), only limited quantitative information on the kinetics of intracellular trafficking of siRNA is available. Therefore, an accurate and quantitative methodology is needed to better understand the intracellular trafficking of siRNA or miRNA containing nanocomplexes. Fluorescence resonance energy transfer (FRET) can discern spatial separation and has been used to measure lipid/DNA or lipid/siRNA condensation and dissociation processes by many researchers (17,19,22–25). As dual-labeled oligonucleotide probes with a reporter fluorophore at one end and a quencher at the other, molecular beacons (MB) can be used as probes for intracellular mRNA targets (19). MB has been used as a probe to track endosomal release in our recent publication (21). qRT-PCR based methods are also available for the analysis of intracellular nucleic acids. For example, Hayashi *et al.* (26) reported that there is good correlation between intracellular siRNA molecules and their RNAi activities both in cultured cells and in mouse liver.

Cellular delivery of siRNA and miRNA mediated by LNPs is a dynamic and complicated multi-step process that includes highly inter-connected cellular uptake, intracellular processing, and gene silencing activities. Currently, there lacks a suitable method to decouple the cellular uptake and the endosomal escape processes. In this study, we demonstrate a simple and non-invasive method that can easily and reliably track cellular uptake and endosomal release events by combining fluorescently labeled siRNA with MB or miRNA mimics. We used this method to compare four commercially available cationic lipids formulated as LNPs for siRNA delivery, and found that potent gene silencing could be achieved through the combination of permanently and conditionally ionized cationic lipids. Our work provides new insight into the mechanisms of cellular uptake and intracellular release of LNPs.

MATERIALS AND METHODS

Materials

1,2- Dioleyloxy-3-trimethylammonium propane (DOTMA), 2-dioleoyl-3-trimethylammonium-propane (DOTAP), and *N*-palmitoyl-sphingosine-1-succinyl [methoxy(polyethylene glycol) 2000] (Ceramide-PEG2000) were obtained from Avanti Polar Lipids, Inc (Alabaster, AL, USA). 1,2-Dioleyloxy-N,N-dimethyl-3-aminopropane (DODMA) was purchased from Genzyme Pharmaceuticals (Cambridge, MA, USA). Egg phosphatidylcholine (egg PC) was obtained from Lipoid (Newark, NJ, USA). α-(3β)-Cholest-5-en-3-yl-hydroxy [methoxy (polyethylene glycol)] (Chol-PEG) was from NOF America Corporation (White Plains, NY). Other chemicals and reagents were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA) and were of analytical grade. All tissue culture media and supplies were obtained from Invitrogen (Carlsbad, CA, USA). All other reagents were of analytical grade. Luciferase-targeted and negative control siRNA as well as FAM-siRNA scramble were obtained from Applied Biosystems (Austin, TX, USA). The silencer® GL2+GL3 luciferase siRNA (siLuc, AM4629), control #1 siRNA (siNC, AM4611), FAM™-labeled negative control #1 siRNA (FAM-siRNA, AM4620), Cy3-labeled negative control #1 siRNA (Cy3-siRNA, AM4621), Quant-iT™ RiboGreen® RNA reagent, and Quant-iT™ OliGreen® ssDNA reagent were supplied by Applied Biosystems (Austin, TX, USA). Cy3-labeled GAPDH molecular beacon (Cy3-MB) was custom-synthesized by Eurofins MWG Operon (Huntsville, AL, USA). The sequence was 5′- Cy3-CGACGGAGTCCT TCCACGATACCACGTCG-BHQ2-3′. miRNA-122 mimic (Cat. No. C-300591-05) was purchased from Dharmacon (Pittsburgh, PA). TaqMan MicroRNA reverse transcription kit was provided by Applied Biosystems (Cat. No. 4366596).

Preparation of siRNA or miRNA Loaded LNPs

An ethanol dilution method was modified for the synthesis of LNPs containing siRNA or miRNA (27). Briefly, an ethanolic lipid solution composed of various cationic lipids/eggPC/Cholesterol/Chol-PEG at 45:35:48:2 (molar ratio) was mixed with si/miRNA in citrate buffer (20 mM, pH=4) at an RNA/lipid ratio of 1/10 (*w*/*w*) (7,28). The resulting complexes contained 30% ethanol, which was removed by dialysis using a MWCO 20,000 Dalton Float-A-Lyzer (Spectrum Laboratories Inc., Ranco Dominguez, CA, USA) against HEPES buffer (20 mM HEPES, pH 7.4) for 2 h at room temperature.

Measurement of Size, Zeta Potential and siRNA Incorporation Efficiency

The particle size of siRNA loaded LNPs was determined by dynamic light scattering using a particle sizer BI-200SM (Brookhaven Instruments Corp., Holtsville, NY, USA) in an intensity-weighted mode. Following dilution in water, the zeta potentials (ζ) of siRNA-LNPs was measured on a ZetaPALS zeta potential analyzer (Brookhaven Instrument Corp.,

Holtsville, NY). The siRNA incorporation efficiencies of the formulations were measured using the Quant-iT™ RiboGreen® RNA Kit, following manufacturer's instruction (7,21).

Cell Culture and *In Vitro* Transfection

SK-HEP-1 cells with stable luciferase expression (SK Hep-1 Luc) were grown in MEM culture medium supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin, at 37°C in a humidified atmosphere containing 5% CO_2. SK-HEP-1 cells were plated at a density of 1×10^4 cells per well in 96-well plates and grown to 60–70% confluent prior to transfection. Luciferase specific siRNA (Luci-siRNA) and negative control (NC siRNA) were formulated into various cationic lipids based LNPs. In all transfection studies, cells were treated with various siRNA-LNPs at the concentration of 100 nM in the medium with 10% FBS. The SK-HEP-1 cells were incubated for another 24 h at 37°C and 5% CO_2 and then washed with PBS and lysed. The luciferase activity for each well was determined using Luciferase Reagent (Promega) on a Berthold MicroLumatPlus LB96V plate luminometer. The resulting luciferase activity was then normalized to the amount of protein determined by the Micro BCA assay kit (Pierce, Rockford, IL). Luciferase down-regulation relative to controls was then determined for each condition. Lipofectamine 2000 (Invitrogen, CA, USA) was used as a positive control. Untreated cells were used as a negative control.

Cellular Uptake and Endosomal Release of siRNA Measured by Flow Cytometry

Cy3 or FAM-siRNA was used to study cellular uptake of LNPs. A total of 6×10^4 cells/well were seeded in 24-well plates prior to treatment. At 4 h post transfection with the Cy3 or FAM-siRNA loaded Lipofectamine 2000, LNPs-Cy3 or FAM-siRNA, the cells were rinsed three times with 500 μl phosphate buffered saline (PBS, pH = 7.4) and fixed in 4% para-formaldehyde solution. The cell suspension was directly introduced into a Beckman Coulter EPICS XL (Beckman Coulter Inc., CA, USA) to determine the fluorescence intensity of Cy3 (550 nm exCitation, 570 nm emission) or FAM (492 nm exCitation, 518 nm emission). For each cell sample, a minimum of 10,000 events were collected under the LIST mode.

For the endosomal release study, Cy3-MB was complexed with various cationic LNPs following the same method of preparing siRNA loaded LNPs. For transfection, 6×10^4 SK Hep-1 cells were seeded in a 24-well plate one day before treatment. Cells were treated with the LNPs containing Cy3-MB (100 nM) at 37°C. At indicated time points, the cells were collected, washed three times with PBS, and fixed in 4% para-formaldehyde. The mean fluorescence intensities (MFI) of Cy3-MB were measured by flow cytometry on a Becton Dickinson FACS Calibur cytometer. For each cell sample, a minimum of 10,000 events were collected under the LIST mode.

Laser-Scanning Confocal Microscopy Analysis

For confocal analysis of the trafficking of LNPs, FAM-siRNA and Cy3-MB at a molar ratio of 50/50 were first complexed with DOTAP or DODMA based LNPs following the same method of preparing siRNA loaded LNPs. The seeded SK-HEP-1 cells were treated with FAM-siRNA (50 nM) and Cy3-MB (50 nM) for 2 h. The nuclei were stained with 5 mg/ml of Hoechst 33342 for 10 min at room temperature. After washing twice with PBS, the cells were fixed with 4% para-formaldehyde prior to confocal analysis.

To determine the endocytic pathways that may be involved in siRNA delivery by DOTAP or DODMA based LNPs, co-localization experiments of Cy3-labeled siRNA with common endocytic pathway markers were performed by confocal microscopy. Briefly, 4×10^4 cells were seeded on a glass-bottom dish (Fisher Scientific, 12-545-82) in 24-well plates overnight and then incubated with various LNPs formulated with Cy3-siRNA (100 nM) and markers for macropinocytosis (dextran-FITC (490 nm exCitation, 525 nm emission), 2.5 mg/ml, Sigma), clathrin-mediated endocytosis (Transferrin-AlexaFluor 488 (499 nm exCitation, 519 nm emission), 100 mg/ml, Invitrogen), or caveolae-mediated endocytosis (cholera toxin subunit B-AlexaFluor 488, 1 mg/ml, Invitrogen). After incubation of 30 min at 37°C, the cells were washed twice with PBS followed by fixation with 4% para-formaldehyde for 20 min. Nuclei were stained with Hoechst 33342 (Invitrogen) for 5 min at room temperature. Red fluorescence of Cy3-siRNA and blue fluorescence of Hoechst were observed on an Olympus FV1000 Filter Confocal Microscope (Olympus Optical Co., Tokyo, Japan).

Quantitative Real-Time PCR Analysis of miR-122 in Cells

The cells were washed with cold 1X PBS twice and then treated with TRIzol reagent (Invitrogen, 15596–018). Total RNA was extracted according to manufacturer's instructions. To measure mature miR-122 level, the total RNA was first reverse transcribed into cDNA using the TaqMan MicroRNA reverse transcription kit (Applied Biosystems, 4366596). qRT-PCR amplification of cDNA was then performed using TaqMan MicroRNA assay (Applied Biosystems, Assay ID 002247). miR-122 level was determined by the ΔΔCT method and normalized to RNU6B (Applied Biosystems), which was an endogenous control (28).

Statistical Analysis

The results were presented as the mean ± standard deviation (SD) of three repeat studies unless otherwise indicated. Statistical significance of the differences in fluorescent intensity data obtained by flow cytometry was examined using the Student's t-Test.

RESULTS

Cellular Uptake of LNPs Does not Closely Correlate with the Efficacy of Gene Down-Regulation

To achieve successful target gene silencing, exogenous synthetic siRNAs should be efficiently encapsulated in LNP that have high cellular uptake and efficient endosomal escape, along with timely intracellular disassemble and effective loading into the RNA-induced silencing complex (RISC) (Fig. 1). Therefore, cellular uptake of siRNA encapsulated LNPs (LNP-siRNA) is a critical step for delivery. It is generally accepted that higher cellular uptake generates better efficacy of nanocarrier mediated nucleic acid delivery (8,9,21,26,29). To identify the correlation between cellular uptake and final siRNA gene silencing, we first evaluated the gene silencing efficiency of four LNP formulations differing in cationic lipids on SK-HEP-1 cells that stably express luciferase gene. Luciferase specific and negative control siRNA were delivered with various LNPs and luciferase expression was determined 24 h after transfection.

Four cationic lipids were classified into two groups: permanently ionized cationic lipids (DOTAP and DOTMA) and conditionally ionized (ionizable) cationic lipids (DODAP, and DODMA), which contain a quarternary amine and a tertiary amine head-group, respectively (Fig. 2a). Since the LNPs were designed for *in vivo* applications, other components such as EggPC, Chol (helper lipids) and Chol-PEG (PEG-lipid) were included in LNPs besides cationic lipids. The particle size and zeta potential of the four resulting LNP-siRNAs are listed in Table I. The siRNA encapsulation efficiencies of all LNPs were higher than 90% (data not shown). Among all tested LNPs, DOTMA and DODMA based LNPs demonstrated better luciferase silencing at 100 nM after 24 h transfection (Fig. 2b). Compared to Lipofectamine 2000, DODMA based formulation, when used to transfect cells, resulted in 15% lower luciferase expression.

The cellular uptake studies were carried out using Cy3 as the fluorophore for siRNA labeling because its fluorescence is relatively insensitive to pH changes that occur in the endosome or lysosome. The percentage of positive cells in the flow experiments was more than 90%. As shown in Fig. 2c, the mean fluorescence intensity (MFI) values of permanently ionized cationic lipids (DOTAP and DOTMA) based LNPs were

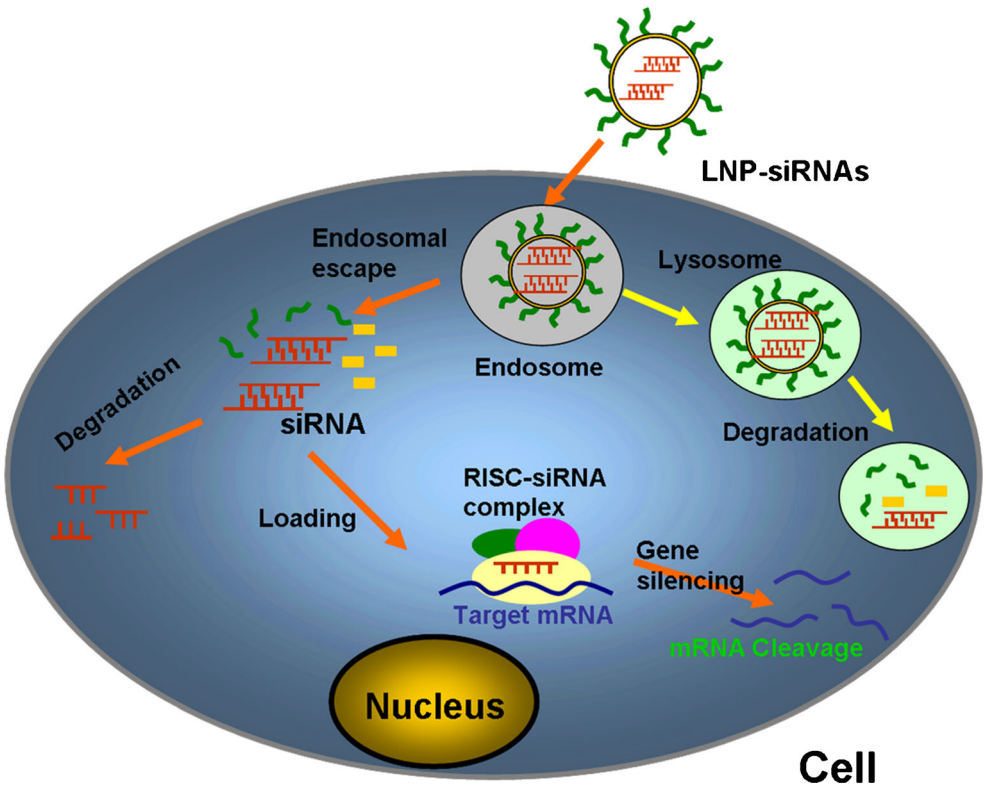


Fig. 1 Schematic diagram of the intracellular trafficking of siRNA loaded in lipid nanoparticles (LNPs). siRNA loaded LNPs are internalized by cells via endocytosis. A fraction of the siRNA escape from the endosomes into the cytosol, whereas the rest are transported into lysosomes and undergo degradation. The condensed siRNA was released from LNPs into the cytosol. The released siRNA engage with RNA-induced silencing complex (RISC), leading to target mRNA cleavage.

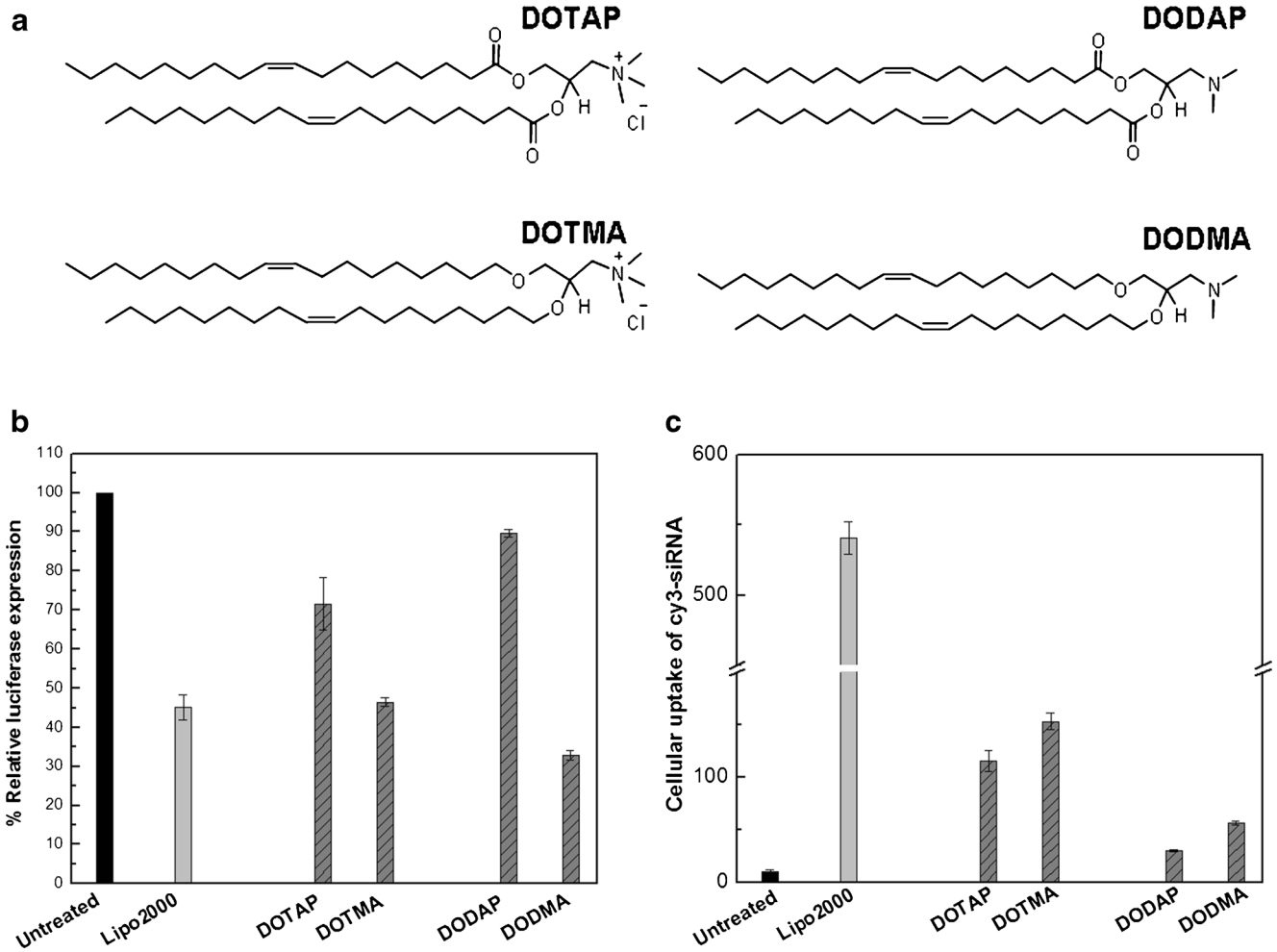


Fig. 2 Poor correlation between siRNA down-regulation and cellular uptake. (**a**) Chemical structures of four tested cationic lipids; (**b**) Evaluation of luciferase gene silencing efficacies of four cationic lipids based LNPs. SK-HEP-1 cells were transfected with 100 nM luciferase siRNA. After 24 h incubation, the luciferase gene silencing was determined. (**c**) Cellular uptake determined by flow cytometry. Cy3-labeled nonspecific siRNA (control siRNA) was complexed with various LNPs or with Lipofectamine 2000. The mean fluorescence intensity (MFI) of Cy3-siRNA was determined by flow cytometry to study the cellular uptake of Cy3-siRNA in LNPs after 4 h treatment. Data are represented the mean ± S.D. ($n = 3$).

relatively higher than those of conditionally ionized cationic lipids (DODAP, and DODMA) based LNPs. For example, the MFI of 56 for DODMA LNPs was much lower than the MFI of 153 for DOTMA LNPs (Fig. 2c) though their gene silencing activities were comparable (Fig. 2b). The MFI for Lipofectamine 2000 mediated Cy3-siRNA delivery was significantly higher than those of four cationic LNPs mediated Cy3-siRNA. However, its luciferase gene silencing efficacy was lower than that of DODMA based LNPs.

Keeping the remaining components the same in the LNP formulation, the efficiency of luciferase gene silencing for the four cationic lipids decreased in the following order: DODMA > DOTMA > DOTAP > DODAP (Fig. 2b). However, the oligonucleotide cellular uptake determined by Cy3-siRNA reveals the following trend: DOTMA > DOTAP > DODMA > DODAP (Fig. 2c). Thus, we can conclude that higher cellular uptake of siRNA does not directly translate into better siRNA gene silencing efficacy.

Table I Characterization of Particle Size and Zeta Potential for siRNA Loaded LNPs

Formulation	Components	Particle size (nm)	Zeta potential (mV)
DOTAP-LNP	DOTAP/eggPC/Chol/Chol-PEG=45/18/35/2	106.1	15.32 ± 1.24
DOTMA-LNP	DOTMA/eggPC/Chol/Chol-PEG=45/18/35/2	99.7	17.05 ± 1.06
DODAP-LNP	DODAP/eggPC/Chol/Chol-PEG=45/18/35/2	132.2	2.22 ± 0.22
DODMA-LNP	DODMA/eggPC/Chol/Chol-PEG=45/18/35/2	85.2	2.07 ± 0.85

Probing Mechanisms of Cellular Uptake and Endosomal Escape Using a Combination of FAM-siRNA and Cy3-MB

MBs become fluorescent only when they enter into the cytoplasm and bind to target mRNAs. Because of the comparable physiochemical properties of siRNA and MB molecules, we can use the MB as a mimic of siRNA to measure the amount of siRNA delivered into the cytoplasm. A quantitative analysis of the nanocarrier-mediated disposition of siRNA through a combination of FAM-siRNA and Cy3-MB (21) was used to investigate the influence of cellular pharmacokinetics (uptake and endosomal release) on transfection efficiency of siRNA mediated by ionized or permanently conditionally ionized cationic lipids. DOTAP and DODMA were selected as the representative cationic lipids because they are the most commonly cationic lipids used in previous studies. As illustrated in Fig. 3, cellular uptake and cytoplasmic release of siRNA over an 8 h-period were quantified by measuring the fluorescence intensity of FAM-siRNA and Cy3-MB using flow cytometry, respectively. DOTAP based LNPs showed continuous increase of cellular uptake (measured by MFI of FAM) and endosomal release (measured by MFI of Cy3) over a 4 h-period (Fig. 3a). For DODMA based LNPs, the highest uptake (FAM signal) was also observed at 4 h, whereas the Cy3 signal for endosomal release increased continuously over the 8 h period (Fig. 3b). Thus, the majority of intracellular cellular uptake took place within the first 4 h. In comparison to DODMA based LNPs, the overall MFI for DOTAP based LNPs was much higher indicating that the cellular uptake capacity for DOTAP LNPs was greater than that for DODMA LNPs. This result was in good agreement with the observation shown in Fig. 2b. Although the cellular uptake signal of DOTAP LNPs was stronger, the endosomal release signal of DOTAP LNPs was lower than that of DODMA LNPs during the period of 8 h. Hence, DODMA was better than DOTAP with respect to the endosomal release capacity. The time frame for intracellular trafficking events shown in Fig. 3 can explain the gene silencing activity difference between DOTAP and DODMA LNPs shown in Fig. 2b.

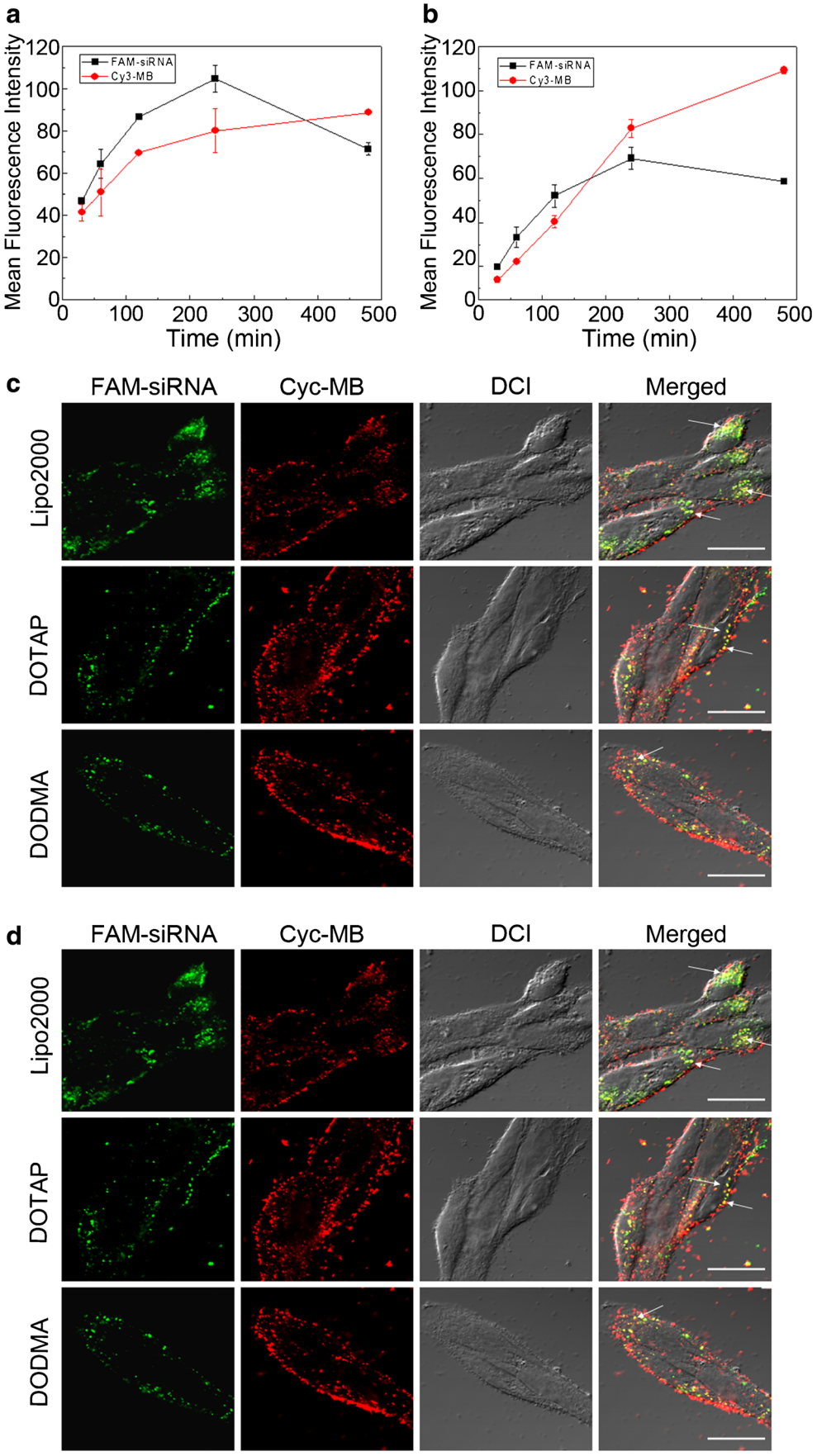
To further investigate intracellular trafficking of DOTAP and DODMA based LNPs, we performed the co-localization study on LNPs loaded with FAM-siRNA and Cy3-MB at the time point of 4 h. As seen in Fig. 3c, cells treated with DOTAP-LNPs exhibited a higher green signal (cellular uptake) but a comparable red signal (endosomal escape) relative to SK-HEP-1 cells treated with DODMA-LNPs, which was consistent with the results shown in Fig. 3a and b. However, we also observed some yellow dots (indicated by white arrows in Fig. 3c), suggesting the separation of the reporter fluorophore (Cy3) probes from the quencher on MB in the absence of endosomal release. In other words, there existed some non-specific biodegradation of Cy3-MB and FAM-siRNA associated with the LNPs.

Fig. 3 Cellular uptake and endosomal escape studied by a combination of FAM-siRNA and Cy3-MB. (**a**) Time-dependent measurement of FAM and Cy3 signals for DOTAP based LNPs; (**b**) Time-dependent measurement of FAM and Cy3 signals for DODMA based LNPs; (**c**) Co-localization study of LNP-FAM-siRNA (*green*) with Cy3-MB (*red*). White arrows indicate the co-localization. Scale bar = 20 μm. (**d**) Endocytoic uptake mechanism of siRNA delivery by DODMA or DOTAP based LNPs. SK-HEP-1 cells were treated with 100 nM Cy3-labeled siRNA (*red*) in the presence of various labeled markers for 1 h. Markers for macropinosomes (70 kDa dextran), clathrin-coated pits (transferrin), and caveosomes (CT-B) were used. Scale bar = 10 μm.

Nanocarriers such as LNPs were generally internalized through endocytosis and the specific endocytoic pathway utilized may determine the final delivery efficacy (11–13). Endocytic mechanisms of siRNA delivery for DOTAP and DODMA based LNPs were investigated using co-localization approaches in SK-HEP-1 cells. SK-HEP-1 cells were transfected with the Cy3-siRNA-LNPs (Red) and were co-labeled with specific markers of endocytic pathways (Green): clathrin-mediated endocytosis (A488-Tf), caveolae-mediated endocytosis (A488-CT-B), and macropinocytosis (FITC-dextran) (7,21). The yellow clusters in Fig. 3d show that siRNAs in DODMA and DOTAP based LNPs strongly co-localized with A488-Tf, a common pathway marker for the classical clathrin mediated endocytosis. The red LNPs did not show detectable co-localization with either FITC-dextran or A488-CT-B. So both DOTAP and DODMA based LNPs entered the SK-HEP-1 cells through the clathrin-mediated endocytic pathway.

Comparison of siRNA Delivery Mediated by Four Different Cationic Lipids

Based on the above findings, a comparison of four different cationic LNPs was performed, via the combination of FAM-siRNA and Cy3-MB at the time point of 8 h. The mean fluorescence intensity was quantified by flow cytometry and summarized in Fig. 4. The efficiency of overall cellular uptake (determined by FAM signal) for the tested four cationic lipids decreased in the following order: DOTMA > DOTAP > DODMA > DODAP, which is consistent with the observation in Fig. 2c. The efficiency of endosomal release determined by Cy3-siRNA reveals the following trend: DOTMA ≈ DODMA > DOTAP > DODAP, which correlates with the efficacy of gene silencing trend (DODMA > DOTMA > DOTAP > DODAP) observed in Fig. 2b. Overall, LNPs based on permanently ionized cationic lipids (DOTAP and DOTMA) showed much stronger cellular uptake than those based on conditionally ionized cationic lipids, including DODAP, and DODMA.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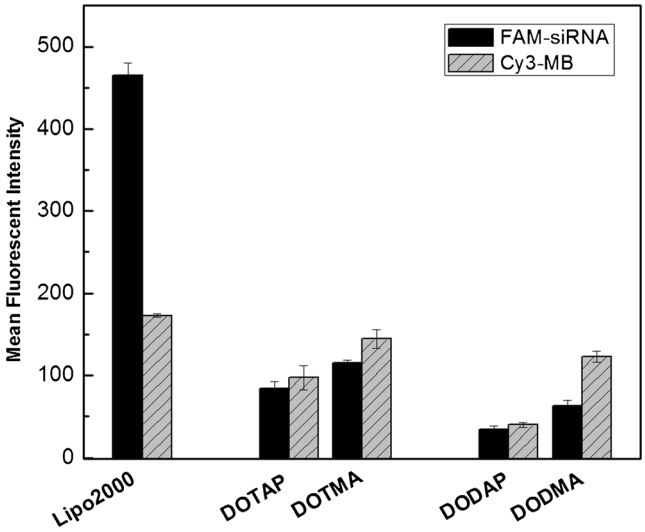

Fig. 4 Comparison of siRNA delivery mediated by four different cationic lipids. The analysis of four different cationic LNPs mediated siRNA delivery in SK-HEP-1 cells were performed via the combination of FAM-siRNA and Cy3-MB at the time point of 8 h. Mean fluorescence intensity was quantified by flow cytometry. Data are represented the mean ± S.D. ($n = 3$).

Compared with Lipofectamine 2000, both FAM and Cy3 signals for four cationic lipids were lower. However, the luciferase gene silencing of Lipofectamine 2000 in Fig. 2b did not match with the observation in Fig. 4. In other words, the Cy3 signal reflecting endosomal release efficiency did not closely correlate with the efficacy of siRNA-mediated gene silencing. We noted an inconsistency in the relationships between endosomal release and gene silencing comparing DOTMA and DODMA. A possible reason for this inconsistency may be the non-specific bio-degradation of MB in endosome prior to the escape (Fig. 3c). Hence, we need a more precise approach or probe to better describe the intracellular trafficking process in order to explain the gene silencing activities of various cationic LNPs.

Comparison of Cellular Pharmacokinetics Using microRNA Mimics as Probe

To precisely determine the cellular pharmacokinetics, we used miRNA mimics as a probe. miRNA mimics are structurally similar to siRNAs and both of them function through an RNAi mechanism. The kits for measuring microRNA mimics are commercially available. It has to be noted that this PCR quantification method can determine cell-internalized miRNA but cannot discriminate endosomal/vesicular (inactive) miRNA from cytosolic (active) miRNA. We chose the liver specific miR-122 in this study because it is drastically reduced in human HCC cells and holds great promise for miRNA based cancer therapy (30–32). DOTAP and DODMA were selected as the representatives for permanently ionized and ionizable cationic lipids, respectively. miR-122 level was evaluated over a 8 h-period in SK-HEP-1 cells after transfection with miR-122 mimics containing DOTAP or DODMA based LNPs (Fig. 5a). The miR-122 level of DOTAP-LNPs mediated delivery increased during the period of 0 ~ 2 h but decreased after 2 h. The level of miR-122 in SK-HEP-1 cells treated with DODMA based LNPs reached the maximum at the time point of 4 h and then decreased to 67% at 8 h. In addition, the overall level of miR-122 in DODMA based LNPs was higher than that of DOTAP based LNPs at the same time point, suggesting that the delivery of siRNA or miRNA mediated by DODMA containing LNPs can lead to better cellular uptake and endosomal escape, followed by higher target gene silencing.

Next, we examined miRNA-122 delivery by various cationic LNPs at the time point of 8 h (Fig. 5b). Among all tested groups, cells treated DODMA based LNPs showed the highest level of miR-122. The trend of miR-122 delivery by various LNPs and Lipofectamine 2000 was consistent with the gene down-regulatory efficacy shown in Fig. 2b. These sets of experiments successfully demonstrated the utility of the miRNA mimic probes for characterizing the cellular uptake and endosomal release processes.

A Combinational Strategy for LNP Mediated Delivery

Given that permanently ionized cationic lipids are better for cellular uptake, while ionizable cationic lipids are better for endosomal release, we hypothesize that a combination of permanently and conditionally ionized cationic lipids for siRNA delivery may achieve the most potent gene silencing. From the gene silencing data shown in Fig. 2a, we selected DOTMA and DODMA to verify this hypothesis. In this experiment, DOTMA and DODMA were mixed at various molar ratios to form different DOTMA/DODMA based LNPs, where the total cationic lipid amount was kept as 45% for all lipids. Luciferase silencing by siRNA was determined in SK-HEP-1 cells to assess the effect of combination on the delivery efficacy of various DOTMA/DODMA based LNPs. Figure 6 shows that the luciferase level was significantly reduced with the increase of DOTMA/DODMA ratio from 5/40 to 40/5. The luciferase expression could be inhibited up to 90% when the DOTMA/DODMA ratio was at 5/40. These results indicated that the combination of DOTMA/DODMA can significantly improve the gene silencing efficiency of siRNA.

DISCUSSION

Cationic lipids based LNPs are attractive delivery systems for small RNAs (siRNA and miRNA) based therapy because a wide variety of LNPs can be produced easily and

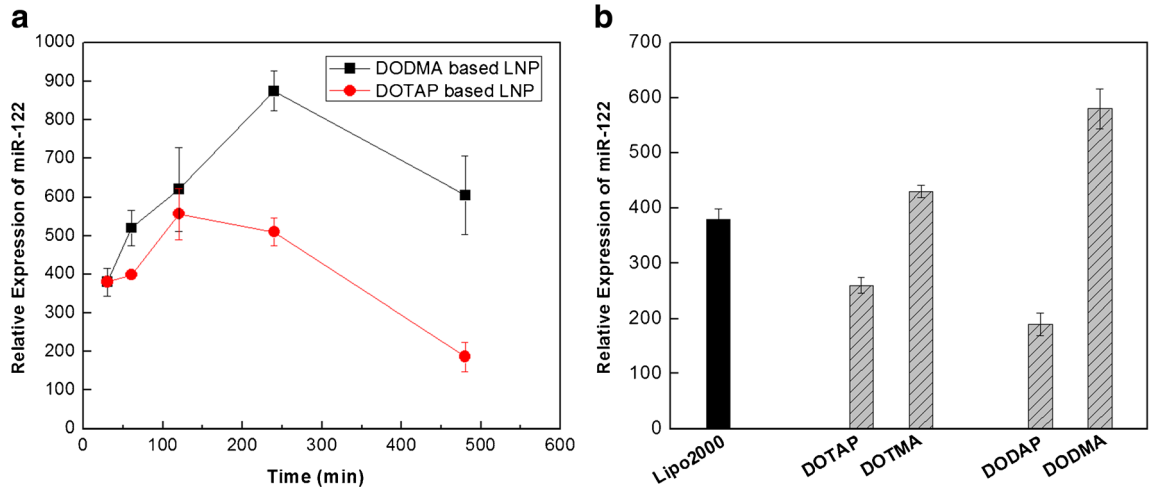

Fig. 5 Comparison of endosomal release using microRNA mimics as probe. (**a**) Time-dependent release of miRNA-122 mediated by DODMA or DOTAP based LNPs; (**b**) Comparison of endosomal release for four LNPs with different cationic lipids using miRNA-122 as probe. Data are represented the mean ± S.D. ($n = 3$).

the safety and effectiveness of this class of nanocarriers have been demonstrated in clinical trials. A large number of cationic lipids have been synthesized for nucleic acid delivery (5–9,19,21,26,33). However, the selection of cationic lipids is still largely based on trial-and-error. A better understanding of cellular uptake and intracellular release mechanisms would enable improved design of LNP formulation for siRNA and miRNA delivery.

Generally, LNP-mediated siRNA delivery can be divided into three steps: (i) binding to the cell surface, (ii) entry of LNP-siRNA into the cells via endocytosis, and (iii) siRNA release from endosome into the cytosol (Fig. 1). To date, the efficiency of RNAi based therapeutic agents delivered by these nanocarriers still remains low, mainly because of our limited understanding of the mechanisms involved and the lack of reliable methods to study the cellular events. Recently, the FRET technology has been used to provide information on the complexation status, uptake, release and degradation of LNPs mediated nucleic acid delivery (17,19,22,24,25,29). For example, the design of FRET systems incorporating quantum dots as energy donors has led to improved signal stability, allowing prolonged measurements and increased sensitivity (24,29). Two different siRNA-based FRET probes have been used to investigate the disassembly behavior of various LNPs containing lipidoids (19). These new probes can measure the disassembly properties without significantly perturbing the structure of the LNPs because it does not require the labeling on LNP themselves.

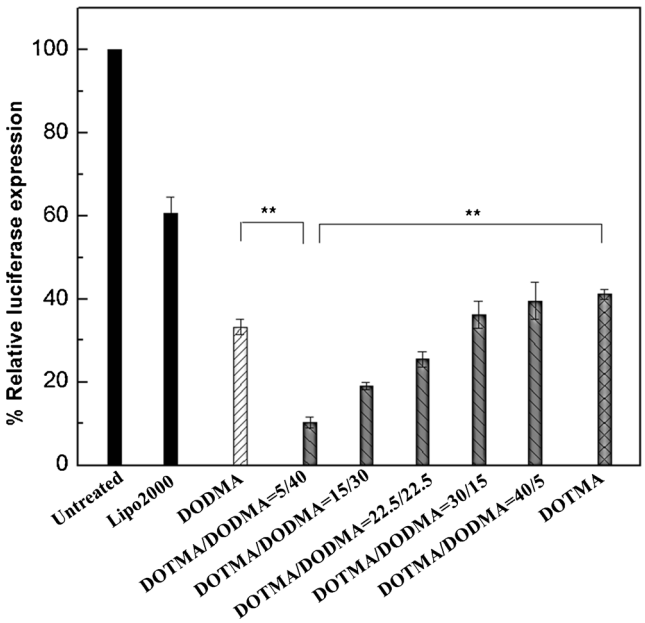

Fig. 6 Enhanced gene silencing by combination of DOTMA and DODMA in LNPs. The gene silencing activities were examined in SK-HEP-1 cells after transfection of 100 nM luciferase siRNA. After 24 h incubation, luciferase gene silencing was determined. Data are represented the mean ± S.D. ($n = 3$, **: $P < 0.01$).

Cationic lipid based LNPs have a positive surface charge, which can improve their interactions with the cell membrane and subsequently facilitate endocytosis mediated cellular uptake. Typically, cationic lipids are classified into two groups according to the amine moiety on the head group (Fig. 2a). Conditionally ionized cationic lipids (DODAP, and DODMA) have a tertiary amine group with a moderate pKa value that shows a positive charge at low pH and are able to efficiently encapsulate nucleic acids during LNP synthesis through temporary reduction of pH, and yield LNPs with neutral or low zeta potential at pH 7.4, i.e., the physiological pH (6–9). The permanently ionized cationic lipids (DOTAP and DOTMA) are not able to change their charge by varying the pH because of the quarternary amine head-group, which has a high pKa value. Besides the cationic lipids, our LNP formulations also included helper lipids and PEG-lipid. PEGylation can increase the stability of siRNA against RNase and prolong the residence

time of LNPs in the blood circulation by avoidance of opsonization. Overall, the cellular uptake by permanently ionized cationic lipid based LNPs was stronger than that by the LNPs containing ionizable cationic lipids because of the higher net positive charge (Table I). However, the gene silencing efficacy results did not agree with cellular uptake findings (Fig. 2).

The role of the cationic lipid in the LNP formulation is to enable the formation of complexes with the negatively charged siRNAs by electrostatic interaction. After the internalization of siRNA-LNP by endocytosis, cationic lipids can form ion pairs with anionic phospholipids within the endosomal membrane, destabilizing the membrane by promoting bilayer transition to an inverted micelle or hexagonal (H_{II}) phase. The transformation to H_{II} phase is expected to trigger endosomal escape and cytoplasmic localization of the cargo siRNA. This has been described as the "flip-flop" mechanism of endosomal escape. Helper lipids, including Egg PC, cholesterol, and dioleoyl-phosphatidylethanolamine (DOPE), are often used to improve the stability of the LNPs or facilitate endosomal escape. Since DOPE is a fusogenic lipid, incorporation of DOPE in LNP formulations could facilate the transition from a lamellar phase into a non-lamellar structure such as the H_{II} phase.

Endosomal escape is widely considered a major rate-limiting step (34). Several strategies have been proposed to enhance the escape from endosome, including the controlled lysosomal destabilization (e.g., triggered by the lysosomal pH value or enzymes) and the incorporation of membrane-disruptive or fusogenic moieties (e.g., the TAT peptide) (2,34,35). Since a molecular beacon (MB) only shows fluorescence when it combines with the target mRNA, MB can be used as a readout for endosomal release. In our study, the FAM-siRNA and Cy3-MB for GAPDH were complexed with various LNPs. The green signal of FAM-siRNA and the red signal of Cy3-MB were able to describe the efficiency of cellular uptake and endosomal release, respectively. Kinetics of cellular uptake and endosomal release were examined for DOTAP and DODMA lipids based LNPs. At 4 h post-transfection, the FAM signal reached the maximum for both DOTAP and DODMA, and afterwards the FAM signal started to decrease, presumably by the biodegradation of the oligonucleotide in endosome and excretion of the fluorophore.

The confocal images (Fig. 3c) further confirmed the relative levels of FAM and Cy3 signals at 4 h (Fig. 3a and b).

The co-staining approaches for the endocytoic pathway study showed that clathrin-mediated endocytosis was used by both DODMA and DOTAP based LNPs. Although the FAM signal of DODMA-LNP was much lower than that of DOTAP-LNP, the Cy3 signal of DODMA-LNP was significantly higher than that of DOTAP-LNP, which may represent a higher available amount of released siRNA that can be loaded onto RISC. Therefore, the superior RNAi activity of DODMA delivered siRNA compared with DOTAP can be attributed to its ability to promote endosomal release. We compared the siRNA delivery mediated by four different cationic lipids through the combination of FAM-siRNA and Cy3-MB (Fig. 4). The capacities of endomal release determined by Cy3-MB correlated well with the gene silencing observed in Fig. 2b.

The miRNA mimics are small, chemically modified double-stranded RNAs that mimic endogenous miRNAs. They are usually used to elucidate the functional roles of individual microRNAs in human, mouse, or rat (36). Due to its structural similarity with siRNA and availability of kits for determination of mature miRNA, a miR-122 mimic was used as a probe to investigate the cellular pharmacokinetics of LNPs. Upon miR-122 is internalized by cells, the guide strand should be detected, the passenger strand was degraded and the guide strand loaded into RISC. Therefore, the guide strand loaded into RISC should be detected. The cellular pharmacokinetics evaluation data based on miR-122 correlated well with the gene silencing efficiency obtained in Fig. 2b. Our analysis reveals that the amount of siRNA internalized by cells is important for efficient gene silencing.

Based on this simple and non-invasive method, which determines the cellular uptake and endosomal release simultaneously, we found that the permanently ionized cationic lipids are better for cellular uptake, while conditionally ionized cationic lipids have better cellular endosomal release. A combination of permanently ionized and conditionally ionized cationic lipids in LNP formulations can achieve more potent gene silencing compared to the two parent cationic LNPs (Fig. 6).

CONCLUSIONS

Our work presents a mechanistic view on the transfection pathway of LNPs by decoupling the steps of cellular uptake and the endosomal release. This study provides a versatile tool that enhances our ability to measure the two important aspects of cellular trafficking in a non-invasive manner. These findings would have direct implication on the future design of LNPs for RNAi therapeutics.

ACKNOWLEDGMENTS AND DISCLOSURES

This work was supported by NSF Nanoscale Science and Engineering Center (NSEC) grant EEC-0914790 and the Chinese National 863 Project (No. 2012AA020804).

REFERENCES

1. Fire A, Xu S, Montgomery MK, Kostas SA, Driver SE, Mello CC. Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. Nature. 1998;391:806–11.
2. Yu B, Zhao X, Lee LJ, Lee RJ. Targeted delivery systems for oligonucleotide therapeutics. AAPS J. 2009;11:195–203.
3. Pecot CV, Calin GA, Coleman RL, Lopez-Berestein G, Sood AK. RNA interference in the clinic: challenges and future directions. Nat Rev Cancer. 2011;11:59–67.
4. Kim DH, Rossi JJ. Strategies for silencing human disease using RNA interference. Nat Rev Genet. 2007;8:173–84.
5. Love KT, Mahon KP, Levins CG, *et al.* Lipid-like materials for low-dose, in vivo gene silencing. Proc Natl Acad Sci U S A. 2010;107:1864–9.
6. Semple SC, Akinc A, Chen J, *et al.* Rational design of cationic lipids for siRNA delivery. Nat Biotechnol. 2010;28:172–6.
7. Yu B, Hsu SH, Zhou C, *et al.* Lipid nanoparticles for hepatic delivery of small interfering RNA. Biomaterials. 2012;33:5924–34.
8. Wang X, Yu B, Ren W, Mo X, Zhou C, He H, *et al.* Enhanced hepatic delivery of siRNA and microRNA using oleic acid based lipid nanoparticle formulations. J Control Release. 2013;172:690–8.
9. Lin PJ, Tam YY, Hafez I, *et al.* Influence of cationic lipid composition on uptake and intracellular processing of lipid nanoparticle formulations of siRNA. Nanomedicine. 2013;9:233–46.
10. Wang X, Yu B, Wu Y, Lee RJ, Lee LJ. Efficient down-regulation of CDK4 by novel lipid nanoparticle-mediated siRNA delivery. Anticancer Res. 2011;31:1619–26.
11. Sahay G, Alakhova DY, Kabanov AV. Endocytosis of nanomedicines. J Control Release. 2010;145:182–95.
12. Duncan R, Richardson SC. Endocytosis and intracellular trafficking as gateways for nanomedicine delivery: opportunities and challenges. Mol Pharm. 2012;9:2380–402.
13. Hsu CY, Uludag H. Cellular uptake pathways of lipid-modified cationic polymers in gene delivery to primary cells. Biomaterials. 2012;33:7834–48.
14. Raemdonck K, Remaut K, Lucas B, Sanders NN, Demeester J, De Smedt SC. In situ analysis of single-stranded and duplex siRNA integrity in living cells. Biochemistry. 2006;45:10614–23.
15. Chen HH, Ho YP, Jiang X, Mao HQ, Wang TH, Leong KW. Quantitative comparison of intracellular unpacking kinetics of polyplexes by a model constructed from quantum dot-FRET. Mol Ther. 2008;16:324–32.
16. Abrams MT, Koser ML, Seitzer J, *et al.* Evaluation of efficacy, biodistribution, and inflammation for a potent siRNA nanoparticle: effect of dexamethasone co-treatment. Mol Ther. 2010;18:171–80.
17. Jiang S, Zhang Y. Upconversion nanoparticle-based FRET system for study of siRNA in live cells. Langmuir. 2010;26:6689–94.
18. Shi B, Keough E, Matter A, *et al.* Biodistribution of small interfering RNA at the organ and cellular levels after lipid nanoparticle-mediated delivery. J Histochem Cytochem. 2011;59:727–40.
19. Alabi CA, Love KT, Sahay G, *et al.* FRET-labeled siRNA probes for tracking assembly and disassembly of siRNA nanocomplexes. ACS Nano. 2012;6:6133–41.
20. Hirsch M, Strand D, Helm M. Dye selection for live cell imaging of intact siRNA. Biol Chem. 2012;393:23–35.
21. Zhou C, Zhang Y, Yu B, Phelps MA, Lee LJ, Lee RJ. Comparative cellular pharmacokinetics and pharmacodynamics of siRNA delivery by SPANosomes and by cationic liposomes. Nanomedicine. 2013;9:504–13.
22. Jarve A, Muller J, Kim IH, *et al.* Surveillance of siRNA integrity by FRET imaging. Nucleic Acids Res. 2007;35:e124.
23. Schneider S, Lenz D, Holzer M, Palme K, Suss R. Intracellular FRET analysis of lipid/DNA complexes using flow cytometry and fluorescence imaging techniques. J Control Release. 2010;145:289–96.
24. Shaheen SM, Akita H, Yamashita A, *et al.* Quantitative analysis of condensation/decondensation status of pDNA in the nuclear subdomains by QD-FRET. Nucleic Acids Res. 2011;39:e48.
25. Shin S, Kwon HM, Yoon KS, Kim DE, Hah SS. FRET-based probing to gain direct information on siRNA sustainability in live cells: Asymmetric degradation of siRNA strands. Mol Biosyst. 2011;7:2110–3.
26. Hayashi Y, Noguchi Y, Harashima H. Non-linear pharmacokinetics of octaarginine-modified lipid nanoparticles: barriers from in vitro to in vivo. J Control Release. 2012;161:757–62.
27. Yang X, Koh CG, Liu S, *et al.* Transferrin receptor-targeted lipid nanoparticles for delivery of an antisense oligodeoxyribonucleotide against Bcl-2. Mol Pharm. 2009;6:221–30.
28. Hsu SH, Yu B, Wang X, *et al.* Cationic lipid nanoparticles for therapeutic delivery of siRNA and miRNA to murine liver tumor. Nanomedicine. 2013;9:1169–80.
29. Wu Y, Ho YP, Mao Y, *et al.* Uptake and intracellular fate of multifunctional nanoparticles: a comparison between lipoplexes and polyplexes via quantum dot mediated Forster resonance energy transfer. Mol Pharm. 2011;8:1662–8.
30. Wang B, Hsu SH, Wang X, Kutay H, Bid HK, Yu J, *et al.* Reciprocal regulation of miR-122 and c-Myc in hepatocellular cancer: Role of E2F1 and TFDP2. Hepatology. 2014;59:555–66.
31. Bai S, Nasser MW, Wang B, *et al.* MicroRNA-122 inhibits tumorigenic properties of hepatocellular carcinoma cells and sensitizes these cells to sorafenib. J Biol Chem. 2009;284:32015–27.
32. Hsu SH, Wang B, Kota J, *et al.* Essential metabolic, anti-inflammatory, and anti-tumorigenic functions of miR-122 in liver. J Clin Invest. 2012;122:2871–83.
33. Whitehead KA, Sahay G, Li GZ, *et al.* Synergistic silencing: combinations of lipid-like materials for efficacious siRNA delivery. Mol Ther. 2011;19:1688–94.
34. Dominska M, Dykxhoorn DM. Breaking down the barriers: siRNA delivery and endosome escape. J Cell Sci. 2010;123:1183–9.
35. Stanton MG, Colletti SL. Medicinal chemistry of siRNA delivery. J Med Chem. 2010;53:7887–901.
36. Garzon R, Marcucci G, Croce CM. Targeting microRNAs in cancer: rationale, strategies and challenges. Nat Rev Drug Discov. 2010;9:775–89.