Co-localization of fluorescent labeled lipid nanoparticles with specifically tagged subcellular compartments by single particle tracking at low nanoparticle to cell ratios

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ABSTRACT

We utilized quantitative high-resolution single particle tracking to study the internalization and endosomal sorting of lipid nanoparticles (LNPs) by HeLa cells \textit{in vitro} to gain a better understanding of how cells process LNPs that are used for siRNA delivery. We compared the trafficking of three formulations that have been demonstrated to deliver siRNA into cells. They were composed of either a tritratable anionic lipid, formulation of cholesterol hemisuccinate (CHEMS), or a tritratable cationic lipid formulation of 1,2-dilinoleoyx-3-dimethylamino propane (DLinDMA) or a non-tritratable cationic formulation lipid formulation of 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP). They also contained either a substantial percentage of 1,2-dioleoyl-sn-glycerol-3-phosphoethanolamine (DOPE) or cholesterol and 5 mole percent 1,2-dimyristoyl-sn-glycerol-[methoxy(polyethylene glycol)-2000 (PEG-DMG). We optically measured the endosomal pH experienced by individual LNPs, observed the internalization pathways used and tracked the particles as they co-localized with fluorescent protein tags on compartment-specific proteins, during endosomal sorting to the lysosome. The data revealed significant differences in the accumulation in subcellular compartments among the three formulations, which help to explain the observed effects LNP composition exerts on \textit{in vitro} delivery efficiency.

Introduction

Lipid nanoparticles (LNPs) a system pioneered by Pieter Cullis, developed through his long-standing collaborations with his colleagues, have emerged as one of the most effective delivery vehicles for siRNA therapeutics [1]. Continued efforts at screening novel lipids and optimization of existing architectures have produced highly efficient formulations that have progressed into the clinic. Well-established chemical structure-function relationships have been identified for many of the most promising LNPs used to date, primarily through empirical observation and large-scale screening [2,3]. However, unlike the specific and established chemical criteria for efficient LNP formulations that contain unsaturated lipid tails, which prefer the inverted hexagonal phase and amino headgroups with a pKa of ~6.8, the biological criteria have not progressed as rapidly. Avoidance of the lysosome has remained the most important biological criteria for the past three decades, but the underlying biological mechanism for what differentiates effective from ineffective LNP formulations has remained largely undefined. This is in part due to the continuously growing complexity of our understanding of cellular endocytosis [4].

High-resolution single particle tracking (SPT) has been used to study endocytosis of a wide variety of carriers that mediate cellular nucleic acid delivery, including polymers and LNPs [5–12]. These studies have begun to identify the cellular-vector interactions and subsequent cellular responses leading to delivery. It is clear that signal cascades dictate the entry and flux through endocytosis [10,13,14]. These signals, therefore, have the potential to render an effective vector ineffective by shunting it to a nonproductive pathway and vice-versa. Greater understanding of these signals will allow us to manipulate them to our advantage [15]. Added to the cellular-vector interactions is the understanding that the delivery vector is not static \textit{in vivo}. Vectors can be modified with endogenous ligands \textit{in vivo} that significantly alter the cellular interactions resulting in efficiency alterations [16]. These factors allow for the selection and design of delivery vectors that are more efficient at traversing the endosomal network, once greater understanding of biological structure-function is achieved. Therefore, continued investigation of the internalization pathways utilized by LNPs will aid in the improvement of siRNA delivery, by identifying subcellular compartments and cellular responses that are more amenable for delivery.

In this work, we have focused on three formulations containing three different chemicals that are reported to assist in nucleic acid delivery: cholesterol hemisuccinate (CHEMS), 1,2-dinoleoleoxy-3-dimethylamino propane (DLinDMA) and 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) (references needed). These lipids impart distinct biophysical characteristics to LNPs, namely, a carboxylate with a pKa of 5.5 that is negatively charged, a titratable tertiary amine with a pKa of 6.8, and a quaternary amine that is always cationic, respectively. As a consequence, the delivery mechanism is hypothesized to be different between the three formulations. Using quantitative high-resolution SPT, we have investigated how these three formulations alter endosomal pH and are internalized and sorted \textit{in vitro}. 

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Supplemental data for this article can be accessed here.
Results

LNP characterization

The physical characteristics of each formulation were determined by measuring the size and zeta potential (Table 1). The data show that the formulations are between 60 and 90 nm in diameter and have zeta potentials between −11 and +15 mV. The differences in size between the three formulations are significant, but it has been demonstrated that particles between 50 and 100 nm are internalized by cells by similar mechanisms [17]. Thus, the surface structure, including charge, lipid head group and opsonized proteins, rather than size are likely the dominant characteristics affecting observed differences between the formulations.

DOTAP LNPs efficiently deliver siRNA in vitro

The ability of each formulation to deliver siRNA was tested in vitro. An siRNA against luciferase and a scrambled control were formulated in LNP treatments comprised of CHEMS, DLinDMA, or DOTAP using a modified method of dilution [18]. The complexes were incubated with HeLa-Luc cells for 4 h in reduced serum media, washed, and incubated in full media for an additional 20 h before the level of luciferase activity was determined (Figure 1(A)). The DOTAP formulation displayed the highest knockdown in vitro with an IC50 of 1.30 nM ±0.33. When ApoE3 is added exogenously with DLinDMA LNPs, the formulation is able to deliver siRNA, as previously demonstrated [16]. However, toxicity limited using higher concentrations of LNPs. In the absence of ApoE, DLinDMA LNPs perform as well as CHEMS LNPs at siRNA delivery with an IC50 of 70 nM. Detailed microscopic analysis of LNP internalization during the 4 h transfection period was performed to investigate how the LNPs were differentially endocytosed and sorted.

Table 1. Physical characteristics of LNP formulations used in this study. Average size, PDI and zeta potential are from three replicate formulations.

| Formulation | Size (nm)/PDI | Zeta (mV) |
|-------------|--------------|-----------|
| CHEMS       | 67.1/0.21    | −10.7     |
| DOTAP       | 89.4/0.13    | +15.1     |
| DLinDMA     | 80.8/0.18    | +5.4      |

LNP formulations containing DOTAP and DLinDMA increase endosomal pH

A luciferase siRNA labeled with TEX-615 was formulated into LNP complexes with DOTAP. The fluorescent formulations were pulsed into HeLa cells for 30 mins in the presence of 12 mg/ml FITC-dextran to co-label endosomes. The cells were then washed with PBS containing 1% heparin sulfate and PBS supplemented with 6 mM glucose and 1.1 mM pyruvate (PBSgp) at 4°C, followed by imaging in PBSgp at 37°C for 4 h. The pH of endosomal compartments was determined by ratiometric fluorescence imaging of FITC-dextran, which had been previously calibrated (Figure S1(A) and validated Figure S1(B)). Population averages of a large number of endosomes containing and lacking LNP formulations are shown in Figure 1(B). The results indicate that DOTAP and DLinDMA LNPs cause a 0.62 ± 0.04 and 0.38 ± 0.03 (mean ± SEM) pH unit increase, respectively, in endosomal pH, where as CHEMS LNPs do not alter endosomal pH. In the presence of ApoE, the average pH of endosomes labeled with FITC-dextran increases by 0.35 ± 0.04 pH units and endosomes containing ApoE-opsonized DLinDMA LNPs increase by 0.46 ± 0.04 pH units.

To further analyze at which point during the 4 h imaging experiment endosomal pH is altered, the images were binned into 30 min intervals and population averages were determined for endosomes containing and lacking LNPs formulations (Figure 2(A–C)). The results indicate that both DOTAP and DLinDMA cause an increase in endosomal pH at all time points during a 4 h transfection. Finally, the correlation of the labeled siRNA contents and bilayer lipid with pH was determined on a per particle basis for DOTAP LNPs (Figure 2(D)). The results demonstrate that both the pH-insensitive siRNA and lipid fluorescence signals decrease with lower pH, suggesting that both signals are lost during endosomal acidification. The DLinDNA and CHEMS formulations tested did not display the same correlation with pH (data not shown).

LNP formulations utilize clathrin-, caveolin- and flotillin-mediated internalization to a similar extent

To determine if the LNPs preferentially utilized a specific internalization pathway, the labeled LNP formulations were incubated with HeLa cells expressing mTurquoise fusions to CltA, Rab5a, Cav1 and Flot1. The complexes were incubated with cells for 30 mins in PBSgp at 4°C, followed by extensive washing to remove unbound complexes and were imaged at 37°C for 1 h.

![Figure 1](image-url) LNP in vitro luciferase knockdown and intracellular pH measurements. (A) LNPs containing siRNA against luciferase were incubated with HeLa-Luc cells for 4 h and luciferase activity was determined after 24 h. The data represent mean ± SD (n = 3) and the curves were fit to log (inhibitor) versus normalized response with a variable slope in Prism. (B) LNPs were incubated with HeLa cells in the presence of FITC-Dextran and co-localized during endocytosis for 4 h. Population averages of a large number of endosomes containing and lacking LNP formulations are shown with 95%CI. CHEMS (n = 2617; ±n = 2110), DOTAP (n = 1321; ±n = 1083), DLinDMA (n = 3153; ±n = 2301), DLinDMA + ApoE (n = 4598; ±n = 2474).
The results demonstrate that all examined internalization pathways are utilized by the formulations tested (Figure 3(A–D)), with the exception that DOTAP demonstrates a significant increase in utilization of Flot1-mediated endocytosis (Figure 3(D)). There is also a slight preference of DLinDMA for clathrin-mediated endocytosis during the first ~15 mins of transfection (Figure 3(A)), which is coupled with the early endosomal marker, Rab5a (Figure 3(B)). In contrast, the CHEMS formulation does not display a preference for any of the internalization pathways tested.

**LNP formulations display differential endosomal sorting and co-localization with endosomal compartments**

The labeled LNP formulations were incubated with HeLa cells expressing mTurquoise fusions to Rab11a, CD82 and LAMP1 to determine how the LNPs are sorted during endocytosis. The LNPs were incubated with HeLa cells at 37°C for 30 mins followed by extensive washing to remove unbound complexes and then were imaged for 4 h. The results demonstrate that the DOTAP formulation co-localizes significantly with the recycling endosomal marker Rab11a and the multivesicular body marker CD82 (Figure 4(A,B)). In addition, the DOTAP formulation also displays a slower accumulation into LAMP1 positive lysosomes, relative to the other tested formulations (Figure 4(C)). In contrast, CHEMS colocalizes rapidly and to a greater extent with LAMP1 positive lysosomes than do the other formulations tested (Figure 4(C)). The DLinDMA formulation does not display significantly higher accumulation in the markers tested, suggesting that it co-localizes with other compartments that are not labeled (Figure 4(A–C)).

To capture the initial 30 mins of endocytosis and confirm the results of the live cell imaging experiments, the LNP formulations were incubated with cells at 4°C and fixed at 10, 20 and 30 mins after incubation at 37°C. The results largely confirm the observations in live cells that the CHEMS formulation co-localizes extensively with LAMP1 positive lysosomes (Figure 5(C)), and the DOTAP formulation co-localizes with CD82 positive multivesicular bodies (Figure 5(B)). However, the increased co-localization of the DOTAP formulation with Rab11a positive recycling endosomes is not observed (Figure 5(A)). The discrepancy between the live cell imaging and the fixed cell experiments could be a result of the fixation process or the different time points analyzed.

**Discussion**

The *in vitro* potency demonstrated by DOTAP (Figure 1(A)), was further investigated by analysis of cellular trafficking during endocytosis and compared to two other formulations that have markedly different chemistries and delivery efficiencies. The results demonstrate that both DOTAP and DLinDMA formulations, which are characterized by a net positive charge arising from quaternary ammonium and tertiary amines, respectively, increase the pH of endosome by ~0.5 pH units (Figure 1(B)). These data are consistent with the observation that polyethyleneimine (PEI) and polyamidoamine (PAM) increase endosomal pH [5]. In the presence of ApoE, DLinDMA exhibits an overall increased pH that is likely the result of increased recycling caused by the presence of ApoE, which has been demonstrated to increase cellular recycling [19]. The exact mechanism causing the increased endosomal pH is currently unknown, however, the mechanism could be similar to the one proposed for the proton sponge effect [20] whereby endosomal pH is increased by H+ buffering and Cl− accumulation.

In contrast to the greater buffering capability of polyamine polymers, LNPs containing mono-amines can not lead to an osmotic-induced rupture of the compartment. Consistent with this interpretation, endosomes containing DOTAP or DLinDMA LNPs exhibit increased overall pH during the 4 h incubation (Figure 2(A–C)), which is in contrast to the more rapid pH increase
demonstrated by PEI and PAM polyplexes [5]. These data demonstrate that LNPs containing tertiary and quaternary amines are able to increase endosomal pH and that LNPs that contain carboxylates have no effect on endosomal pH.

The DOTAP formulation uniquely demonstrates a significant pH dependent disassembly of the LNPs (Figure 2D). Both the lipid membrane, DiD, and siRNA fluorophore, TEX615, decrease in signal proportional to the measured pH. Neither fluorophore is pH

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Figure 3. LNP co-localization with early endosomal markers. HeLa cells expressing mTurquoise fusions to (A) CltA (CHEMS, n = 14439; DOTAP n = 12397; DLinDMA, n = 6431), (B) Rab5a (CHEMS, n = 12303; DOTAP n = 12805; DLinDMA, n = 6840), (C) Cav1 (CHEMS, n = 9644; DOTAP n = 11230; DLinDMA, n = 5947), and (D) FLOT1 (CHEMS, n = 18462; DOTAP n = 2989; DLinDMA, n = 7659) were incubated with LNPs at 4°C for 30 mins. Cells were washed to removed unbound LNPs followed by image acquisition for 1 h at 37°C in PBSgp. Lines represent 5 min running averages of liposome co-localization with each marker and data points at 5 min intervals showing average and SEM.

Figure 4. LNP co-localization with late endosomal markers. HeLa cells expressing mTurquoise fusions to (A) Rab11a (CHEMS, n = 24670; DOTAP n = 12662; DLinDMA, n = 92395), (B) CD82 (CHEMS, n = 39223; DOTAP n = 64332; DLinDMA, n = 69238), and (C) LAMP1 (CHEMS, n = 18209; DOTAP n = 28250; DLinDMA, n = 130719) were incubated with LNPs at 37°C for 30 mins. Cells were washed to removed unbound LNPs followed by image acquisition for 4 h at 37°C in PBSgp. Lines represent 10 min running averages of liposome co-localization with each marker and data points at 10 min intervals showing average and SEM.
dependent, which suggests that the particles are dissociating as pH decreases. Importantly, this is the only formulation that displayed this type of behavior, suggesting that DOTAP measurably disassembles during endocytosis within the observed 4 h time frame. These data suggest that the DOTAP formulation is more susceptible to destabilization in endosomes compared to the other the other two formulations tested. This may, in part, account for the better performance of DOTAP in the luciferase knockdown assay. These data are consistent with the observed gradual disassembly of Lipofectamine 2000 lipoplexes in vitro [12]. However, it must be noted that particle disassembly is not a direct measure of siRNA delivery, but is rather an a priori step in the delivery mechanism.

Analysis of LNP co-localization with the early internalization markers revealed that all of the tested formulations utilized clathrin- and caveolin-dependent endocytosis to a similar extent. These data agree with published results demonstrating the importance of clathrin-mediated endocytosis in DOTAP and DLin-MC3-DMA cellular internalization [10,21]. The DOTAP formulation, however, also exhibited a unique utilization of flotillin-1 dependent endocytosis (Figure 3(D)). It documented in several cell types that recycling endosomes are enriched in flotillin-1 and that flotillin-1 delivers GPI-anchored proteins to recycling endosomes [22,23]. This observation provides a link between the increased co-localization of the DOTAP formulation with flotillin-1 and Rab11a.

The observed increased co-localization of the DOTAP formulation with the recycling endosomal marker Rab11a, suggests that the recycling endosome confers an advantage to LNPs during transfection (Figure 4(A)). These data agree with the results of Sahay et al. that demonstrated an ~2-fold decrease in cellular LNP uptake in cells treated with Rab11a siRNA concomitant with a 2-fold increase in the IC50 [11]. These results indicate that not all endocytic recycling compartments are detrimental to efficient siRNA delivery, and at least the Rab11a compartment can be acting as a beneficial intracellular reservoir for LNPs. Neither of the poor performing LNPs formulated with CHEMS or DLinDMA, display increased co-localization with Rab11a, consistent with the proposed beneficial role of that recycling compartment.

DOTAP LNPs also extensively co-localize with the multivesicular body marker, CD82, early during the transfection process (Figure 4(B)). RISC recycling has been demonstrated to occur on the cytoplasmic face of multivesicular bodies [24–26]. The increased co-localization of the DOTAP LNP with multivesicular bodies raises the potential for localized siRNA delivery at this site, which is primed for loading guide strands into RISC. For this to be occur, both increased localization and delivery of the siRNA across the endosomal membrane into the cytoplasm is required. We have only demonstrated that the first step in the delivery process is not limiting. However, if siRNA delivery is occurring at this site, and, if it is a general causative feature for efficient formulations remains to be tested.

The time course for co-localization of the LNP formulations with the lysosomal marker LAMP1 demonstrates that DOTAP formulation accumulates into this compartment slower than the other formulations tested (Figure 5(C)). These data suggest that DOTAP either slows down normal endocytic traffic, or traverses through endosomal maturation slowly or disassembles in an earlier compartment. At this time, we are unable to distinguish among these possibilities In contrast CHEMS is found to co-localize with LAMP1 positive lysosomes within 30 mins in the live cell experiments and within 10 mins in the fixed cells experiments. These data suggest that the main cause for the inability of CHEMS to efficiently deliver siRNA is due to the high proportion and rapid entry of CHEMS LNPs into the lysosomes.

**Conclusion**

With the continued but marginal gains in transfection efficiency derived from screening new lipids and polymers, it has become...
increasingly important to understand how cells internalize and process nanoparticles. The complexity of cellular endocytosis highlights the necessity to fully characterize how nanoparticles traverse the endosomal network during delivery. Given the diversity of nucleic acid drugs that are now available i.e. miRNA, siRNA, antisense oligonucleotides, mRNA and DNA it effective delivery requires that systems be devised that target not only the cell types, but also subcellular compartments if continued gains in delivery of therapeutic nucleic acids are to be realized. In this work, we have confirmed that cells endocytose LNPs formulations differentially. The observations of DOTAP LNPs being sequestered into multivesicular bodies and recycling endosomes to a greater extent than the other formulations tested suggest that these compartments are in part responsible for the higher efficiency of the DOTAP formulation.

Future research to specifically perturb the complex endocytosis pathways will aid in the development and selection of more effective nanoparticles. We expect the Cullis consortium of collaborations will continue to lead in identifying chemical and biophysical pathways to improved nucleic acid delivery. This will help usher in the personalized medicine revolution that has found a voice from many in the biomedical field including Pieter Cullis [27].

Methods
Materials
All cell culture reagents were supplied by the UCSF Cell Culture Facility. 1,2-Dioleoyl-3-trimethylammonium-propane (DOTAP), 1,2-dioleoyl-sn-glycerol-3-phosphoethanolamine (DOPE), 1,2-dipalmityloctadecyl-sn-glycerol-3-phosphocholine (DPPC), 1,2-distearoyl-sn-glycerol-3-phosphoethanolamine-N-[amino(polyethylene glycol)-2000] (PEG-DSPE), cholesterol (Chol) and cholesteryl hemisuccinate (CHEMS) were obtained from Avanti Polar Lipids (Alabaster, AL). 1,2-dimyristoyl-sn-glycerol-[methoxy(polyethylene glycol)-2000] (PEG-DMG) was obtained from NOF-Corporation (Tokyo, Japan). 1,2-dilinoleoyloxy-3-dimethylaminopropane (DLinDMA) was provided by Pfizer (Cambridge, MA). Luciferase sense and antisense TEX615 were obtained from Promega (Madison, WI). 1,1-dioctadecyl-3,3,3,3-tetramethylindodicarbocyanine perchlorate (DiD), Fluorescein isothiocyanate (FITC), amino dextran (40 kDa), and Lysosensor Blue DND-167 were obtained from Molecular Probes (Eugene, OR). The Steady-Glo luciferase assay kit was purchased from Promega (Madison, WI).

Cell culture
HeLa and all HeLa derivatives containing endosomal marker fusions were maintained in T75 flasks between 10 and 90% confluency (100% confluent T75 yields ~10 x 10^6 total cells) in RPMI 1640 supplemented with 10% heat inactivated FBS (Gibco, Life Technologies, Benicia, CA) and 25 mM HEPES in a humidified atmosphere with 5% CO2 at 37°C. HeLa cell derivatives expressing the endosomal markers were constructed by lentiviral transduction of HeLa cells with the respective markers. HeLa cells were first transduced with the rTA construct from pLenti-UbC-rTA and selected using 400 µg/mL G418. Pooled cells were then transduced with constructs expressing the marker fusions and selected with 2 µg/mL puromycin. Pooled cells were used for all further microscopy studies.

For microscopy studies, HeLa and HeLa derivative cells were cultured in RPMI 1640 supplemented with 10% defined FBS (HyClone, Thermo Fisher, Waltham, MA) and 25 mM HEPES. Forty eight hours prior to microscopy, cells were seeded at 300,000 cells per 35 mm uncoated glass coverslip bottom FluoroDish (World Precision Instruments, Sarasota, FL). The media was changed 24 h later and if induction was used, 10 µg/mL doxycycline was added at that time to induce fluorescent marker expression. On the day of imaging, the plate was 90% confluent at ~1,200,000 cells per dish. During imaging experiments, the cells were maintained in PBS supplemented with 6 mM glucose and 1.1 mM pyruvate (PBSgp).

Plasmids
mturquoise was amplified from pRSSET-mTurquoise by PCR and replaced GFP between the BamHI and NotI sites in pEGFP-N1 or the Agel and BspEI sites in pECEG-P1 to create pmTurquoise-N1 and pmTurquoise-C1, respectively. pClta-mTurquoise, pCav1-mTurquoise, and pLAMP1-mTurquoise were constructed by PCR amplifying the sequences from a cDNA library made from HeLa cells using gene specific primers. For Cav1 and LAMP1, the PCR products were cloned between the Xhol and BamHI sites in pmTurquoise-N1. For CltA, the PCR product was cloned between the Xhol and Agel sites in pmTurquoise-N1, pmTurquoise-CDB2, pmTurquoise-FLOT1, pmTurquoise-Rab5a, pmTurquoise-Rab7a, and pmTurquoise-Rab11a were constructed by PCR amplifying the sequences from a cDNA library made from HeLa cells using gene specific primers. For Rab5a, Rab7a and Rab11a the PCR products were cloned between the Xhol and BamHI sites in pmTurquoise-C1. For FLOT1, the PCR product was cloned between the Xhol and EcoRI sites in pmTurquoise-C1. For CD82, the PCR product was cloned between the Xhol and Xbal sites in pmTurquoise-C1.

To create the lentiviral constructs under doxycycline induction, pLVX-Tight-Puro (Clontech, Mountain View, CA) was first modified to remove the XhoI site 5’ to the Pigtight promoter by QuikChange mutagenesis (Agilent, Santa Clara, CA). Then a new multiple cloning site was added between the BamHI and MluI sites to introduce the marker fusions and selected with 2 µg/mL puromycin. Pooled cells were used for all further microscopy studies.

LNP preparation
The formulations tested were as follows: DOTAP:DOPE:PEG-DMG:DiD (56:38:5:1 mole percent), CHEMS:DOPE:PEG-DMG:DiD (38:56:5:1 mole percent) and DLinDMA:DPPC:Cholesterol:PEG-
DMG:DID (40:14:40:5:1 mole percent). In all, 1 µM of total lipid was dried down by rotary evaporation under reduced pressure in a glass test tube for 4 h then dried overnight under high vacuum. The lipids were dissolved in 200 µL of methanol and rapidly injected into 300 µL of 50 mM citrate pH 4 aqueous buffer containing 35 µg of siRNA-TEX615 stirring at 300 rpm at 60 °C. The mixture was extruded (11x) through 100 nm pores in a polycarbonate membrane (Nucleopore, Whatman, Clifton, NJ), followed by rotary evaporation under reduced pressure to remove the methanol. Liposome preparations were then dialyzed overnight ~4000-fold against 5 mM HEPES, pH 7, 145 mM NaCl, 3.4 mM EDTA with 100 kDa MWCO membrane (Spectrum, New Brunswick, NJ). For studies on RNA knockdown, the formulations were prepared identically except that DiO replaced DiD. The particle size of the DiO-labeled formulations was measured using a Malvern Zetasizer NanoZS (Westborough, MA).

For microscopy studies, liposomes were further purified by equilibrium density centrifugation in a linear, 0–18% sucrose gradient. The gradients were prepared by layering 2 mL of 15% sucrose dissolved in PBS under 2 mL of PBS in Beckman Polylamellar ½ × 2 in. tubes, type 326819 (Beckman Coulter, Indianapolis, IN). The gradients were then spun for 30 revolutions at 45 °C in a cell culture rotor. After mixing the two layers, the tubes were frozen at −80 °C, and thawed at 4 °C before use. Liposome preparations were added and spun at 100 000 × g for 16 h at 4 °C in a Beckman SW-55Ti rotor (Beckman Coulter, Indianapolis, IN). A needle was used to poke a hole in the bottom of the centrifuge tube and fractions were collected and analyzed for DiD and TEX615 fluorescence. The fractions with the highest levels of fluorescence for both markers were used for microscopy studies. The liposome preparation was stored at −80 °C in 15% sucrose.

Luciferase assay

Twenty four hours prior to the start of the experiment, 8000 HeLa-Luc cells were seeded into each well of a 96-well plate such that the cells were ~80% confluent during the transfection. Lipoplex containing either an anti-luciferase siRNA or a nonspecific control were added to the cells and incubation proceeded for 4 h at which time the media was exchanged and incubation proceeded for 20 h until luciferase activity was measured. Luciferase expression was measured using the Steady-Glo assay kit (Promega, Madison, WI). Luminescence was measured using an Infinite m1000 Pro microplate reader (Tecan, San Jose, CA).

pH calibration of FITC-dextran

An 8-well chamber slide (Lab-Tek, Thermo Fisher Scientific Inc., Waltham, MA) was seeded with 10 000 cells per chamber. 24 h later 12.5 mg/mL FITC-dextran in PBS was added to the cells and incubated for 30 mins at 37 °C. The cells were subsequently washed extensively with PBS to remove unbound fluorescence. The intracellular pH in all cellular compartments was clamped by incubation in high K⁺ solutions containing the ionophores bafilomycin A1 (100 nM), nigericin (10 µM) valinomycin (10 µM) and carboxyl cyanide m-chlorophenyl hydrazone (CCCP, 10 µM). The pH buffers all contained 120 mM KCl, 20 mM NaCl, 1 mM CaCl₂, 1 mM MgCl₂, pH 4 and 5 buffers contained 50 mM acetic acid. pH 5.5, 6 and 6.5 buffers contained 50 mM MES. pH 7 and 8 buffers contained 50 mM HEPES. pH 9 buffer contained 20 mM Tris. The cells were incubated for 5 mins before imaging to equilibrate the intracellular pH. Fluorescence ratios at the clamped pH values were fitted to the equation:

\[
\frac{F_{491\text{nm}}}{F_{430\text{nm}}} = B + \frac{T - B}{1 + 10^{p\text{Ka} - p\text{H}/n_H}}
\]

Where, F is the fluorescence signal in the 491 nm and 430 nm channels, B and T are the amplitude and offset factors, respectively, pKa is the acid dissociation constant and n_H is the Hill constant.

Live-cell microscopy

Images were acquired on a Nikon Eclipse Ti-E inverted microscope equipped with an In Vivo Scientific temperature controlled chamber with humidification and CO₂ control, infrared autofocus, 100 × 1.4 numerical aperture Plan Apo objective, Photometrics Cooolsnap HQ2 CCD camera, and Sutter Lambda XL lamp. Filters used were 89000 Sedat Quad, 89021 GFP/mCherry and 89002 Dual CFP/YFP (Chroma Technology, Bellows Falls, VT). Image acquisition was performed using NIS Elements software and image analysis was performed using NIH ImageJ (NIH, Bethesda, MD). For ratiometric pH determination, images were taken using the ET490/20, ET525/36m and ET430/24x, ET535/30m filters of the Sedat Quad and Dual CFP/YFP sets, respectively, with 100 ms exposure. CFP fusions were imaged using the ET430/24x, ET470/24m filters of the Dual CFP/YFP set with a 100 ms exposure. DiD was imaged using the ET645/30x and ET705/72m filters of the Sedat Quad set with 500 ms exposure and 2 × 2 binning. TEX615 labeled siRNA was imaged using the ET572/35x and ET632/60m filters of the GFP/mCherry set with 500 ms exposure and 2 × 2 binning.

Image analysis was performed using freely available Fiji plugins and some were written de novo. Images were first deconvolved using the Parallel Spectral Deconvolution plugin (Piotr Wendykier) using a point spread function generated by the Diffraction PSF 3D plugin (Bob Dougherty). Deconvolved images were then background subtracted and 3D Gaussians were fit to the image using a modified GaussianFit plugin (Nico Stuurman). The X and Y coordinates of each fitted Gaussian spot in each frame were then co-localized with spots in other channels. Co-localization was defined as the center point of one Gaussian spot fitting within the width of a Gaussian spot from another channel.

Immunofluorescence microscopy

50 000 HeLa cells expressing mTurquoise fusion proteins to endosomal compartment markers were seeded into 24-well flat bottom plates with acid washed 12 mm #1 cover slips (Chenglass Life Sciences, Vineland, NJ). The cells were allowed to adhere for 24 h in media with defined FBS. The media was replaced with fresh media containing 10 µg/mL doxycycline and allowed to incubate overnight to induce expression of mTurquoise fusion proteins. The cells were pre-incubated with liposomes for 45 mins at 4 °C at concentrations between 1 and 5 µM. The cells were then washed with cold PBS containing 1% heparin and PBSgp to remove unbound liposomes. PBSgp at 37 °C was added to the cells and incubated for various times. The cells were then fixed with 4% paraformaldehyde for 30 mins on ice, followed by a quench with 0.1 M glycine pH 7.4 for 30 mins. The fixed cells were then rinsed with PBS and stored in PBS until further used. Fixed cells were blocked with PBS containing 3% BSA for 30 mins at 25 °C. The cells were then incubated with Alexa Fluor 488 conjugated AffiniPure goat anti-rabbit IgG (111–545-003, Jackson Immuno Research Laboratories, West Grove, PA) for 30 mins.
PA in blocking buffer. For mTurquoise-Rab11a protein, the incubation with primary and secondary antibodies were done at 25°C for 60 mins, while for CD82-mTurquoise and LAMP1-mTurquoise the incubations were at 4°C for 18h. The cells were then rinsed with PBS and incubated with 0.2 μg/ml Hoescht 33342 (Molecular Probes, Eugene, OR) in PBS for 30 mins at 25°C. The cells were then mounted using ProLong Gold Antifade Reagent (Molecular Probes, Eugene, OR) that cured for 24h before imaging.

Images were acquired on a Nikon Eclipse Ti-E inverted microscope equipped with a 100×1.4 numerical aperture Plan Apo objective, Photometrics Coolsnap HQ2 CCD camera and Sutter Lambda XL lamp. Filters used were 89000 Sedat Quad, and 89021 GFP/mCherry (Chroma Technology, Bellows Falls, VT). Image acquisition was performed using NIS Elements software and image analysis was performed using NIH ImageJ. Images were taken using the ET402/25× and ET455/50m (Hoescht 33342), ET490/20× and ET525/36m (Alexa Fluor 488), and ET645/30× and ET705/72m (DiD) filters of the Sedat Quad, and ET572/35× and ET632/60m (TEX615) filters of the GFP/mCherry, all with 800 ms exposure. DiD and TEX615 were additionally exposed using 2 × 2 binning. Image stacks were acquired spanning 3 μm at 0.23 μm spacing to achieve Rayleigh resolution.

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Disclosure statement

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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References

1. Tam P, Monck M, Lee D, et al. Stabilized plasmid-lipid particles for systemic gene therapy. Gene Ther 2000;7:1867–74.
2. Semple SC, Akinc A, Chen J, et al. Rational design of cationic lipids for siRNA delivery. Nat Biotechnol 2010;28:172–6.
3. Jayaraman M, Ansell SM, Mui BL, et al. Maximizing the potency of siRNA lipid nanoparticles for hepatic gene silencing in vivo. Angew Chem Int Ed Engl 2012;51:8529–33.
4. Huotari J, Helenius A. Endosome maturation. EMBO J 2011;30:3481–500.
5. Sonawane ND, Szoka FC, Jr., Verkman AS. Chloride accumulation and swelling in endosomes enhances DNA transfer by polyamine-DNA polyplexes. J Biol Chem 2003;278:44826–31.
6. Vercauteren D, Deschout H, Remaut K, et al. Dynamic co-localization microscopy to characterize intracellular trafficking of nanomedicines. ACS Nano 2011;5:7874–84.
7. Suh J, An Y, Tang BC, et al. Real-time gene delivery vector tracking in the endo-lysosomal pathway of live cells. Microsc Res Tech 2012;75:691–7.
8. Akita H, Enoto K, Tanaka H, Harashima H. Particle tracking analysis for the intracellular trafficking of nanoparticles modified with African swine fever virus protein P54-derived peptide. Mol Ther 2013;21:309–17.
9. Lin PJC, Tam YYC, 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. Gilleron J, Querbes W, Zeigerer A, et al. Image-based analysis of lipid nanoparticle-mediated siRNA delivery, intracellular trafficking and endosomal escape. Nat Biotechnol 2013;31:638–46.
11. Sahay G, Querbes W, Alabi C, et al. Efficiency of siRNA delivery by lipid nanoparticles is limited by endocytic recycling. Nat Biotechnol 2013;31:653–8.
12. Rehman ZU, Hoekstra D, Zuhorn IS. Mechanism of polyplex- and lipoplex-mediated delivery of nucleic acids: real-time visualization of transient membrane destabilization without endosomal lysis. ACS Nano 2013;7:3767–77.
13. Rehman Z, Hoekstra D, Zuhorn IS. Protein kinase A inhibition modulates the intracellular routing of gene delivery vehicles in HeLa cells, leading to productive transfection. J Control Release 2011;156:76–84.
14. Lonez C, Vandenbransen M, Ruysschaert JM. Cationic lipids activate intracellular signaling pathways. Adv Drug Deliv Rev 2012;64:1749–58.
15. Tam YYC, Chen S, Zaifman J, et al. Small molecule ligands for enhanced intracellular delivery of lipid nanoparticle formulations of siRNA. Nanomedicine 2013;9:665–74.
16. Akinc A, Querbes W, De S, et al. Targeted delivery of RNAi therapeutics with endogenous and exogenous ligand-based mechanisms. Mol Ther 2010;18:1357–64.
17. Rejman J, Oberle V, Zuhorn IS, Hoekstra D. Size-dependent internalization of particles via the pathways of clathrin- and caveolae-mediated endocytosis. Biochem J 2004;377:159–69.
18. Nguyen J, Walsh CL, Motion JPM, et al. Controlled nucleation of lipid nanoparticles. Pharm Res 2012;29:2236–48.
19. Heeren J, Grewal T, Laatsch A, et al. Recycling of apoprotein E is associated with cholesterol efflux and high density lipoprotein internalization. J Biol Chem 2003;278:14370–8.
20. Behr JP. The proton sponge: a trick to enter cells the viruses did not exploit. CHIMIA Int J Chem 1997;51:34–6.
21. Rejman JJ, Bragonzi AA, Conese MM. Role of clathrin- and caveolae-mediated endocytosis in gene transfer mediated by lipo- and polyplexes. Mol Ther 2005;12:7.
22. Gagescu R, Demaurex N, Parton RG, et al. The recycling endosome of Madin-Darby Canine kidney cells is a mildly acidic compartment rich in raft components. Mol Biol Cell 2000;11:2775–2791.
23. Sabharanjak S, Sharma P, Parton RG, Mayor S. GPI-anchored proteins are delivered to recycling endosomes via a distinct Cdc42-regulated, clathrin-independent pinocytic pathway. Dev Cell 2002;2:411–423.
24. Gibbings DJ, Claudio C, Erhardt M, Voinnet O. Multivesicular bodies associate with components of miRNA effector complexes and modulate miRNA activity. Nat Cell Biol 2009;11:1143–1149.
25. Gibbings D, Voinnet O. Control of RNA silencing and localization by endolysosomes. Trends Cell Biol 2010;20:491–501.
26. Lee YS, Pressman S, Andress AP, et al. Silencing by small RNAs is linked to endosomal trafficking. Nat Cell Biol 2009;11:1150–1156.
27. Cullis P. The personalized medicine revolution. Vancouver/ Berkeley: Greystone Books; 2015:1–161.