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Cholesterol transport from liposomal delivery vehicles

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Abstract

Rapid internalization of drugs from delivery vehicles via non-endocytotic pathways is an important goal. The transport of imaging probes attached to cholesterol and introduced via a liposomal formulation is considered here, in order to evaluate the intracellular distribution and kinetics of small molecular cargo that might be attached to cholesterol or phospholipids. The internalization efficiencies of two fluorescent cholesterol analogues, one carrying a fluorophore on the head of the cholesterol molecule (BODIPY-cholesteryl ester) and the other on the tail (NBD-cholesterol), were compared with those of other phospholipid molecules (NBD-PC and NBD-PE) using a liposomal formulation (DPPC, 85.5 M%; DSPE-PEG2k, 9.5 M%; fluorescent analogue, 5 M%). The rate and transfer efficiency were NBD-cholesterol > BODIPY-cholesteryl ester > NBD-PC > NBD-PE. NBD-cholesterol, delivered by liposomes with an average diameter of 100 nm, localized in the perinuclear region and lipid storage droplets, with transfer observed in as little as 5 minutes. NBD-cholesterol transport was approximately constant with time, suggesting a unidirectional mode of entry. In the absence of PEG within the liposome, the transfer rate decreased. Filipin, a caveolae-blocking agent, caused 70% inhibition of cholesterol internalization in treated cells, suggesting that cholesterol internalization follows a caveolae-mediated pathway.

Keywords

Cholesterol internalization; Endocytosis pathway; Caveolae; Liposome; Drug Delivery

1. Introduction

Design and development of a long-circulating and efficient drug/gene delivery vehicle requires an understanding of the molecular mechanism by which lipid carriers mediate delivery [1,2]. Delivery of the cargo to and through the cell membrane with high transportation efficiency and low cytotoxicity is required. Although the details of internalization mechanisms are still poorly understood, evidence supports the hypothesis that lipid carriers, such as liposomes and lipoplexes, enter non-phagocytic eukaryotic cells by endocytosis with the materials being processed by the endosomal-lysosomal pathway [3–5]. Escape from the endosome-lysosome is known to be a limiting step. A “perturbation” of the endosomal membrane causing release of the product in the cytosol is the approach pursued in new formulations such as pH sensitive liposomes [6], fusogenic systems [7], and liposome-bearing fusogenic peptides [8]. However, alternative intracellular delivery pathways could be advantageous in carrier-mediated drug/gene delivery systems. One of these systems is the cholesterol transport system studied in lipoprotein delivery systems [9–12]. In one study, siRNAs conjugated to derivatives of

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cholesterol were shown to enhance cellular uptake of siRNA, and therefore, to improve gene silencing in liver cells [13]. In another, intravenous injection of the most potent cholesterol-siRNA conjugate, selected through an *in vitro* evaluation, silenced an endogenous gene encoding apolipoprotein B in liver and jejunum, decreased plasma levels of apoB protein and reduced total cholesterol [14]. Intracellular trafficking and kinetics of lipid-drug/gene conjugates have not been systematically investigated and require additional fundamental studies.

Cholesterol is a major lipid component of the plasma membrane of mammalian cells, estimated to compose 30–40% on a molar basis, supplied to cells through either endogenous synthesis or by the uptake of exogenous cholesterol or cholesteryl ester from circulating lipoproteins. Cholesterol is efficiently trafficked through cells, which is important as it can be utilized immediately in cellular metabolism, stored by the cells in lipid storage droplets, or returned to the cell surface [15]. Flip-flop of cholesterol across the cell membrane is also very rapid and has been reported in the millisecond time range in a simple phospholipid bilayer [16,17].

The transport of imaging probes attached to cholesterol and introduced via a liposomal formulation is considered here, in order to evaluate the intracellular distribution and kinetics of small molecular cargo that might be attached to cholesterol. Recent papers regarding cholesterol and its internalization pathway have been primarily focused on receptor-mediated pathways using lipoprotein formulations (HDL, LDL, or artificial lipoprotein emulsions), mimicking the native delivery mode of cholesterol or cholesteryl ester into cells [9,12]. A study of cholesterol transport from an alternative delivery system (a liposomal formulation) is performed here for the following reasons: the effect of a stealth layer on the transport of cholesterol from liposomes to cells has not been determined; liposomes provide a lipid bilayer structure for the accommodation of cholesterol and its analogues and can be prepared uniformly in different sizes; liposomes are known to internalize into cells via endocytotic pathways, therefore, they provide a suitable system to study uptake and intracellular distribution of cholesterol; liposomes can be prepared using a simple lipid formulation and in the absence of apoprotein; and an understanding of the cholesterol internalization pathway will highlight the potential application of cholesterol conjugates in drug/gene delivery agents. Fluorescent analogues of cholesterol which mimic the native orientation of cholesterol in the biomembrane were used to monitor the cellular uptake and internalization of cholesterol and also to model the concept of cargo attachment to both the head and tail of cholesterol and phospholipid molecules [10,18]. Since our goal is to deliver therapeutics to diseased sites and since the transport and metabolism of cholesterol by cancerous cell lines has been reported in some (but not all) studies to differ from normal cells (19,20), the internalization of cholesterol conjugates was compared in cancerous and normal cell lines.

2. Materials and Methods

The fluorescent analogues, NBD-cholesterol, BODIPY-cholesteryl ester, NBD-phosphatidylcholine (NBD-PC) and NBD-phosphatidylethanolamine (NBD-PE), were incorporated into liposomes composed of DPPC, DSPE-PEG2k. Of these probes, NBD-cholesterol and NBD-PC attach the fluorophore at the end of the alkyl chain, while BODIPY-cholesteryl ester and NBD-PE are attached to the head group.

2.1 Materials

DPPC (1,2-dipalmitoyl-sn-glycero-3-phosphocholine); DSPE-PEG2k (1,2 distearoyl-sn-glycero-3-phosphoethanolamine-N-[Methoxy(Polyethylene glycol)-2000]); cholesterol, NBD-cholesterol (25-[N-[(7-nitro-2-1,3-benzoxadiazol-4-yl)methyl]amino]-27-norcholesterol); 16:0–12:0 NBD-PC (1-Palmitoyl-2-[12-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]dodecanoyl]-sn-Glycero-3-Phosphocholine); and NBD-DPPE (1,2-Dipalmitoyl-sn-

Glycero-3-Phosphoethanolamine-N-(7-nitro-2-1,3-benzoxadiazol-4-yl) Ammonium salt), were purchased from Avanti Polar Lipids Inc. (Alabaster, AL). Cholesteryl 4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-dodecanoate (cholesteryl BODIPY® FL C₁₂) was supplied by Invitrogen Co. (Carlsbad, CA). Filipin, nocodazole, and chloroquine diphosphate were obtained from Sigma-Aldrich (St. Louis, MO). Unless otherwise stated, cell culture media and supplements were from Invitrogen (Carlsbad, CA). All other reagents were of analytical grade and were used as received.

2.2 Methods

2.2.1 Cell culture—Two cancerous and two non-cancerous cell lines were used in this study. PC-3, a human prostate cancer cell line and A-375, a malignant melanoma cell line, were purchased from ATCC (CRL-1435 and CRL-1619, respectively). The two non-cancerous cell lines, a rat aortic endothelial cell (RAOEC), and a human umbilical vein endothelial cell (HUVEC), were obtained from Cell Applications (R 304-05, San Diego, CA) and Cascade Biologics (C-003-5C, Portland, OR), respectively. PC-3 cells were cultured as a monolayer in Ham's F12K medium with 2 mM L-glutamine adjusted to contain 1.5 g/L sodium bicarbonate. The medium was then supplemented with fetal bovine serum, 10% and penicillin/streptomycin, 1%. For A-375, Dulbecco's modified Eagle's medium (high glucose), which contains 4.5 g/L D-glucose, L-glutamine, and 110 mg/L sodium pyruvate, was used with 10% fetal bovine serum, 1% 10 mM non-essential amino acids, and 1% penicillin/streptomycin. A rat endothelial cell growth medium kit (R211K-500, Cell Applications) was used for RAOEC culture. HUVEC culture was performed using Medium 200 (M-200-500, Cascade Biologics) and Low Serum Growth Supplement (S-003-10, Cascade Biologics). Cells were then incubated in a humidified atmosphere containing 5% CO₂ at 37°C.

2.2.2 Liposome preparation—Figure 1 shows the structure of the four fluorescent lipids used in the liposome formulations, each with the label on the head or tail of single and double acyl chains. DPPC, DSPE-PEG2k, cholesterol, and the fluorescent lipid (in a molar ratio of 85.5:9.5:0.5 in case of fluorescent cholesterol analogues and 80.5:9.5:5:5 in case of fluorescent phospholipids) in chloroform were added to a test tube with total lipid mass of 1 mg. As described previously, chloroform was removed under N₂ and evaporated under a vacuum for at least 2 h [21]. A volume of 1 mL of Dulbecco's phosphate buffered saline (DPBS, Invitrogen) was added to the dried lipids. The lipid suspension was mixed well above the phase transition temperature of the lipids (50–55°C) to form a milky solution of multilamellar vesicles. The multilamellar lipid solution at a final concentration of 1 mg/mL was then extruded through a polycarbonate membrane with a pore diameter of 100 nm, with the extrusion also performed above the phase transition temperature of the lipid mixture. Liposomal diameter after extrusion was 97 nm ± 20 nm as measured using a Particle Analyzer, Microtrac 150 (Microtrac Inc., Montgomeryville, PA).

2.2.3 Cholesterol internalization—Glass coverslips with a cell monolayer were mounted into a stainless steel frame, which provided a 2 mm well depth above the cell layer. The well was filled with approximately 1 mL of a DPBS buffer solution containing 50 µL of liposome solution. The well was then sealed with a 25 mm glass coverslip, and the sample was placed on a heating block incubator. After incubation the cell monolayer was then gently rinsed with DPBS to remove remaining liposomes and transferred to a fluorescence microscope (Mikron IV 600L) for optical observation. Samples were analyzed optically with 20x and 63x objective magnification.

To alter the membrane fluidity and investigate temperature effects on uptake and internalization of NBD-cholesterol, the cell monolayer was incubated in the presence of NBD-cholesterol

liposomes at 4°C for 1h, observed under the microscope, and then warmed to 37°C with continued incubation for an additional hour before repeat observation.

2.2.4 Observation of liposome internalization—Confocal microscopy using a confocal microscope (LSM-510, Zeiss, Thornwood, NY) was performed in order to determine the intracellular location of fluorescent probes after incubation. We used the 63x, 1.4 NA lens. With the pinhole adjusted to 126 microns for the 488 nm excitation and 505 nm Long Pass emission, the system calculates an optical slice of 0.9 μ m. To avoid rapid bleaching of the sample, we took 6 x/y images every 4 μ m in z-stacks.

2.2.5 Treatment with inhibitors—In the investigation of the mechanism of cholesterol internalization, cells were pre-incubated with blocking agents including nocodazole (10–15 μ g/ml), filipin (5–25 μ g/ml), or chloroquine diphosphate (100 μ M, 51.5 μ g/ml) in DPBS for 1 h at 37°C. Subsequently, fluorescent liposomes (50 μ g/ml) were added in the presence of inhibitors and the incubation was continued for an additional hour at 37°C.

2.2.6 Quantification of internalized NBD-cholesterol—The relative level of NBD-cholesterol internalized into PC-3 cells was determined using a FACScan flow cytometer/CELLQuest software system (Becton Dickinson; San Jose, CA). The cells were incubated with fluorescent liposomes at 37°C, and then rinsed with DPBS several times prior to the addition of 0.05% Trypsin-EDTA solution. When a single cell suspension was obtained, trypsin was neutralized using the complete media. After centrifugation, the cells were washed with DPBS buffer and resuspended in 0.4% (w/v) Trypan Blue solution in DPBS to quench the extracellular fluorescence. The treated samples were washed twice and analyzed by the flow cytometer using logarithmic amplification. 5000–10,000 cells were measured in each sample, excluding cell debris by gating on characteristic forward and side scatter. Fluorescence intensity of the cells with internalized fluorescent cholesterol conjugates was measured upon excitation at 488 nm and emission at 530 $\pm$ 30 nm at a constant intensity in all experiments. A single peak corresponding to the intracellular distribution of the fluorescent conjugates with a coefficient of variation (CV) less than 10% was observed. The geometric mean values were calculated as the magnitude of internalized fluorescence and plotted.

2.2.7 Statistical analysis—Data points represent the average of triplicate measurements and the error bars are the standard deviations of the triplicate measurements. Statistical analysis was performed using two-tailed *t*-test assuming unequal variances. Statistical significance was assumed at $p < 0.05$.

3. Results

3.1 Kinetic studies of internalization of all four fluorescent lipid analogues

PC-3 cell internalization of NBD-cholesterol and BODIPY-cholesteryl ester was compared with the internalization of two phospholipid fluorescence analogues: NBD-PC, in which NBD was attached to the fatty acid tail of the molecule; and NBD-PE, with NBD attached to the head (Figs. 2A–D). We found that cholesterol analogues were transported faster than phospholipid analogues, with NBD-PE internalization substantially lower than any of the other fluorescent analogues. Flow cytometry indicated linear internalization with time for all analogues, with the internalization of NBD-cholesterol > BODIPY-cholesteryl ester > NBD-PC > NBD-PE (Figure 2E). The cellular fluorescent intensity observed for NBD-cholesterol after 2h incubation was significantly higher than any of the other lipid analogues, $p < 0.01$. Treating PC-3 cells after trypsinization with Trypan Blue 0.4% (w/v) in PBS, as a surface fluorescence quencher, did not affect the kinetics of cellular internalization of lipid fluorophores used here (data not shown).

3.2 Effect of cholesterol concentration

The effect of cholesterol concentration on transfer from liposomes to cells was examined after incubation of the liposomes with a confluent monolayer of PC-3 cells at 37 °C for 1h. The intracellular labeling was more intense with increasing liposome concentration (images 3A–E). Similarly, the quantified fluorescence intensity of labeled cells increased with liposome concentration, showing a tendency to saturation at higher liposome concentrations (Fig. 3F). Fluorescent images (Fig. 3D, 3E) show labeled plasma membrane and perinuclear regions. The perinuclear regions were assumed to be the Golgi compartments, as previously reported [22, 23].

3.3 Effect of incubation time

At a lipid concentration of 50 μ M, we investigated the time course of NBD-cholesterol internalization. We found that PC-3 cells were increasingly labeled with time (Figs. 4A–4E). By 2h after incubation (Fig. 4E), the number of lipid droplets had increased, perinuclear regions became more highlighted, and the cell background was more intense. Figures 4F–4I present confocal images of NBD-cholesterol internalization into PC-3 cells at two z-axis positions of 4 and 8 μ m and compare the images acquired after 5 min incubation with those after 1 h incubation. The accumulation of fluorescent cholesterol was observed in the form of droplet structures increasing over time. These lipid storage structures were identified previously by Reaven et al. as “lipid droplets” [24]. With our confocal microscopy parameters, the light in every plane is independent of the planes above or below and therefore the bright foci visualized within the images were not associated with the surfaces.

3.4 Effect of temperature

The internalization of NBD-cholesterol by both A-375 and PC-3 cells was dramatically reduced following incubation at 4°C (Figs. 5A–D). Upon warming to 37°C, the fluorescence intensity approached that of the control cells with a similar intracellular distribution (Figs. 5B and 5D). FACS showed that the relative fluorescence intensity of PC-3 cells incubated with NBD-cholesterol at 4°C for 2h diminished to 6.7% as compared to incubation at 37°C, $p < 0.01$ (Fig. 5E).

3.5 Effect of DSPE-PEG2k in liposome formulation on internalization of NBD-cholesterol

In order to determine whether the presence of PEG in the liposome alters transfer, fluorescence was compared for PC3 cells incubated with liposomes containing 5% NBD-cholesterol in two formulations (DPPC, DSPE-PEG2k, NBD-cholesterol 85.5:9.5:5.0 and 95:0:5.0) and with liposomes containing NBD-PC (DPPC, DSPE-PEG2k, cholesterol, NBD-PC 80.5:9.5:5.0:5.0 and 90:0:5:5). Both the NBD-cholesterol and NBD-PC probes demonstrated higher internalization rates when PEG lipids were included in the liposome formulation, ($p < 0.01$) (Figs. 6A–E). Cytoplasmic staining was observed with both the NBD-cholesterol and NBD-PC probes (with or without PEG) (Figs. 6C, D) However, lipid droplet formation was observed only with the NBD-cholesterol probe (Figs. 6A, B).

3.6 Effect of cell type

In order to determine whether the NBD-cholesterol internalization rate and distribution differ between cancerous cell lines and non-cancerous cells, NBD-cholesterol liposomes were incubated with various cell lines at a 50 μ M concentration, and the fluorescence intensity of the trypsinized cells was measured over time using flow cytometry. Fluorescent images of NBD-cholesterol internalization into the cell lines studied are provided in Figs. 7A–7D. Although the internalization rate of NBD-cholesterol varied across the cell lines tested, the intracellular distribution pattern was similar in all cell lines studied. NBD-cholesterol internalized linearly with time during the 2h-time period (Fig. 7E) in each cell line with a slope

varying from 1.33 % RFI/min for rat aortic endothelial cells to 0.53 % RFI/min for the A375 cell line.

3.7 Mechanism of internalization of NBD-cholesterol

To clarify the mechanism by which NBD-cholesterol is transferred from liposomes, PC-3 cells were pretreated with three inhibitors of cellular function: filipin, nocodazole, or chloroquine diphosphate. Filipin was used to specifically block caveolae-mediated uptake without affecting the function of coated pits [25,26]. Nocodazole interferes with the microtubule assembly, inhibiting the clathrin-mediated pathway [27,28] and disrupting Golgi membranes. Chloroquine diphosphate blocks lysosomal functions [15]. Incubation of PC-3 cells with filipin at 5 and 10 $\mu\text{g/mL}$ produced a significant inhibition of NBD-cholesterol uptake. As shown by confocal images (8A, 8B) acquired at the z position of 4 μm in Figure 8, the number of lipid droplets was dramatically reduced by treatment with filipin at 5 $\mu\text{g/mL}$, as compared to the control cells (Figure 8E, $p < 0.01$). Following incubation with filipin at a concentration of 10 $\mu\text{g/mL}$, droplets were absent and the number of viable cells was reduced ($p < 0.01$). Nocodazole had a significant but less marked effect than filipin ($p < 0.05$) (Figs. 8C, 8D). Although the number of lipid droplets was reduced relative to the control cells at a nocodazole concentration of 10 $\mu\text{g/mL}$, a noticeable number of droplets remained. Quantification of the degree of inhibition by the blocking agents indicates approximately 50% and 70% inhibition by filipin at concentrations of 5 $\mu\text{g/mL}$ and 10 $\mu\text{g/mL}$, respectively, compared with 15% and 25% inhibition with nocodazole at concentrations of 10 $\mu\text{g/mL}$ and 15 $\mu\text{g/mL}$, respectively (Fig. 8F). Chloroquine diphosphate had no significant effect on the uptake and cellular distribution of NBD-cholesterol.

Discussion

A study of the transport of imaging probes attached to cholesterol and introduced via a liposomal formulation was considered here, in order to evaluate the intracellular distribution and kinetics of small molecular cargo that might be attached to cholesterol. We hypothesize that an improved understanding of the cholesterol internalization pathway for components of delivery vehicles will highlight the potential application of cholesterol conjugates in drug/gene delivery agents. To this end, we used a simple liposomal formulation (DPPC, DSPE-PEG2k, fluorophore 85.5:9.5:5.0), with PEG lipids used to stabilize liposomes from aggregation and prevent rapid systemic clearance [29–32].

Incubation of liposomes incorporating NBD-cholesterol with cells at 37°C resulted in a rapid and lipid concentration-dependent uptake and intracellular distribution of the fluorophores (Figs. 2 and 4). The pattern of intracellular distribution of NBD-cholesterol was characterized by early cytoplasmic and perinuclear staining, followed by the development of highly fluorescent spots previously shown to be lipid droplets [24]. The fluorescent perinuclear regions were assumed to belong to the Golgi compartment based on their location when stained with an antibody to Golgi membrane coat protein, β -COP in [22] or their location in granulose cell preparations provided in [23]. The bright regions throughout the cytoplasm, found by Reaven et al., correspond in size and location with “lipid droplets” visualized on images by Nomarski [24].

To determine whether the internalization of cholesterol by cancerous cell lines is associated with their rapid proliferation [33,34], we extended our study to non-cancerous cell lines as well. As shown in Fig. 7E, all four cell lines internalized NBD-cholesterol with a similar distribution pattern, although the transfer rate varied. No conclusion can be drawn regarding the relative internalization of particles by cancerous cell lines, but we infer that the rapid internalization of cholesterol is not unique to highly proliferative cells.

Quantification of the fluorescence intensity after incubation with NBD-cholesterol liposomes for prolonged periods at 37°C shows that internalization proceeds at a constant rate (Fig. 2E). The linear increase in fluorescence intensity suggests unidirectional entry of cholesterol. Internalization of NBD-cholesterol was much slower at 4°C compared to 37°C, indicating that the delivery pathway is highly dependent on the fluidity of the membrane (Fig. 5). We hypothesize that this change was due to changes in the rigidity of the lipid bilayer, its functionality, and/or the reduced level of cellular energy. Cholesterol motion, known to be restricted by the membrane [35], has been reported to be capable of rapid motion at temperatures higher than 36°C [36,37].

We observed the internalization of NBD-cholesterol, in which the fluorophore attached to the cholesterol tail, and that of BODIPY-cholesteryl ester, in which the cargo is esterified to the head of cholesterol (Fig. 2). Both NBD-cholesterol and BODIPY-cholesteryl ester have been shown to retain some aspects of the native function of cholesterol and cholesteryl ester, respectively [10,18]. BODIPY-cholesteryl ester is hydrolysable by acid cholesteryl esterases in lysosomal compartment, but not hydrolyzable with neutral cholesteryl esterases of the cells [24]. Therefore, this probe can specifically distinguish between endocytic and non-endocytic pathways. We found that BODIPY-cholesteryl ester internalization was slower than NBD-cholesterol when incubated with identical lipid concentration, liposome diameter, and incubation time ($p < 0.01$). However, BODIPY cholesteryl ester demonstrated a similar pattern of uptake and intracellular distribution compared to NBD-cholesterol. The perinuclear localization and formation of lipid droplets of both probes suggest that internalization of cholesterol is not routed through lysosomes, and thus is not likely to be mediated by an endocytosis pathway. Pretreatment of cells with chloroquine diphosphate, which blocks lysosomal function, had no noticeable effect on internalization of NBD-cholesterol. This also refutes the hypothesis of endocytic-mediated delivery of NBD-cholesterol.

Phospholipid analogues carrying the NBD fluorophore (NBD-PC, with NBD attached to the fatty acyl tail of the molecule, and NBD-PE, with NBD on the head of the molecule) were used to compare internalization of phospholipid molecules with that of cholesterol analogues (Fig. 2). The internalization of phospholipid analogues was significantly slower than fluorescent cholesterol molecules ($p < 0.05$). Increasing the concentration of NBD-PC to 3 times than that of NBD-cholesterol showed comparable fluorescence intensity throughout cytoplasm at 2h (Data not shown). It should be noted that attachment of NBD to the head of PE makes the molecule negatively charged, while NBD-PC has a net charge of zero. Therefore, the slower internalization of NBD-PE compared to NBD-PC ($p < 0.002$) might be due to the negative charge of liposomes and/or due to the attachment of NBD to the head of the molecule as observed for cholesterol analogues.

The addition of PEG lipids (DSPE-PEG2k) to the liposomal formulation sterically stabilizes liposomes and prolongs their circulation in blood [38]. For this reason, one might expect PEG to produce a steric barrier for the interaction of liposomes with cells. However, our results indicated that the uptake of NBD-cholesterol as a result of liposome-cell interaction was higher with 9.5% PEG in the liposomal preparation. Similar results were observed for NBD-PC, which has a slower internalization rate, as compared with NBD-cholesterol. The faster internalization in the presence of PEG may result from the stabilization of the membrane against vesicle-vesicle fusion, which inhibits the formation of multi-lamellar liposomes. Large liposomes have been shown to slow down the cellular uptake [39–41]. However, the mechanism for rapid internalization in the presence of PEG will be investigated in a follow-up study.

We treated cells with various potent inhibitors of cellular function to get more detailed insight into the mechanism of the cholesterol transport system. Filipin suppressed NBD-cholesterol internalization considerably more than nocodazole (70% with filipin at a concentration of 10

µg/mL compared to 25% with nocodazole at a concentration of 15 µg/mL) supporting the hypothesis that NBD-cholesterol internalization is mediated by caveolae. The fact that lipid droplets are still formed despite disruption of the Golgi membrane in nocodazole-treated cells suggests that an intact Golgi compartment might be not an absolute requirement for development of lipid droplets.

Conclusions

Cellular uptake and internalization of cholesterol, delivered by liposomes, is characterized by a fast, non-endocytic, and unidirectional mode of entry, which is likely to be mediated by caveolae. The internalization of NBD-cholesterol delivered by liposomes is observed after a 5-minute incubation period. Cholesterol carrying a fluorophore on the tail group was transported faster than cholesterol with a cargo on the head group. Although similar results were obtained for phospholipids in regards to the position of cargo attachment, phospholipids showed a lower transport rate compared to NBD-cholesterol. This study highlights potential of cholesterol molecule for delivery of therapeutics into cells via a rapid and non-lysosomal pathway.

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NBD-cholesterol **NBD-PC** **BODIPY-cholesteryl ester** **NBD-PE** **Fig 1.**

Structure of fluorescent lipids used in liposome preparation. Each fluorescent probe was incorporated separately in liposomes prepared, as described in the Methods section.

Fig 2.

Internalization of fluorescent lipid analogues in PC-3 cells after incubation with a liposome concentration of 50 μM as a function of time. (A–D) Fluorescence images (63x) of PC-3 cells incubated with NBD-cholesterol, BODIPY-cholesteryl ester (BODIPY-CE), NBD-PC, and NBD-PE for 2 h at 37°C, respectively. (E) Kinetics of internalization of the fluorescent lipid analogues delivered by liposomes into PC-3 cells, as quantified by flow cytometry. (Relative fluorescence intensity, RFI).

Fig 3. Internalization of NBD-cholesterol delivered by liposomes into PC-3 cells as a function of liposome concentration. (A–E) Fluorescence images (63 $\times$) of trypsinized PC-3 cells after incubation with NBD-cholesterol liposomes for 1 h at 37°C at liposome concentrations of 5, 25, 35, 50, and 100 μM , respectively. (F) Relative fluorescence intensity (RFI) of PC-3 cells after incubation with NBD-cholesterol liposomes for 1 h at 37°C, quantified by flow cytometry.

Fig 4.

Uptake and intracellular distribution of NBD-cholesterol delivered by liposomes at a 50 μM concentration with varying incubation time. (A–E) Fluorescence images (63x) of trypsinized PC-3 cells after incubation with NBD-cholesterol liposomes for 0 min, 15 min, 30 min, 1 h, and 2 h. (F, G) Confocal images (63x) of A-375 cells incubated with NBD-cholesterol liposomes for 5 min at z-sections of 4 and 8 μm , respectively. (H, I) Confocal images (63x) of A-375 cells after 1 h incubation at z-sections of 4 and 8 μm , respectively.

Fig 5. Effect of temperature on NBD-cholesterol transport at liposome concentration of 50 μ M. Fluorescent images of A-375 cells incubated with NBD-cholesterol liposomes at 4°C for 1 h (A) and then warmed to 37°C and incubated for another 1 h (B), 20x. Fluorescent images of trypsinized PC-3 cells incubated with NBD-cholesterol for 2h at 4°C (C) and 37°C (D), 63x. E) Quantification of fluorescence intensity of PC-3 cells incubated at 4°C compared to 37°C for 2h.

Fig 6. Effect of PEG incorporation within liposomes on the internalization of NBD-cholesterol and NBD-PC into PC-3 cells. Fluorescence images (63x) of trypsinized PC-3 cells incubated for 2 h with (A, B) NBD-cholesterol liposomes with and without 9.5% DSPE-PEG2k and (C, D) NBD-PC liposomes with and without 9.5% DSPE-PEG2k. (E) Relative fluorescence intensity of NBD-cholesterol and NBD-PC delivered by liposomes prepared in the presence and absence of 9.5% mole ratio of DSPE-PEG2k at a liposome concentration of 50 μ M as a function of time.

Fig 7. Internalization of NBD-cholesterol in various cell types. (A–D) Fluorescent images of Raoec, PC-3, Huvec, and A-375 cell lines treated with NBD-cholesterol liposomes at 37°C for 2h. (E) Relative fluorescence of various cell lines incubated with NBD-cholesterol as a function of time, as quantified by flow cytometry.

Fig 8.

Effect of various inhibitors on internalization of NBD-cholesterol delivered by liposomes at 50 µM. Cells were incubated with each inhibitor for 1 h, followed by 1 h with the inhibitor plus NBD-cholesterol. (A–E) Four-µm z-section confocal images (63x) of A-375 cells (A, B) incubated with filipin, a caveolae inhibitor, at 5 and 10 µg/mL, respectively. (C, D) incubated with nocodazole (disrupts the microtubule assembly and Golgi membrane) at 10 and 15 µg/mL, respectively. (E) incubated with NBD-cholesterol without inhibitors. (F) Relative fluorescence intensity of PC-3 cells treated with inhibitors, as quantified by flow cytometry.