Uptake and trafficking of liposomes to the endoplasmic reticulum

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ABSTRACT Liposomes are vesicular structures consisting of an aqueous core surrounded by a lipid bilayer. Apart from the cytosol and lysosomes, no other intracellular compartment has been successfully targeted using liposomal delivery. Here, we report the development of liposomes capable of specific targeting to the endoplasmic reticulum (ER) and associated membranes. Using competition and inhibitor assays along with confocal microscopy, we have determined that ER liposomes utilize scavenger and low-density lipoprotein receptors for endocytosis and enter cells through a caveolin- and microtubule-dependent mechanism. They traffic intact to the ER, where fusion with the ER membrane occurs after 22–25 min, which was confirmed by fluorescence-dequenching assays. Once inside the ER, tagged lipids intercalate with the ER membrane and are subsequently incorporated into ER-assembling entities, such as the ER-budding viruses hepatitis C virus (HCV) and bovine viral diarrhea virus (BVDV), lipid droplets, and secreted lipoproteins. ER liposomes are superior to cytosolic liposome formulations for the intracellular delivery of aqueous cargo, such as HIV-1 antivirals, and are especially suited for the prolonged delivery of lipids and lipophilic drugs into human cells.—Pollock, S., Antrobus, R., Newton, L., Kampa, B., Rossa, J., Latham, S., Branza Nichita, N., Dwek, R. A., Zitzmann, N. Uptake and trafficking of liposomes to the endoplasmic reticulum. FASEB J. 24, 1866–1878 (2010). www.fasebj.org

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Liposomes have been used as carriers for both soluble and insoluble material for over two decades, and more recently, they have entered the clinic for the treatment of several cancers and fungal infections (1). The liposome formulations used are biologically inert, biocompatible, and cause little or no adverse toxic or antigenic reactions, making liposomal drug delivery attractive for the treatment of other human diseases and infections.

One common strategy for the intracellular delivery of encapsulated and/or intercalated material via liposomes exploits intracellular pH gradients (2). Following endocytosis via clathrin-mediated processes, these pH-sensitive liposomes are subjected to acidification along the endosomal pathway from early endosomes to lysosomes, which leads to destabilization of the liposome bilayer and release of encapsulated material. Because of the mechanism necessary for mediating lipid destabilization, and therefore release of encapsulated material, delivery by pH-sensitive liposomes is restricted to the lumen of endosomal vesicles and the cytosol. In addition, lipophilic material intercalated between lipids constituting the liposome bilayer is targeted to lysosomes and degraded (3).

Until now, no other intracellular compartments have been successfully targeted using liposome-mediated delivery systems. An important intracellular target is the endoplasmic reticulum (ER), which is responsible for the export of membrane-bound and soluble molecules and is involved in signaling pathways regulating cell growth and differentiation. The ability to deliver compounds (drugs, peptides, or markers) specifically to this organelle will have an effect on many areas of intense scientific interest. One key application presented here is the delivery of iminosugars known to inhibit the ER resident enzymes α-glucosidase I and II, which play an important role in glycoprotein folding. These iminosugars are antiviral against a range of human pathogens, including human immunodeficiency virus (HIV), hepatitis C virus (HCV), and hepatitis B virus (HBV), among others (4–7). Free delivery of iminosugars is currently not possible in vivo, as therapeutically relevant antiviral concentrations are excessive (8, 9). Liposome delivery via pH-sensitive liposomes has already proven to lower the effective dose by several orders of magnitude (10), and direct ER delivery should further reduce the drug concentrations necessary for treatment.

In recent years, several alternative pathways in addition to the classical clathrin-mediated uptake route have been explored, and it has become apparent that different cellular routes offer opportunities for selective delivery. There are multiple pathways for internal-
ization of vesicles with a diameter of 50–300 nm, including clathrin-mediated endocytosis, caveolae-mediated endocytosis, phagocytosis, macropinocytosis, and nonclathrin–noncaveolae-dependent endocytosis. For targeted cargo delivery to intracellular localizations other than the cytosol and endosomal vesicles, exploiting caveolae-mediated uptake is thought to be most promising, since it does not automatically transport internalized material to endosomes and lysosomes. In contrast to this theory, others have shown that the final destination of cargo endocytosed by either clathrin or caveola-dependent mechanisms is not only decided by route of uptake, but rather by the cargo itself following further sorting within early or sorting endosomes (11, 12).

We have developed liposomes that localize to the ER and ER-associated membranes on internalization into coincubated cells. ER liposomes are the first class of liposomes that target specific cellular subdomains other than lysosomes for improved intracellular delivery of both hydrophilic and hydrophobic material, without causing cytotoxicity when used at concentrations up to 500 μM.

**MATERIALS AND METHODS**

**Lipids and liposomes**

1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (PE), 1,2-dioleoyl-sn-glycero-3-phosphocholine (PC), 1,2-distearoyl-sn-glycero-3-phosphoinositol (PI, from bovine liver), 1,2-distearoyl-sn-glycero-3-phosphoarginine (PS, from porcine brain), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (rh-PE), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (ammonium salt) (PEG-PE), and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-cap biotinyl (sodium salt) (b-PE) were all purchased from Avanti Polar Lips (Alabaster, AL, USA). Cholesteryl hemisuccinate (CH) was purchased from Sigma-Aldrich (Gillingham, UK). Liposomes were prepared fresh before each experiment, as described previously (10). The distribution of liposome diameter within the final preparation was measured by dynamic light scattering.

**Cell culture**

Huh7.5 cells (Apath, LLC, St. Louis, MO, USA) were grown in complete DMEM (100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, and 1 × MEM) with or without 10% FBS. Peripheral blood mononuclear cells (PBMCs) from 4 uninfected donors were isolated using Histopaque density gradient centrifugation (Sigma-Aldrich), pooled, and stimulated with phytohemagglutinin (PHA; 5 μg/ml) for 48 h, followed by interleukin-2 (IL-2, 40 U/ml) for 72 h in complete RPMI (RPMI plus 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine) before starting experiments. All incubations were at 37°C/5% CO₂, unless stated otherwise. Cell toxicity of various treatments was determined by an MTS-based cell proliferation assay (CellTiter 96; Promega, San Luis Obispo, CA, USA). Blood monocyte-derived macrophages were isolated and grown as described previously (13).

**JC-1 HCV cell culture**

Huh7.5 cells were grown in complete DMEM/10% FBS. Cells were infected for 1 h at a multiplicity of infection (MOI) of 0.5 using a viral stock of known titer, and liposome treatments were started when >50% of cells tested positive for HCV cell culture infection, as determined by HCV core protein immunofluorescence. The quantification of viral RNA from supernatant was determined using quantitative PCR.

**Bovine viral diarrhea virus (BVDV) cell culture**

Madin-Darby bovine kidney (MDBK) cells were grown in complete RPMI/10% FBS, and infected with BVDV strain Pe515 (National Animal Disease Laboratory, Woking, UK) at MOI = 0.1. Liposome treatments were begun after a stable infection was achieved, as determined by RT-RCR to quantify secreted BVDV particles (14).

**HIV-1 primary isolate cell culture**

PBMCs were infected with 100 TCID₅₀ (tissue culture infectious dose 50%) of HIV-1 primary isolate stock, and HIV secretion and infectivity assays were performed as described previously (10). The HIV-1 primary isolate LAI was obtained from the U.K. National Institute for Biological Standards and Control (Ridge, U.K.).

**Fixed-cell confocal imaging**

Huh7.5 cells were coincubated with liposomes added to a final lipid concentration of 50 μM. After a 5-min incubation at 4°C, medium-containing liposomes were removed; cells were washed and incubated in fresh medium for the stated time period before being fixed and used in immunofluorescence assays. Goat primary antibodies against ER degradation-enhancing α-mannosidase-like protein (EDEM) and early endosomal antigen-1 (EEA1) were purchased from Insight Biotechnology (Wembley, UK). Mouse primary antibody against golgin97 was purchased from Invitrogen (Paisley, UK). Mouse primary antibody against CD-81 was purchased from Ancell Corp. (Bayport, MN, USA). Donkey anti-goat and goat anti-mouse secondary antibodies labeled with AlexaFluor488 were purchased from Invitrogen. For visualization of cellular lipid droplets, cells were incubated with BODIPY493/503 (Invitrogen). Cells were stained with Vectashield DAPI (Vector Laboratories, Burlingame, CA, USA) prior to mounting. Confocal images were taken using a Carl Zeiss laser scanning microscope (LSM) microscope, and image analysis was done using the LSM 5.10 software (Carl Zeiss, Oberkochen, Germany).

Percentage colocalization was measured using MetaMorph 7 software (Molecular Devices, Downingtown, PA, USA). Images were filtered using a median filter set to 3 × 3 pixels, and thresholds used to determine integrated colocalization between two images (rh-PE/red images and EDEM/green images) were set at the mean + 1 SD intensity for each. Reported values represent means ± SD of 30 cells.

**Live-cell confocal imaging**

All incubations were done in complete DMEM/10% FBS without phenol red. To label the ER membrane, cells were preincubated with blue-white ER tracker (Invitrogen). For imaging colocalization of liposomes with cholera toxin subunit B or transferrin (both from Invitrogen, AlexaFluor488-labeled), the manufacturer’s protocols were followed. Cells were incubated while still on ice with rh-PE-labeled liposomes, final lipid concentration of 50 μM, for 5 min with or without 100 μM calcein. Cells were then incubated in prewarmed DMEM/10% FBS prior to imaging. All images were
taken using a Carl Zeiss laser LSM inverted microscope located inside a chamber set at 37°C/5% CO₂, and image analysis was done using LSM 5.10 software.

**Purification and quantification of secreted biotin-labeled particles**

Virus-infected cells were coincubated with 50 μM b-PE-labeled liposomes and left for 48 h. Cells were then washed and incubated without liposomes for a further 24 h. High-performance streptavidin Sepharose (GE Healthcare, New York, NY, USA) resin was used to capture biotinylated particles. To quantify the amount of b-PE-labeled virions, culture supernatant was split in two, one half was used for total virus quantification, and the other half captured on streptavidin-sepharose, washed, and used directly for RNA quantification by incubating beads with viral RNA lysis buffer (Qiagen, Valencia, CA, USA) for HCVcc and BVDR RT-PCR analysis, or by incubating in 1% emgiphen for p24 HIV ELISA assays. All b-PE labeled material in the supernatant of treated cells was captured separately on streptavidin resin and analyzed by SDS-PAGE and mass spectrometry (Supplemental Data).

**Liposome uptake and lipid retention assays**

Rh-PE-labeled liposomes (1% mol) were added to Huh7.5 cells at a final phospholipid concentration of 50 μM in serum-free complete DMEM without phenol red, and left to incubate for 1 h. For lipoprotein competition assays, low-density lipoprotein (LDL; Invitrogen), acLDL (Invitrogen), high-density lipoprotein (HDL), and fucoidin (both a kind gift from Dr. Claudine Neyen, University of Oxford, Oxford, UK) were coincubated with liposomes and cells. For assays in the presence of LDL receptor (LDLr) expression regulators, each inhibitor was incubated with Huh7.5 cells to determine the maximum concentration leading to <5% cell death, which was then used in addition to the half-maximum concentration to incubate cells for 1 h prior to the addition of rh-PE-labeled liposomes. For assays in the presence of endocytosis and trafficking inhibitors, each inhibitor was incubated with Huh7.5 cells to determine the maximum concentration leading to <5% cell death, which was then used in addition to the half-maximum concentration to incubate cells for 1 h prior to the addition of rh-PE-labeled liposomes. Following incubation with liposomes, cells were washed, counted using trypan blue staining, and read in a spectrofluorometer at λ_em = 550 nm, λ_ex = 520 nm.

For assays to determine liposome uptake in the presence of different human serum (with Huh7.5 cells) or autologous plasma (with macrophages) concentrations, 1% rh-PE-labeled liposomes were incubated with cells at a final lipid concentration of 50 μM for 4 d before cells were washed in PBS and lysed in PBS/1% Triton X-100. Lysed samples were read in a spectrofluorometer at λ_em = 550 nm, λ_ex = 520 nm, and results were normalized to total cell content determined by trypan blue staining.

For long-term (4 d) liposome uptake assays, 1% rh-PE-labeled liposomes were added to cells to a final phospholipid concentration of 50 μM and left to incubate for 2, 24, 48, 72, and 96 h. Following incubation times, cells were harvested and analyzed as described above. To measure the retention of rh-PE lipids inside Huh7.5 cells following the 96 h incubation, cells were washed and replaced in fresh medium for a further 8, 24, 30, and 48 h prior to harvesting and analysis.

**Uptake assays with caveolin-1- and dynamin-2-dominant negative mutants of HepaRG cells**

Creation of these dominant-negative cells lines has been previously described, along with a detailed protocol for monitoring the uptake of fluorescently labeled material (15). Briefly, to monitor changes in ER liposome uptake in these cell lines, 1% rh-PE-labeled liposomes were incubated with serum-starved cells for 1 h at a final lipid concentration of 50 μM. Cells were harvested by trypsin/EDTA to remove any plasma membrane-bound liposomes, washed 2–3 times in PBS, and lysed in a HEPES/CHAPS buffer. Lysates were centrifuged at 12,000 g for 30 min, and clear supernatants were kept. The rh-PE-labeled lipids released from the cells were quantified by spectrofluorometry (λ_ex = 540 nm, λ_em = 625 nm) using unlabeled liposome-treated cell lysates as blanks. The fluorescence was further normalized to the total protein content/sample, as determined by BCA.

**R18 and calcine dequenching assays**

Liposomes were prepared incorporating 10% mol of the self-quenching lipid dye octadecyl rhodamine B (R18; Invitrogen) or encapsulating 80 mM calcine, a self-quenching aequorin fluorophore. R18-labeled or calcine-loaded liposomes were separated from unincorporated R18/calcine by size exclusion chromatography using Sephadex G-50 (medium) resin (G.E. Healthcare). To measure R18 lipid and calcine dequenching, liposomes were added to cells at a final lipid concentration of 1 and 50 μM, respectively, and were incubated within a SpectroMax M-5 spectrofluorometer (Molecular Devices, Sunnyvale, CA, USA) for 45 min at 37°C, with readings taken every 30 s. R18 dequenching was measured at λ_ex = 550 nm, λ_em = 590 nm, and calcine dequenching was measured at λ_ex = 490 nm and λ_em = 520 nm. The addition of Triton X-100 to a final concentration of 1% allows disruption of all membranes, achieving 100% R18 lipid/calcine dequenching to calibrate the fluorescent scale.

**Leakage of calcine from liposomes in human serum**

To monitor the stability of liposomes in the presence of various concentrations of human serum, calcine-loaded liposomes were prepared, separated from unencapsulated calcine by size-exclusion chromatography, and added to complete DMEM with 5–50% serum in the absence of cells, final phospholipid concentration of 50 μM. Liposomes were left to incubate for 4 d, at which time a sample of liposome-containing medium was taken to monitor calcine dequenching at λ_ex = 490 nm, λ_em = 520 nm as a result of liposome destabilization and leakage of calcine into the surrounding medium. Addition of Triton X-100 to a final concentration of 1% following the 4-d incubation disrupts the liposome membranes, achieving 100% calcine dequenching in order to calibrate the fluorescent scale.

**Quantifying intracellular calcine delivery**

Liposomes containing 1% rh-PE and encapsulating 80 mM calcine were incubated with Huh7.5 cells for 30 min at 37°C or 4°C (final lipid concentration of 50 μM). Following incubations, calcine dequenching was measured at λ_ex = 490 nm and λ_em = 520 nm, and rhodamine fluorescence was measured at λ_ex = 550 nm and λ_em = 590 nm. The initial calcine to rhodamine fluorescence ratio of liposomes bound to cells in the absence of endocytosis was obtained by incubating the liposomes with cells at 4°C and is used to adjust values at 37°C.

**Statistical analysis**

All statistical analyses were performed by using Microsoft Excel software. (Microsoft, Redmond, WA, USA). All graphs
RESULTS

ER-like liposomes are targeted to the ER and ER-associated membranes

In an attempt to localize endocytosed liposomes to the ER membrane of human cells, a series of liposome formulations was designed based on the combination of pH-sensitive PE:CH liposomes and the approximate phospholipid composition of the ER membrane. Purified rough ER membrane from rat liver comprises 57% PC, 24% PE, 13% PI, 3% PS, and 3% sphingomyelin (16). The lipid compositions of the liposome formulations used in our study are listed in Supplemental Table 1, along with their molar lipid ratios and size distributions. To visualize colocalization of the liposome preparations with the ER membrane, liposomes containing 1% rh-PE were used to incubate with Huh7.5 cells, and their intracellular localization was determined 30 min following treatment.

![Figure 1](https://example.com/figure1.png)

**Figure 1.** Liposomes containing the lipids PI and PS colocalize with the ER and ER-associated membranes. A) EDEM staining of rh-PE liposome-treated cells 30 min postincubation. DAPI is used as a nuclear stain. Confocal images from one representative experiment are shown. B) Calculated colocalization of liposome-delivered rh-PE with the EDEM antibody. Results represent mean ± sd colocalization for the 30 cells. C) Capture and quantification of HCV (black), BVDV (red), and HIV (blue) viral particles associated with b-PE lipids delivered by either ER liposomes (solid lines) or pH-sensitive liposomes (dashed lines). Results are displayed as percentage of tagged viral particles captured by streptavidin in relation to total amount of secreted virions within same sample (100%). D) LD staining of rh-PE liposome-treated cells 24 h postincubation. A confocal image of one representative experiment is shown. E) SDS-PAGE of b-PE-associated material from serum captured on streptavidin resin (gel lane 1). A control for cell-free association of b-PE lipids with medium components is also shown for both ER liposomes (gel lane 2) and 3% PEGylated ER liposomes (gel lane 3). Visible gel bands were excised and identified by mass spectrometry, and protein IDs are listed (hu, human; bv, bovine).
such that PE:CH:PI:PS liposomes demonstrated 76 \pm 8.7\% ER membrane colocalization and 88 \pm 3.5\% colocalization was observed with PE:PC:PI:PS liposomes (herein referred to as ER liposomes).

To demonstrate that lipids delivered by ER liposomes specifically intercalate within the ER membrane of treated cells, we monitored the incorporation of a tagged lipid into the lipid envelopes of viruses that bud from the ER and compared it to results obtained with a plasma membrane budding virus. Cells infected with the ER budding HCV and BVDV were incubated with b-PE tagged ER liposomes for 48 h. B-PE lipids delivered by ER liposomes were positively identified in complex with both secreted ER-budding viruses, HCV and BVDV, following a 48-h incubation. B-PE was complex with both secreted ER-budding viruses, HCV and BVDV, and the optimal concentration for tagging secreted ER liposomes were positively identified in complex with both secreted ER-budding viruses, HCV and BVDV, for 48 h. B-PE lipids delivered by ER liposomes were positively identified in complex with both secreted ER-budding viruses, HCV and BVDV, following a 48-h incubation. B-PE was incorporated into ER liposomes at 0.1, 0.5, 1, 5, or 10 mol\%, and the optimal concentration for tagging secreted HCV and BVDV was determined to be 1%, capturing 90 \pm 3.6 and 91 \pm 1.5\% of the total number of secreted virions, respectively (Fig. 1C). PBMCs infected with a primary isolate of HIV-1 (LAI) were treated similarly; however, HIV-1 particles did not contain detectable amounts of the tagged lipid. When experiments were repeated with b-PE-tagged pH-sensitive liposomes at the same concentrations, a maximum of 18.7 \pm 2.9\% of HCV particles and 11.3 \pm 3.5\% of BVDV particles were captured (Fig. 1C). B-PE-tagged pH-sensitive liposomes delivered to HIV-infected PBMCs led to the subsequent tagging of only 3.0 \pm 2.0\% of secreted HIV particles. These results highlight the specificity of ER liposomes for delivering lipids to the ER and ER-associated membranes.

Lipid droplets (LDs) are intracellular organelles used for lipid storage and are surrounded by a monolayer of ER-derived phospholipids (17). To investigate whether lipids delivered via ER liposomes eventually colocalize with LD membranes, cells were treated with 1% rh-PE-labeled ER liposomes for 1 h, at which point, cells were washed and further incubated in liposome-free medium for 24 h to allow rh-PE lipid distribution within the intracellular membranes. In BODIPY 493/503-stained cells, 96 \pm 8.6\% of LDs colocalized with lipids from ER liposomes following a 24-h incubation (Fig. 1D), highlighting the close association between LDs and the ER membrane, as well as the ability of lipids delivered by ER liposomes to localize to these ER-associated membranes.

In an attempt to identify all of the secreted and/or serum proteins that associate with lipids delivered by ER liposomes, the supernatant of Huh7.5 cells treated with 1% b-PE-labeled ER liposomes was captured on streptavidin-sepharose resin and separated by SDS-PAGE, and proteins were subsequently identified using mass spectrometry (Fig. 1E). Secreted material captured by this method comprises apolipoproteins apoAI, apoB, and apoE. Although most apolipoproteins identified were human proteins, the majority of apoAI identified was of bovine origin, and likely due to lipid exchange of b-PE lipids onto these FBS-derived serum proteins by scavenger receptors (SRs) within the intracellular vesicles of treated cells (18). To identify secreted or serum proteins capable of association with liposomes in the absence of cells, supernatant from untreated Huh7.5 cells was incubated with 1% b-ER liposomes for 24 h and analyzed in the same manner. As shown in Fig. 1E, gel lane 2, the only protein that interacts with liposomes is fibronectin, a known serum opsonin. In addition to ER liposomes, we also tested a 3% (mol) PEGylated version of these liposomes (1.35: 1.5:1:1.0.15 PE:PC:PI:PS:PEG-PE), as the addition of PEGylated lipids increases in vivo circulation times by limiting the recognition of these liposomes by serum opsonins (19). PEGylation of ER liposomes does not affect their trafficking through the cell, as these liposomes still localize to the ER in Huh7.5 cells (Supplemental Fig. 1). When incubated with cell supernatant only, these liposomes demonstrated a weaker association with fibronectin (Fig. 1E, gel lane 3), confirming a reduced interaction with opsonins that may otherwise clear liposomes from serum. Again, there were no detectable interactions with serum lipoproteins; therefore, liposomes do not associate with extracellular lipoproteins in the absence of cells.

Overall, these results suggest that ER liposomes are taken up by cells and trafficked intact to the ER membrane, where lipids are then free to incorporate into ER-budding entities, such as certain viruses, lipid droplets, and secreted lipoproteins.

**ER liposomes are endocytosed by the LDL receptor and scavenger receptors**

To distinguish between the various mechanisms of endocytosis potentially utilized by liposomes to gain entry into cells, a series of liposome uptake assays was performed in the presence of inhibitors known to block each individual pathway (Fig. 2A). All inhibitors were used at the maximum concentrations, which caused <5\% cell death. Wortmannin and amiloride inhibit the process of macropinocytosis, and at the highest tolerated concentrations, liposome uptake was inhibited by 23 \pm 11.7\% (P=0.02) and 15 \pm 4.2\% (P=0.02), respectively. Chlorpromazine inhibits clathrin-mediated endocytosis but can also lead to liposome disruption and nonclathrin-mediated uptake of lipids if not removed prior to the incubation with liposomes. In this modified assay, chlorpromazine-treated cells demonstrated a 13 \pm 7.7\% (P=0.02) decrease in liposome uptake at a final concentration of 25 \mu M. Treatment of cells with indomethacin and filipin, two inhibitors of caveolae-mediated endocytosis, led to a 49 \pm 6.9\% (P<0.001) and 73 \pm 7.8\% (P<0.001) decrease in liposome uptake, respectively. Further inhibitors that block intracellular trafficking pathways such as endosomal acidification (with chloroquine), actin-dependent movement (with cytochalasin D), and microtubule-dependent movement (with nocodazole) were assayed. Only the disruption of microtubule-dependent trafficking led to a significant, dose-dependent decrease in liposome uptake (maximum decrease of 63 \pm 11.3\%; P<0.001), suggesting a role for microtubules in liposome trafficking through the cell.
To confirm the role of caveolae in ER liposome uptake, HepaRG cell lines with dominant-negative (DN) mutations in either caveolin-1 or dynamin-2 were used in rh-PE-labeled liposome uptake assays along with wild-type (WT) versions of the same cell lines. As shown in Fig. 2B, the caveolin-1 DN mutation led to a decrease in ER liposome uptake by 58.5 ± 4.0% (P=0.009), and the dynamin-2 DN mutation demonstrated a 44.9 ± 12.9% (P=0.03) decrease. These results confirm the use of caveolae for ER liposome uptake into cells.

PS-containing liposomes have been previously suggested to utilize scavenger receptors (SRs) for uptake into various cell types (20). To assess whether ER liposomes utilize one or several SRs or LDLr for uptake into Huh7.5 cells, 1% rh-PE-labeled ER liposomes were incubated with cells in the presence of various SR or LDLr ligands, including LDL, acetylated LDL (acLDL), HDL, and fucoidin (Fig. 2C). LDL, a ligand of both LDLr and SR-BI, reduced liposome uptake by 70 ± 7.4% (P<0.001) at a final concentration of 100 μg/ml in the medium. At the same final concentration, acLDL and HDL, ligands of SR classes A and B, inhibited liposome uptake by 79 ± 5.8 and 52 ± 9.9% (P<0.001), respectively. Fucoidin, which specifically blocks uptake by SR-A, but not SR-BI or LDLr, demonstrated the least effective inhibition of liposome uptake, reducing uptake by 40 ± 10.0% (P<0.001) at a final concentration of 100 μg/ml. These experiments were repeated using blood monocyte-derived macrophages with similar results (Supplemental Fig. 2).

To distinguish whether the presence of LDL was blocking ER liposome uptake in Huh 7.5 cells by either SR-BI or LDLr-mediated uptake, we used drugs that specifically up- and down-regulate the expression of LDLr in Huh7 cells (squalestatin, SQ, and 25-hydroxycholesterol, 25-HC, respectively) (21) but that have no effect on SR-BI expression. Cells were cultured in the presence of either SQ or 25-HC for 24 h (final concentrations of 10 and 1 μM) prior to the addition of 1% rh-PE-labeled ER liposomes for 1 h. Treatment of cells with 10 μM 25-HC led to a 69 ± 4.9% (P=0.001) decrease in liposome uptake, whereas 10 μM SQ significantly increased liposome uptake to 117 ± 10.7% (P=0.04) of the untreated control, suggesting a dependence on LDLr for uptake of ER liposomes into Huh7.5 cells (Fig. 2D). Live-cell confocal images of Huh7.5 cells treated with either 10 μM SQ or 10 μM 25-HC and then incubated in the presence of 0.1% rh-PE-labeled ER liposomes are shown as Supplemental Fig. 3. Images confirm an increase and decrease in liposome uptake within Huh7.5 cells following the 24-h treatments with SQ and 25-HC, respectively.

Taken together, these results suggest that ER liposomes primarily use SR-BI and LDLr, and to a lesser extent, SR-A, for uptake into human liver cells and macrophage.

**Intracellular trafficking of ER liposomes to the ER**

In the uptake pathway used by cholera toxin, the cholera toxin subunit B (CTB) directly interacts with the glycolipid GM1 located within lipid rafts at the plasma membrane. Following binding to these mem-

**Figure 2.** ER liposomes primarily utilize a caveolae-dependent uptake mechanism in combination with scavenger and lipoprotein receptors for entry into target cells. Uptake of 1% rh-PE liposomes in the presence of inhibitors of endocytosis and vesicle trafficking in Huh7.5 cells (A), HepaRG cells containing dominant-negative (DN) mutations in either caveolin-1 or dynamin-2 in addition to the wild-type (WT) cell lines (B), ligands of scavenger and lipoprotein receptors in Huh7.5 cells (C), and drugs used to up- and down-regulate the expression of lipoprotein receptors in Huh7.5 cells (D). Data represent means ± sd of triplicates from 3 independent experiments. Data are presented in relation to the untreated control (100%).

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In the uptake pathway used by cholera toxin, the cholera toxin subunit B (CTB) directly interacts with the glycolipid GM1 located within lipid rafts at the plasma membrane. Following binding to these mem-

**Figure 2.** ER liposomes primarily utilize a caveolae-dependent uptake mechanism in combination with scavenger and lipoprotein receptors for entry into target cells. Uptake of 1% rh-PE liposomes in the presence of inhibitors of endocytosis and vesicle trafficking in Huh7.5 cells (A), HepaRG cells containing dominant-negative (DN) mutations in either caveolin-1 or dynamin-2 in addition to the wild-type (WT) cell lines (B), ligands of scavenger and lipoprotein receptors in Huh7.5 cells (C), and drugs used to up- and down-regulate the expression of lipoprotein receptors in Huh7.5 cells (D). Data represent means ± sd of triplicates from 3 independent experiments. Data are presented in relation to the untreated control (100%).
brane rafts, the raft/toxin complexes travel to the ER via sorting endosomes and the Golgi. To examine whether ER liposomes utilize a similar retrograde trafficking pathway en route to the ER membrane, we used live-cell confocal microscopy to follow the trafficking of 0.1% rh-PE-labeled liposomes in combination with a fluorescently labeled CTB protein associated with lipid rafts. Representative images of ER liposomes, lipid raft-bound CTB- and ER-tracker-stained cells are shown at 2–5, 5–7, and 20 min following the addition of liposomes to cells (Fig. 3A). Colocalization of liposomes and CTB at the plasma membrane could not be detected prior to their uptake by cells, suggesting that liposomes do not require GM1-containing lipid rafts for cell entry. However, at 5- to 7-min postincubation, liposomes and CTB colocalized within intracellular vesicles (Fig. 3A, second panel, white arrows), which stained positive for EEA-1, and hence were identified as early or sorting endosomes (Fig. 3B). At this same time point, ER liposomes were visualized in combination with a plasma membrane marker (CD-81) in order to confirm no direct fusion of ER liposomes with this

**Figure 3.** ER liposomes traffic to the ER lumen via early endosomes and the Golgi. A) Live-cell confocal imaging of Huh7.5 cells preincubated with ER tracker blue-white, followed by the addition of AlexaFluor488-labeled CTB associated with lipid rafts and 0.1% rh-PE-labeled liposomes. At least 5 images were taken at each time point following liposome incubation. White arrows indicate areas of liposome and CTB colocalization. B–D) Confocal images of 0.1% rh-PE liposome-treated Huh7.5 cells stained with antibodies against: EEA-1 (B), CD-81 (C), and golgin-97 (D). DAPI is used as a nuclear stain. E) Live-cell confocal imaging of Huh7.5 cells preincubated with ER tracker blue-white, followed by the addition of AlexaFluor488-labeled transferrin and 0.1% rh-PE-labeled liposomes. At least 5 images were taken at each time point following liposome incubation. White arrows indicate areas of liposome and transferrin colocalization. All experiments were repeated ≥3 times; images are representative.
membrane prior to endocytosis into the cell (Fig. 3C). After transit through sorting endosomes, CTB reaches the ER via the Golgi (22), and at ~15 min postincubation, liposomes also partially colocalized with the Golgi marker, golgin97 (Fig. 3D). On the basis of these images, liposomes localize to the ER within 20 min postincubation, and specific areas of colocalization between all three markers—ER liposomes, CTB, and ER-tracker—are visible (Fig. 3A, third panel, white arrows). Our results suggest that ER liposomes mainly travel through the cell utilizing a retrograde transport mechanism, rather than the classical endosomal pathway, en route to the ER.

Similar incubations as those described above were repeated with fluorescently labeled transferrin molecules, which are endocytosed by a clathrin-mediated mechanism and trafficked along the endosomal pathway (12). In these experiments, liposomes do not colocalize with transferrin at the cell surface (Fig. 3E, first panel); however, at 5–7 min postincubation, areas of colocalization between liposomes and transferrin can be observed, possibly representing liposome entry into early/sorting endosomes (Fig. 3E, second panel, white arrows). This colocalization is short lived (<7 min), and liposomes start to colocalize with the ER marker at 20 min, while transferrin molecules traffic to a separate and different compartment (Fig. 3E, third panel). These results also confirm the merging of vesicles from both clathrin and caveolae-mediated uptake into the same sorting endosomes.

Fusion of ER liposomes with the ER membrane and release of encapsulated cargo

The rate of lipid fusion and intracellular cargo release was measured in Huh7.5 cells treated with ER-targeting liposomes either labeled with the self-quenching lipid dye R18 (10% mol), or encapsulating the self-quenching aqueous dye calcein at 80 mM. The increase in fluorescence as a result of R18 and calcein dequenching indicates a possible liposome membrane destabilization and/or fusion that was monitored in Huh7.5 cells over 45 min (Fig. 4A). Treatment with ER liposomes containing the R18 tag led to a significant increase in fluorescence at 22–25 min postincubation with cells, demonstrating the start of liposome fusion around the same time point where colocalization of ER liposomes is observed with the ER membrane (Fig. 3A, E; 20-min images). ER liposomes encapsulating calcein exhibited a significant increase in fluorescence after 20–22 min. Together, these results suggest that liposomes travel to the ER intact and begin fusion with the ER membrane at ~20 min postincubation, releasing their encapsulated cargo.

**Figure 4.** ER liposomes are delivered to the ER lumen while still intact and fuse with the ER membrane to release encapsulated cargo. A) Fluorescent dequenching of R18-labeled liposomes and calcein-loaded liposomes measured over a 45-min incubation with Huh7.5 cells. The increase in R18/calcein fluorescence is indicative of liposome membrane destabilization and/or fusion and was monitored in Huh7.5 cells over 45 min (Fig. 4A). Treatment with ER liposomes containing the R18 tag led to a significant increase in fluorescence at 22–25 min postincubation with cells, demonstrating the start of liposome fusion around the same time point where colocalization of ER liposomes is observed with the ER membrane (Fig. 3A, E; 20-min images). ER liposomes encapsulating calcein exhibited a significant increase in fluorescence after 20–22 min. Together, these results suggest that liposomes travel to the ER intact and begin fusion with the ER membrane at ~20 min postincubation, releasing their encapsulated cargo.
Live-cell imaging of 0.1% rh-PE-labeled ER liposomes encapsulating 100 μM calcein (a non-self-quenching concentration) was performed to visualize calcein both inside ER liposomes and intracellular vesicles, as well as released into other intracellular compartments. These images reveal that ER liposomes travel to the ER lumen as intact entities, and by 20 min postincubation, most of the calcein-loaded liposomes are localized to the ER (demonstrated by colocalization with the blue ER marker, Fig. 4B). At this time, calcein localized within the ER appears to be primarily associated with rh-PE lipids (white areas), suggesting liposomes are still mostly intact at this stage; however, areas of calcein release (yellow or green areas) located at or just adjacent to the ER are also evident. At 30 min postincubation, rh-PE-labeled liposomes appear to have fused with the ER membrane (determined by a diffuse staining pattern similar to that of the ER marker), and released calcein is found within the ER lumen and perhaps the cytosol (Fig. 4B, third panel). A potentially interesting observation is that the calcein fluorescence changes from a small/sharp punctate pattern (10 min) to a slightly larger/blurry punctate pattern (20 min). Although this may be due to the start of ER membrane fusion at this time point, it is also possible that ER liposomes have become unstable within the lumen of intracellular trafficking vesicles, leading to the release of encapsulated calcein. This potential leakage might be caused by interactions with certain receptors/proteins that are involved in the sorting and targeting of these liposomes to the ER membrane from early endosomes.

To determine the efficiency of cargo release from ER liposomes compared to pH-sensitive liposomes, rh-PE liposomes encapsulating self-quenching concentrations of calcein were incubated with Huh7.5 cells for 5 min before cells were washed and incubated in fresh medium for 45 min. Mean rh-PE fluorescence in Huh7.5 cells reflects the uptake of liposomes, whereas the mean calcein fluorescence indicates intracellular dequenching, and therefore release of fluorescent dye. The calculated ratio of calcein to rhodamine fluorescence is taken as a measure of the amount of aqueous marker released intracellularly per cell-associated liposome. The calcein/rhodamine ratio for pH-sensitive liposomes was calculated to be 10.3 ± 2.6, whereas the ratio for ER liposomes was 15.7 ± 2.4, an increase of 52% (P=0.02), demonstrating superior release of encapsulated cargo from ER liposomes compared to pH-sensitive liposomes.

**ER liposomes allow prolonged and enhanced delivery of encapsulated cargo to cells**

The cytotoxicity of both ER and pH-sensitive liposome formulations was assessed in Huh7.5 cells following a 4-d treatment period using an MTS-based cell proliferation assay. pH-sensitive liposomes cause ~10–15% cellular toxicity at concentrations > 60 μM, which steadily increases to 85–90% toxicity at concentrations > 250 μM (Fig. 5A). In contrast, ER liposomes cause <5% cellular toxicity, even at a final concentration of 500 μM. No cytotoxicity was observed for either liposome formulation at a concentration of 50 μM; therefore, it was chosen as the best concentration to compare both liposome compositions for long-term incubation assays.

The rate of liposome uptake was monitored in Huh7.5 cells over a 4-d coincubation period with pH-sensitive and ER-targeting liposomes containing 1% rh-PE (Fig. 5B). Cells were seeded at low density, and liposome uptake was measured in relation to cell growth. ER liposomes were taken up continuously by actively dividing Huh7.5 cells over the 4-d incubation period. At d 4, ER liposome-treated cells demonstrated a fluorescence of 1.5 × 10⁻³ ± 3.4×10⁻⁴ AU/cell, which is 6-fold greater than that observed with pH-sensitive liposome treatment (2.5×10⁻⁴±5.5×10⁻⁵ AU/cell). In addition, the maximum fluorescence observed in pH-sensitive liposome-treated cells was reached following a treatment period of only 24 h (5.0×10⁻⁴±1.1×10⁻⁴), after which cell-associated fluorescence slowly decreased, indicating either decreased liposome uptake or increased efflux/degradation of rh-PE lipids, or both. Following the 4-d incubation, treated Huh7.5 cells were washed and returned to medium without any liposomes to monitor the intracellular half-life of rh-PE lipids delivered by both lipid systems. On the basis of these experiments, rh-PE lipids delivered by pH-sensitive liposomes had a half-life in cells of 7 h, whereas the half-life of ER liposomes was extended to 29 h, suggesting greater incorporation of ER liposomes into the cellular membranes of treated cells.

ER liposomes have been developed for the purposes of drug delivery in vivo; therefore, their ability to enter cells and avoid leakage of cargo prior to uptake in cells in the presence of high concentrations of serum was measured. Rh-PE-tagged liposomes were incubated with Huh7.5 cells or blood monocyte-derived macrophages over a period of 4 d in the presence of either 10–50% human serum or autologous human plasma, respectively, and total liposome uptake was compared to that in Huh7.5 cells grown in 10% FBS. When incubated with Huh7.5 cells in the presence of human serum, there are no significant differences in ER liposome uptake compared to data obtained in 10% FBS, even at human serum concentrations up to 50% in the medium (Fig. 5C). Furthermore, although uptake of ER liposomes is reduced in macrophages compared to Huh7.5 cells, again, there is no significant difference in the cellular uptake of these liposomes when incubated in either 10 or 50% human plasma. The stability of liposomes in the presence of human serum was determined by encapsulating self-quenching calcein and monitoring leakage in the presence of various concentrations of serum (5–50% in medium) following a 4-d incubation. As shown in Fig. 5D, ER liposomes still encapsulated >54 ± 9.2% of the self-quenched calcein following the incubation period.
In a previous study, we have shown that treatment with pH-sensitive liposomes encapsulating the iminosugar N-butyldeoxynojirimycin (NB-DNJ) significantly decreased HIV secretion and infectivity compared to the activity of the free drug alone (10). To determine whether NB-DNJ and other DNJ analogues, encapsulated inside ER-targeting liposomes, could further increase the antiviral activity against HIV-1 compared to delivery via pH-sensitive liposomes, could further increase the antiviral activity against HIV-1 compared to delivery via pH-sensitive liposomes, various drug/lipid combinations were tested against the HIV-1 primary isolate, LAI. Effects on both viral secretion and infectivity over a single round of treatment were monitored. Empty liposomes had a similar toxicity profile in human PBMCs compared to Huh7.5 cells (data not shown), and the encapsulation of DNJ derivatives at the concentrations used in this study also did not affect liposome toxicity (data not shown).

Liposomes (final lipid concentration of 50 μM) encapsulating PBS or DNJ compounds were incubated with HIV-1 infected PBMCs for 4 d. Two different encapsulated drug concentrations were tested: 10 μM (the lowest concentration that caused an antiviral effect when encapsulated inside pH-sensitive liposomes) and 1 mM. The liposome-encapsulated drug concentrations are equivalent to final drug concentrations in the medium of 100 nM and 1 μM, respectively, assuming encapsulation efficiency to be 100%. This is unrealistic, as free drug would have been removed from liposome encapsulated drug by size-exclusion chromatography, reducing the actual amount of drug used in treatments. Results for both HIV-1 secretion during treatment, as well as the infectivity of secreted virions, are presented in Fig. 5E. Confirming results of our previous study (10), PBS encapsulating pH-sensitive liposomes had no antiviral activity against HIV-1. In contrast, ER liposome treatment alone decreased both HIV-1 secretion (22 ± 4.6% decrease, P=0.004), as well as the infectivity of secreted particles (50 ± 4.6% decrease, P<0.001), compared to the untreated control. This separate antiviral activity of the ER liposomes, in the absence of encapsulated iminosugars, is likely due to decreased cholesterol biosynthesis in treated cells, an observed effect of ER liposome treatment (unpublished results). HIV depends on increased levels of cholesterol both within the cell and at the cell surface for efficient viral assembly, as well as within the viral particle itself for proper infectivity (23–25).

DNJ iminosugar compounds tested in this study

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include DNJ (solubilized in PBS), NB-DNJ (solubilized in PBS), N-nonyl-DNJ (NN-DNJ; solubilized in ethanol), and N-7-oxadecyl-DNJ (N7-DNJ; solubilized in PBS). pH-sensitive liposomes encapsulating 100 nM NB-DNJ decreased HIV-1 secretion by 19 ± 9.5% (P=0.03) and viral infectivity by 37 ± 5.7% (P<0.001) compared to the untreated control, which is consistent with results reported previously (10) (Fig. 5E). When encapsulated inside ER liposomes, 100 nM NB-DNJ decreased HIV-1 secretion by 62 ± 9.5% (P<0.001) and infectivity by 86 ± 4.4% (P<0.001). ER liposomes therefore lead to enhanced NB-DNJ drug delivery compared to pH-sensitive liposomes, as the decrease in HIV-1 secretion with 100 nM NB-DNJ in ER liposomes is 62%, which is ~1.5-fold greater than the combined activity of 100 nM NB-DNJ in pH-sensitive liposomes (19%) and empty ER liposomes (22%). Analysis of the viral infectivity data showed that there was no such enhancement of the effect on the specific infectivity of secreted virions (86% decrease with 100 nM NB-DNJ ER liposomes vs. a combined decrease of 87% with 100 nM NB-DNJ pH-sensitive liposomes and empty ER liposomes). This likely indicates that the observed effect represents the maximum antiviral activity achievable with a single round of drug treatment.

Similar enhancement of drug activity was observed for the other two water-soluble compounds, DNJ and N7-DNJ. Their antiviral effects on HIV secretion were 1.3- and 1.5-fold greater, respectively, when delivered by ER liposomes compared to pH-sensitive liposomes at a final drug concentration of 100 nM. In the case of the lipophilic DNJ derivative, NN-DNJ, the antiviral enhancement was even more pronounced, with the ER liposome-delivered drug leading to a 3-fold enhancement against HIV secretion; however, given the maximum antiviral activity had been achieved with 100 nM NN-DNJ, the enhancement is likely to be even greater.

The combined results from the antiviral efficacy study of encapsulated iminosugars against HIV-1 (Fig. 5E) and the prolonged intracellular uptake of tagged lipids (Fig. 5B) suggest that ER liposomes are not only better suited for the delivery of lipids or lipophilic compounds to the ER membrane, but are also more efficient at the intracellular delivery of water-soluble drugs compared to other liposomes specifically designed for intracellular drug delivery. Furthermore, the stability and efficient cellular uptake of ER liposomes in the presence of physiologically relevant concentration of human serum/plasma (Fig. 5C, D) suggest these liposomes have the potential to be developed for in vivo drug delivery.

**DISCUSSION**

This report describes for the first time liposomes that traffic directly to the ER, where they fuse and deliver lipids and intercalated lipophilic drugs/markers directly to the ER membrane. These liposomes also deliver encapsulated hydrophilic cargo more efficiently than pH-sensitive liposomes without causing cellular toxicity when used at concentrations up to 500 µM. ER liposomes will be suitable for the intracellular delivery of various types of cargo, including drugs, peptides, and nucleic acids.

Addition of either 20% PI or 20% PS to PE:CH or PE:PC liposomes increased their subcellular localization to the ER membrane by 4- to 6-fold depending on the exact lipid composition. The combination of these two phospholipids within the same lipid composition increased colocalization with the ER membrane marker by up to 8-fold. The highest colocalization value was reached with PE:PC:PI:PS liposomes, which is also the formulation that most closely resembles that of the ER membrane. As ~13% of tagged lipids delivered by pH-sensitive PE:CH liposomes also localize to the ER membrane, it is possible that the addition of PI and/or PS merely increases the shuttling of liposomes through an alternative pathway. This alternative trafficking pathway appears to be similar to that utilized by cholera toxin for trafficking to the ER from the plasma membrane via early endosomes and the Golgi, and may be dependent on an interaction between liposomes and proteins/receptors associated with lipid rafts, which are shuttled between the two membrane compartments.

The specificity of liposome delivery to the ER membrane was highlighted by the presence of tagged lipids from ER liposomes within the membranes of >90% of viral particles assembled at the ER membrane, such as HCV and BVDV, and the absence of these same lipids within HIV particles, which bud from the plasma membrane. Tagged pH-sensitive liposomes were also capable of tagging up to 18% of these same ER-budding viruses, again suggesting that a certain number of these liposomes can reach the ER membrane perhaps via the same trafficking pathway as ER liposomes. Tagged lipids delivered via ER liposomes were positively identified within ER-derived membranes, such as those within cellular LDs and secreted lipoproteins, demonstrating the specificity of ER liposomes to incorporate into the ER, as well as ER-associated membranes.

Using lipoproteins to block liposome entry and drugs to up- and down-regulate LDLr expression, we have shown that both scavenger and lipoprotein receptors are likely required for uptake of ER liposomes into human liver cells. We have shown this uptake mechanism to be primarily dependent on caveolae, based on inhibitor studies and uptake in dominant-negative mutants, as well as cellular microtubules, which mediate the transport of intracellular vesicles. Although microtubule inhibition does not influence the initial particle uptake, a saturation of the early endosome compartment likely prevents further uptake of liposomes into these compartments, as has been shown for other liposome formulations (3). The possibility that clathrin-mediated uptake may also be used as a secondary uptake route is not completely excluded since only low concentrations of the clathrin coat inhibitor chlorpromazine could be used to treat Huh7.5 cells before toxicity became a limiting factor. Regardless of the
The absence of encapsulated drug, the activity of 100 nM DNJ, N-DNJ, and N7-DNJ, the three water-soluble drugs, was between 1.3- and 1.5-times greater than the combined antiviral activity achieved with 100 nM of drug inside pH-sensitive liposomes and empty ER liposomes (measured by differences in viral secretion). When encapsulating the lipophilic drug AN-DNJ, which likely intercalates within the liposome bilayer, a greater enhancement was observed, such that ER liposomes demonstrated an antiviral activity 3-fold greater than pH-sensitive liposomes. We used DNJ-based miminosugars that target the ER α-glucosidases in order to demonstrate the ability of ER liposomes to deliver drugs aimed at ER-resident targets directly to their site of action. The predicted usefulness of this novel approach for the treatment of all viral infections known to be sensitive to this type of therapy, as well as other diseases in which the ER is implicated, such as Alzheimer’s disease and cystic fibrosis, will be studied further. Results of ER liposome stability and intracellular uptake in human serum/plasma concentrations up to 50% further support the development of these liposomes for in vivo use. PEGylation of these liposomes should further increase the observed stability of ER liposomes in vivo by reducing interactions with serum opsonins that ultimately lead to their clearance from the blood (19).

ER liposomes were designed on the basis of the idea that liposomes with a phospholipid composition similar to that of the ER membrane might traffic to this location on endocytosis into target cells. This approach has been successful, as liposomes composed of ER-like phospholipids PE, PC, PI, and PS demonstrated almost complete colocalization with the ER membrane 30 min after uptake into cells. These results encourage similar targeting strategies to be developed for the specific delivery of cargo to other intracellular domains such as the Golgi, mitochondria, and nucleus. Furthermore, the use of ER liposomes is not only limited to drug delivery, as the delivery of tagged lipids using this method can be used for imaging, as well as capturing purposes, both of the ER itself, as well as ER-derived entities, such as lipid droplets, lipoproteins and ER-budding viruses.

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